Effect of Essential Fatty Acid Deficiency on Lipid Metabolism in Isolated Fat Cells of Epididymal Fat Pads of Rats

D.I. DEMEYER,[1] **W.C. TAN,** and **O.S. PRIVETT,** The Hormel Institute, University of Minnesota, Austin, Minnesota 55912

ABSTRACT

Lipogenesis, lipolysis, and stimulation of glucose conversion into lipid by insulin or prostaglandin E_1 were studied in isolated fat cells of the epididymal fat pads of rats fed a fat-free diet or this diet supplemented with 10% hydrogenated coconut oil or 10% safflower seed oil. Changes in fatty acid composition, characteristic of an essential fatty acid deficiency, were well advanced in the neutral lipid but had only started in the polar lipid of the fat cells of the epididymal fat pads of animals 3 months after weaning. Cellularity of the epididymal fat pads, as indicated by protein to lipid ratio of the fat cells, was influenced greatly by hydrogenated coconut oil in the diet irrespective of an essential fatty acid deficiency. Lipogenesis was increased in the fat cells of the animals fed the hydrogenated coconut oil diet 5 weeks after weaning but was not significantly different from that of the safflower fed animals 3 months after weaning. Incorporation of glucose into lipid, oxidation to CO_2, and basal lipolysis were not significantly different in the fat cells of the essential fatty acid deficient animals from those fed safflower oil 3 months after weaning, except in animals of the fat-free group based upon cell lipid. However, conversion of glucose to free fatty acid was significantly greater in the isolated fat cells of animals fed either the hydrogenated coconut oil or the fat-free diet than in those of animals fed the safflower oil supplement. The incorporation of glucose into lipid by isolated fat cells was stimulated significantly by insulin in young animals fed a fat-free diet, but the effect on lipogenesis appeared to be reversed in the fat cells of animals receiving safflower seed oil 3 months after weaning. Prostaglandin E_1 also appeared to stimulate the incorporation of glucose into lipid in the fat cells of the older animals receiving safflower seed oil. Differences in osmolarity produced large differences in utilization of glucose and release of lipid from isolated fat cells, but no significant differences were observed between the cells from animals fed the fat-free diet and those from the controls fed safflower oil. The results demonstrated the effects of diets containing fat or no fat on enzyme activities and membrane properties of fat cells of the epididymal fat pads of essential fatty acid deficient rats.

INTRODUCTION

The effects of an essential fatty acid (EFA) deficiency on fatty acid synthesis have been observed in liver and adipose tissue (1-8). Allmann et al. (2) showed that refeeding starved rats on a fat-free diet or diets containing oleate or palmitate as the sole source of fat in the diet also gave an elevation in activity of enzymes that catalyze fatty acid synthesis. The activity of these enzymes was returned to normal levels by feeding linoleate (2) or polyunsaturated fatty acids (9). Recently, Du and Kruger (4) showed that adipocytes from rats fed a fat-free diet incorporated 8-10 times more radioactivity from uniformly labeled glucose than adipocytes of animals fed a corn oil diet. The above studies suggest that EFA is involved in the regulation of fatty acid synthesis. It also has been suggested (5) that the effect of EFA on enzyme activity in adipose tissue may be due to diminished synthesis of prostaglandins or an effect on membrane properties. That EFA are implicated in the activity of several enzymes has been suggested in a number of studies (10-14), but it is not known if the primary role of these acids is on enzymic processes or membrane properties of the cell. In the present study various ramifications of the effect of an EFA deficiency on lipid metabolism in isolated fat cells of epididymal fat pads were investigated.

MATERIALS AND METHODS

Male weanling rats of the Sprague-Dawley strain (Dan Rolfsmeyer Co., Madison, Wis.) were placed in individual cages and supplied food and water ad libitum. The basic diet consisted of 30% vitamin test casein, 60%

[1] Present address: Department of Nutrition, Faculty of Agricultural Sciences, Ghent, Belgium.

sucrose, 4% cellulose, 4% salt mixture, and 2% vitamin mixture (15). One group was continued on this diet with no fat supplementation (fat-free group). A second group was fed the basic diet supplemented with 10% (by wt) hydrogenated coconut oil (HCO), and a third group was fed the basic diet supplemented with 10% (by wt) safflower oil (saff). Animals were maintained on these diets for up to 5 months and were killed by decapitation at selected intervals. The epididymal fat pads were excised and a suspension of isolated fat cells prepared in a protein-free buffer as described by Lech and Calvert (16).

Incubation procedure: Total ester (17) and protein content (16) of the cell suspensions were determined, and all aliquots were measured and dispensed using polyethylene pipettes. Glucose utilization or lipogenesis was determined by incubation of 0.5 ml cell suspension in a total volume of 2 ml Krebs-Ringer bicarbonate buffer (pH 7.4) containing 4% bovine serum fraction V (Nutritional Biochemicals Corporation, Cleveland, Ohio, dialyzed against the same buffer overnight at 4 C) with 5 μmoles of glucose and 0.2 μci of glucose-U-^{14}C. Additions of insulin, 1 milliunit/ml (Sigma Chemical Co., St. Louis, Mo.), or prostaglandin E_1, 0.1 mg/ml, were made in 10 μl volumes of Krebs-Ringer buffer. Incubations were carried out under O_2 containing 5% CO_2 at 37 C (shaking water bath) in one-armed siliconized Warburg flasks with center wells containing filter paper strips (for measurement of radioactive CO_2). The Warburg flasks were siliconized by dipping them in 5% w/w Dow Corning fluid in $CHCl_3$ and baking at 300 C for 3 hr. The flasks were closed with rubber serum stoppers. Incubations were stopped by the addition of 0.5 ml 2N H_2SO_4 from the side arm of the flask at the end of 2 hr.

CO_2 production: After the 2 hr incubation period, 0.25 ml of hyamine hydroxide (Amersham-Searle, Des Plaines, Ill.) was injected on the filter paper and the incubation stopped by the addition of 0.5 ml 2N H_2SO_4 from the side arm of the flask. The flasks were allowed to stand for 1 hr at room temperature; then the paper strips were transferred to 15 ml scintillation solution (18) and counted in a Packard Tri-Carb model 3002 dual channel scintillation spectrometer.

Radioactivity in total lipid: The incubation mixture was transferred to centrifuge tubes and extracted once with 5 and then with 3 ml chloroform-methanol (2:1, v/v). After centrifugation (clinical centrifuge) for 10 min, the lower layers were withdrawn, pooled, and washed with 2 ml H_2O. Aliquots of the lipid extract were evaporated in scintillation vials, scintillation liquid (15 ml consisting of PPO-POPOP in toluene) added and the radioactivity counted.

Distribution of radioactivity in lipid classes: The lipid extract was fractionated on 20 x 20 cm chromatoplates containing a 0.30 cm layer of Silica Gel G (Brinkman Instruments, Inc., Des Plaines, Ill.). The solvents used for the separation of the neutral lipids were pretroleum ether (60-80 C)-diethyl ether-acetic acid (80:20:1, v/v/v) and isooctane-diethyl ether (20:80, v/v). The positions of the bands were detected by exposing the plates to iodine vapors for just sufficient time to make them visible. The bands were marked, and plates were left for ca. 10 min to evaporate most of the iodine. The bands were scraped directly from the plates into the vials of scintillation solution (18) for counting, except for triglycerides which were eluted from the silica gel on small glass columns with 30 ml $CHCl_3$-methanol (2:1, v/v). Aliquots of the eluate were used for radioactivity counting in toluene scintillation liquid. To determine radioactivity in triglyceride fatty acids, additional aliquots were evaporated and hydrolyzed at 70 C for 3 hr in 5 ml ethanolic KOH. Samples were left overnight, treated with 2 ml 6N H_2SO_4 and extracted with 3 ml hexane (Vortex mixer). Completeness of hydrolysis was checked by thin layer chromatography (TLC). After centrifugation, 2 ml hexane layer was evaporated in scintillation vials and radioactivity counted in toluene scintillation liquid. Determination of radioactivity in individual fatty acids was carried out by radiochromatography as described by Nakamura and Privett (19).

Counting efficiency for carbon 14 was 85% in the toluene solution and 71% in the scintillation solution used for samples recovered from chromatoplates. The amounts of glucose converted to CO_2, total lipid, and lipid classes were calculated from the specific activity of glucose in the original medium and total radioactivity recovered in the products. Results were expressed as mμ moles of glucose/g triglyceride ester or per mg protein. Triglyceride ester was determined by the method of Snyder and Stephens (17) using tristearate as a standard. Free glycerol in the incubation medium was determined, as described by Korn (20). Differences between means were tested for significance using the *t* test, modified when necessary for significant differences in variances (21).

Fatty acid analysis: Epididymal fat pads and livers were extracted twice with 10 volumes chloroform-methanol (2:1, v/v) and fatty acid composition determined by gas liquid chroma-

TABLE I

Fatty Acid Composition of Epididymal Fat Cells and Liver Lipid of Rats Fed 10% Hydrogenated Coconut Oil or Safflower Seed Oil Supplements to a Fat-Free Diet 3 Months After Weaning[a] (% wt)

Fatty acid	Total lipid				Polar lipid[b]			
	Saff diet		HCO diet		Saff diet		HCO diet	
	Fat cells	Liver	Fat cells	Liver	Fat cells	Liver	Fat cells	Liver
16:0	20.3	25.3	28.8	26.9	25.1	22.8	21.8	18.5
16:1	6.7	3.1	17.1	12.0	7.5	3.3	7.8	8.6
18:0	3.3	15.6	3.0	11.2	25.4	24.0	22.6	20.9
18:1	24.2	12.6	50.2	36.2	18.5	6.6	25.2	22.8
18:2	45.5	21.4	0.8	1.4	10.4	12.4	7.6	2.2
20:3	—	—	—	9.3	1.3	—	3.6	17.7
20:4	—	22.0	—	3.0	5.9	31.5	5.3	9.0

[a]Pooled samples of three animals selected on basis of the mean wt of original 10 animals in each group.

[b]HCO = hydrogenated coconut oil, saff = safflower seed oil.

tography (GLC) of methyl esters prepared by interesterification with methanol, as previously described (22). Neutral and polar lipids were separated by chromatography using acid-washed Florisil (23). GLC was carried out with an F&M model 1609 gas chromatograph equipped with a hydrogen flame detector and a 6 ft x 1/4 in. column packed with 8% EGSSX on Gas Chrom P (Applied Science, State College, Pa.) operated with a gas (N_2) flow of 65 ml/min at 185 C.

Effect of osmolarity: A method for determining cell lysis in solutions of different osmolarity was developed based upon techniques described by Rodbell (24). Fat cells (0.5 ml) were incubated for 15 min at 37 C in polyethylene test tubes with 4 ml solution to be tested. After each incubation, 4 ml hexane were layered over the incubation mixture; the tubes were agitated gently for 15 sec, allowed to stand for 15 min, and then agitated again for 15 sec. The triglyceride ester content of the hexane layer then was determined immediately and expressed as per cent of the total amount present in the cell suspension. In all determinations, mean values for at least three replicate flasks are reported.

RESULTS

Changes in fatty acid composition, typical of an EFA deficiency, occurred within 3 months after weaning in the total lipid of the fat cells, as well as livers of animals on the HCO diet. Similar changes occurred in the polar lipids of these tissues during this period but to a lesser degree in the fat cells than in the liver (Table I).

The ratio of protein to lipid in the fat cells appeared to be influenced significantly by fat in the diet, inasmuch as the relative amount of fat in the fat cells increased in both the HCO and control groups and the protein to lipid ratio decreased as the animals became older, except in the fat-free group (Table II). Lipogenesis was significantly greater in the group of EFA deficient animals which were fed the HCO supplemented diet for 5 weeks than in the corresponding controls as illustrated in Table

TABLE II

Lipid and Protein Analyses of Rat Epididymal Fat Cell Preparations

Dietary supplement after weaning[a]	Lipid mg/ml	Protein μg/ml	Protein/lipid ratio
HCO—5 weeks (3)	96 ± 7[b]	380 ± 78	3.96 ± 0.79
Saff—5 weeks (3)	88 ± 10	304 ± 43	3.46 ± 0.18
FF—3.4 months (10)	90 ± 15	369 ± 27	4.11 ± 0.44
Saff—3-4 months (9)	121 ± 6	237 ± 35	1.96 ± 0.29
HCO—4-5 months (7)	138 ± 17	185 ± 19	1.51 ± 0.24
Saff—4-5 months (8)	172 ± 22	196 ± 31	1.42 ± 0.43

[a]HCO = hydrogenated coconut oil, saff = safflower seed oil, and FF = fat-free diet. Number in brackets = number of animals analyzed.

[b]M ± SE.

TABLE III

Conversion of Glucose to Lipid by Epididymal Fat Cells (2 Hr Incubation Period)[a]

5 Weeks after weaning	Lipogenesis mμmoles glucose/mg protein				% Distribution of radioactivity in triglyceride fatty acids						
	Neutral lipid		Polar lipid	Total	Saturated acids				Unsaturated acids		
	Glycerol	FA			14:0	16:0	18:0	Total	16:1	18:1	Total
HCO	1371 ±179[b]	3118 ±533	179 ±24	4668 ±525	8.0 ±1.5	50.0 ±3.9	6.3 ±0.8	64.5 ±6.1	15.9 ±2.0	13.7 ±2.8	29.6 ±4.8
Saff	491 ±101	521 ±58	88 ±10	1100 ±155	9.0 ±0.7	61.0 ±2.2	4.5 ±0.0	74.5 ±1.0	13.1 ±0.9	7.2 ±0.7	20.4 ±0.5

[a]HCO = hydrogenated coconut oil, saff = safflower seed oil, and FA = fatty acids.

[b]Mean ± SE, three animals selected on the basis of the mean wt of 10 animals in the original group.

III. The increase in lipogenesis in these animals appeared to arise mainly from an increase in fatty acid synthesis. The percentage of radioactivity among the fatty acids was higher in the unsaturated fatty acids, particularly oleic acid, and appeared to represent the shift in fatty acid synthesis characteristic of an EFA deficiency. As the animals became older, there was no significant difference in lipogenesis, except for the fat-free group when the results were expressed on the basis of lipid content of the cell; differences were not significant when expressed on the basis of cell protein. Likewise, there was no significant difference in lipolysis, oxidation, or incorporation into lipid of glucose by the isolated fat cells of the epididymal fat pads between the controls and the EFA deficient animals 3 months after weaning, except for the fat-free group when the results were expressed on the basis of cell lipid (Table IV).

Studies on the incorporation of radioactivity into the lipid classes indicated that incorporation into free fatty acids was greater in the isolated fat cells of the EFA deficient animals fed either the fat-free or HCO diets rather than in those of the control group (Table V).

The incorporation of radioactive glucose into lipid was enhanced by insulin in young EFA deficient animals fed a fat-free diet, as illustrated in Table VI. The results in Table VI were obtained in a separate experiment in which animals were sacrificed 6 weeks after weaning. Insulin binding was also greater in the fat cells of these animals as determined with ^{131}I-insulin (67 ± 4 vs. 20 ± 2.5 standard deviation for 10 animals). In the binding experiment, ^{131}I-insulin was used at a concentration of 50 mμg/ml in a plastic vessel containing 5 ml incubation mixture. After incubation, the cells were washed three times on filter paper before being counted. In the studies with older animals, an EFA deficiency had no effect on the response to the incorporation of radioactive glucose into lipid by insulin, as evidenced by the fact that a 10-fold increase in insulin seemed to have a greater effect in experiments with normal animals (Table VII). These experiments also indicated that the response to prostaglandin E_1 of the incorporation of radioactive glucose into lipid similarly was influenced by the age of the animal.

Differences in osmolarity produced marked changes in glucose utilization and lipid release by isolated epididymal fat cells, as illustrated in Table VIII. The differences between the EFA deficient and normal animals were not statistically significant in these experiments, although there was a strong trend in this direction.

TABLE IV

Lipolysis, Oxidation to CO_2, and Incorporation into Lipid of (U-^{14}C)Glucose by Epididymal Fat Cells (2 Hr Incubation Period)

Dietary supplement after weaning[a]	Glycerol	CO_2	Lipid
	(μmoles/mg lipid)	(mμmoles/mg lipid)	
FF[b]–3-4 months (10)	69.4 ± 11.7[c]	5173 ± 673	2589 ± 557
Saff–3-4 months (9)	38.2 ± 6.1	2116 ± 404	623 ± 149
HCO–4-5 months (7)	19.4 ± 1.8	1378 ± 307	1331 ± 368
Saff–4-5 months (8)	20.8 ± 5.0	1013 ± 376	601 ± 124
	(μmoles/mg protein)	(mμmoles/mg protein)	
FF–3-4 months (10)	16.4 ± 7.9	1361 ± 193	664 ± 126
Saff–3-4 months (9)	24.9 ± 6.0	1211 ± 268	365 ± 43
HCO–4-5 months (7)	15.8 ± 3.8	1130 ± 336	847 ± 195
Saff–4-5 months (8)	19.6 ± 4.5	946 ± 291	530 ± 90

[a]FF = fat-free, saff = safflower seed oil, and HCO = hydrogenated coconut oil. Number in brackets = number of animals.

[b]Significant level for FF 3-4 months vs. saff 3-4 months is $P<0.05$.

[c]M ± SE.

DISCUSSION

The present study shows that many factors play a role in the metabolism of lipids in the fat cells of the epididymal fat pads of EFA deficient rats, just as the nutritional status of animals in general has been shown to influence lipid metabolism in adipose tissue (8,25-31). Age, presence or absence of fat in the diet, and the method of expressing the results based on either cell lipid or protein are important. The marked difference of the lipid to protein ratio in the fat cells of the animals fed HCO and the fat-free diets indicated that the size, and perhaps the number, of the cells of these animals were much different in accordance with the general observations of nutritional effects upon the number and size of adipocytes in adipose tissue of rats (27,32-36). Changes of the DNA to lipid ratio in fat cells with age also have been observed and related to cell size (37). The difference in cellularity of the fat pads between animals fed the different diets as they grew older could account for many of the effects observed. The effect of insulin could well be explained on this basis (29,38-45), inasmuch as the response diminished with age and accumulation of fat in the cells. However, because there was a positive response to insulin in the young animals at a time when cell size and lipid content per se were comparable, it appeared that the enhancement of lipogenesis could be associated with an EFA deficiency also. This effect was indicated further by the greater binding properties for insulin of the cells of the EFA deficient animals. Although the mechanism of the action of hormones, such as insulin, in lipid metabolism is not clear (38,46,47), conceivably an EFA deficiency could alter the configural relationship between lipid and protein moieties of enzymes, such as adenyl cyclase, which is located in the fat cell mem-

TABLE V

Incorporation of Radioactivity in Lipid Classes mμmoles of (U-^{14}C)Glucose/mg Protein (2 Hr Incubation Period)[a]

Diet supplement after weaning	TG	DG	FA	PL
FF[b]–3-4 months (10)	510 ± 157[c]	28 ± 13	41 ± 10	84 ± 13
Saff–3-4 months (9)	245 ± 39	20 ± 9	14 ± 4	82 ± 23
HCO[b]–4-5 months (7)	587 ± 160	188 ± 27	27 ± 5	51 ± 10
Saff–4-5 months (8)	324 ± 73	125 ± 48	11 ± 3	66 ± 17

[a]FF = fat-free, saff = safflower seed oil, HCO = hydrogenated coconut oil, TG = triglyceride, DG = diglyceride, FA = fatty acid, and PL = polar lipid. Number in brackets = number of animals.

[b]Significant level for FF 3-4 months vs. saff 3-4 months is $P<0.05$; HCO 4-5 months vs. saff 4-5 months is $P<0.05$.

[c]M ± SE.

TABLE VI

Influence of Insulin Concentration on the Incorporation of (U-^{14}C) Glucose into Lipid by Epididymal Fat Cells of Rats 6 Weeks after Weaning[a,b]

Insulin concentration (μu/ml)	Saff diet (% control values)	Fat-free diet (% control values)
1	121 ± 5	100 ± 3
10	170 ± 6	218 ± 42
30	174 ± 14	309 ± 60
50	194 ± 2	273 ± 55
100	132 ± 9	198 ± 50

[a]% of control values, without added insulin, 2 hr incubation time.

[b]M ± SE from nine rats.

TABLE VII

Influence of Insulin (1 milliunit/ml) and PGE_1 (0.1 μg/ml) on Incorporation of (U-^{14}C) Glucose into Lipid by Epididymal Fat Cells[a]

Dietary supplement after weaning[b]	Insulin	PGE_1
FF–3-4 months (10)	100 ± 3[c]	101 ± 3
Saff–3-4 months (9)	121 ± 5	124 ± 9
HCO–4-5 months (7)	104 ± 6	95 ± 6
Saff–4-5 months (8)	111 ± 8	115 ± 7

[a]% of control values, without added insulin, 2 hr incubation time.

[b]FF = fat-free, saff = safflower seed oil, and HCO = hydrogenated coconut oil. Number in brackets = number of animals.

[c]M ± SE.

branes and related to the action of insulin and prostaglandins (38,46-51).

The present study shows that in young EFA deficient rats, there was a significant increase in fatty acid synthesis expressed on the basis of cell protein (or lipid). Incorporation into free fatty acid was also significantly higher in older EFA deficient animals than the corresponding safflower controls. However, age and the presence or absence of fat in the diet appear to mask effects of an EFA deficiency on lipolysis or lipogenesis. It does not appear to be valid to compare effects on lipolysis or lipogenesis in EFA deficient and normal animals on the basis of cell lipid, inasmuch as the presence of HCO in the diet appears to mask differences due to an EFA deficiency. This conclusion is in accordance with the observation of others who have questioned the validity of expressing data on the basis of cell fat (32,52-55). On the other hand, whether cell protein (16) is a more valid basis for the expression of metabolic data is also open to question, inasmuch as its synthesis may be impaired by an EFA deficiency (14,56).

DNA also may be used as a basis for expression of cellular activity, but it also has drawbacks, inasmuch as it is influenced by age and cell size (37). Hence, although there appears to be little doubt of a role of EFA in the metabolism of lipid in epididymal fat cells, its delineation is still very much a problem.

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TABLE VIII

Influence of Osmolarity on Lipid Relesae and Glucose Utilization of Rat Epididymal Fat Cells

Dietary supplement after weaning[a]	Saff 3-4 months	FF 3-4 months
Lipid release (% of total lipid)		
Hypotonic (in H_2O)	17.7 ± 5.9[b]	8.4 ± 3.4
Hypertonic (in 0.6 M sucrose)	26.1 ± 6.4	13.6 ± 4.3
U-^{14}C glucose oxidation to $^{14}CO_2$ (% of control value)		
Hypotonic (in H_2O)	4.5 ± 0.9	6.2 ± 0.8
Hypertonic (in 0.6 M sucrose)	14.6 ± 3.6	22.8 ± 4.3
U-^{14}C glucose incorporation to lipid (% of control value)		
Hypotonic (in H_2O)	33.0 ± 5.2	28.6 ± 3.2
Hypertonic (in 0.6 M sucrose)	72.6 ± 9.9	62.0 ± 7.7

[a]Saff = safflower seed oil, and FF = fat-free groups.

[b]Mean ± SE from 10 rats.

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Skin Lipids of the Florida Indigo Snake

DAVID G. AHERN[1] and **DONALD T. DOWNING**, Departments of Biochemistry and Dermatology, Boston University Medical Center, Boston, Massachusetts 02118

ABSTRACT

Cast skins of the Florida indigo snake (*Drymarchon corais*) yielded up to 8% chloroform:methanol-extractable lipid, which was found to contain methyl ketones (20%), free secondary alcohols (15%), free primary alcohols (30%), free cholesterol (15%), free fatty acids (5%), and hydrocarbons (5%). The hydrocarbons appeared to be contaminants, because gas chromatography revealed a distribution characteristic of petroleum hydrocarbons. The methyl ketones were predominantly monounsaturated, with double bonds almost exclusively in the $\omega 7$ position. The structures of the secondary alcohols corresponded with the methyl ketones in regard to chain length distribution, location of the oxygen function in the 2 position, and the proportion and position of unsaturation. The primary alcohols were also predominantly straight, odd-carbon, unsaturated compounds, with $\omega 7$ double bonds, but with chain lengths principally of 29 and 31 carbon atoms. The free fatty acids were mainly even-carbon monounsaturated compounds of 16-20 carbon atoms with double bonds mainly in the $\Delta 9$-position. Inspection of the lipid structures obtained from the Indigo snake suggest a biogenetic relationship whereby palmitic and palmitoleic acids are extended in chain length mainly to 32 and 34 carbon-atom fatty acids. Retention or introduction of an oxygen function in the 3 position, followed by decarboxylation, could then yield structures corresponding with the methyl ketones and the related secondary alcohols. Insertion of an oxygen atom between carbons 2 and 3 of the methyl ketones, followed by loss of the two carbon atoms thereby isolated from the chain, would produce the series of odd-carbon primary alcohols that were observed.

INTRODUCTION

In contrast to lipids from internal organs, skin surface lipids from a wide variety of higher animals have been found to differ dramatically in composition (1,2). Unusual classes of lipids are common in skin surface extracts, but no two species have been found yet with similar surface lipid composition. In most species the major part of the surface lipid is produced by specialized epidermal appendages, such as the sebaceous glands in mammals and the uropygeal gland in birds.

This specific variation in surface lipid composition might result from progressive specialization of these glands, and the small amount of lipid produced by the epidermis itself might exhibit a less bizarre variation in composition between species. In humans, the epidermal lipid obtained from areas free from sebaceous glands (the palms and soles) does have a relatively simple composition consisting of cholesterol, cholesterol esters, and triglycerides (3,4). However, the difficulty of locating sebum-free areas has so far precluded a comparison of true epidermal lipid in other species. Therefore, in approaching this question it seemed reasonable to examine the epidermal lipid of reptiles, which mostly appear to lack specialized lipid-producing skin appendages. However, our preliminary examination of lipids from the cast skins of the Florida indigo snake have shown these to be a complex mixture with unusual constituents.

METHODS AND RESULTS

Extraction of Lipids

Freshly cast skins were obtained from specimens of the Florida indigo snake (*Drymarchon corais*) kept at the Museum of Science, Boston, Mass. The skins were extracted by immersion overnight in chloroform-methanol (2:1) under nitrogen. Undissolved material was collected on

[1]Present address: New England Nuclear Corp., 575 Albany St., Boston, Massachusetts 02118

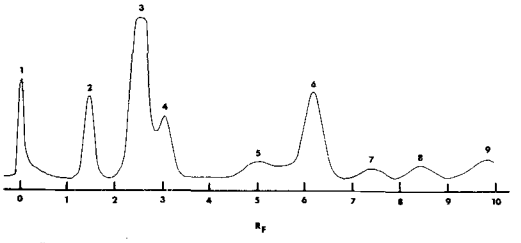

FIG. 1. Photodensitometer scan of a thin layer chromatography separation of the crude lipids from a Florida indigo snake. 1 = phospholipids, 2 = cholesterol, 3 = primary alcohols, 4 = secondary alcohols, 5 = free fatty acids, 6 = methyl ketones, 7 and 8 = unidentified, and 9 = hydrocarbons.

TABLE I

Ca. Composition of Lipids Obtained from Cast Skins of Florida Indigo Snake

Peak number from thin layer chromatography (Fig. 1)	Class	Composition (wt %)
1	Phospholipids (?)	---
2	Cholesterol	15
3	Primary alcohols	30
4	Secondary alcohols	15
5	Free fatty acids	5
6	Methyl ketones	20
7	Unidentified	5
8	Unidentified	5
9	Hydrocarbons	5
Total		100

a sintered-glass filter and re-extracted with the same solvent. The combined extracts were shaken with water, and the chloroform layer was washed twice with water and dried over Na_2SO_4. The solvent was removed on a rotary evaporator at 40 C, leaving a yellow, semisolid residue, representing 5-8% of the dry wt of the skin.

Preliminary Thin Layer Chromatography (TLC) Analysis

An aliquot of ca. 15-20 μg snake skin lipid was chromatographed on a 20 x 20 cm glass plate carrying a 250 μ layer of Silica Gel G, as previously described in detail (5), with the sample applied to a 6 mm-wide lane scored in the adsorbent. The chromatogram was developed with hexane:ether:acetic acid (70:30:1 to 19 cm); the plate was then sprayed with 50% H_2SO_4 and slowly raised to 220 C on a hot plate over 45 min. The chromatogram, which revealed a complex mixture of at least nine lipid classes, was scanned with a recording photodensitometer (Photovolt, Model 530) and the relative proportions of the resolved lipid classes were calculated from the peak areas obtained (Fig. 1 and Table I), as previously described (5).

Free Fatty Acids (Peak 5, Fig. 1)

These were separated from the crude lipid mixture by the procedure of McCarthy and Duthie (6), whereby an ethereal solution of the total lipids was passed through a column of alkali-treated silica gel and flushed with further fresh ether until no further lipid was eluted. The fatty acids were then eluted from the column with 1% formic acid in ether. The recovered fatty acids were methylated with boron trifluoride-methanol and the methyl esters were separated into saturated, monounsaturated, and diunsaturated compounds by TLC on silica gel-silver nitrate. Each of these fractions was analyzed by gas chromatography on a 3% SE-30 silicone stationary phase. The positions of unsaturation in the monoenoic acids were determined by gas chromatographic analysis of the products of periodate-permanganate oxidation, as described by Downing and Greene (7). The results are shown in Table II.

Neutral Lipids

The neutral lipids recovered after removal of the free fatty acids were fractionated by chromatography on a silica gel column (100 mesh), eluting successively with hexane, hexane-benzene, benzene, and benzene-ether. The fractions obtained were analyzed by TLC, and those containing similar lipid classes were recombined. Six lipid classes were recognized among the eluants (see Fig. 2).

Methyl Ketones (Peak 6, Fig. 1)

Functional group: The presence of a car-

TABLE II

Composition of Free Fatty Acids of Florida Indigo Snake Skin (wt %)

Carbon chain	Saturated (66%)	Monoenoic[a] (16%)	Dienoic (8%)
n-C_{12}	0.3		
br-C_{14}	0.2		
n-C_{14}	1.4		
n-C_{15}	1.0		
br-C_{16}	0.4		
n-C_{16}	23.0	2	
n-C_{17}	1.7	2	
br-C_{18}	0.9		
n-C_{18}	46.0	96	99+
n-C_{19}	0.5		
n-C_{20}	16.2		
n-C_{22}	6.8		

[a]Each monoenoic acid was almost exclusively $\Delta 9$-unsaturated. Unsaturation in the dienoic acid was not identified.

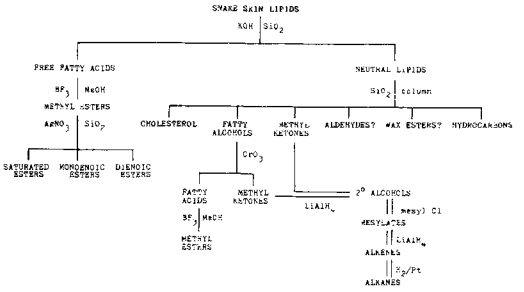

FIG. 2. Scheme employed in the fractionation of lipids from skins of the Florida indigo snake.

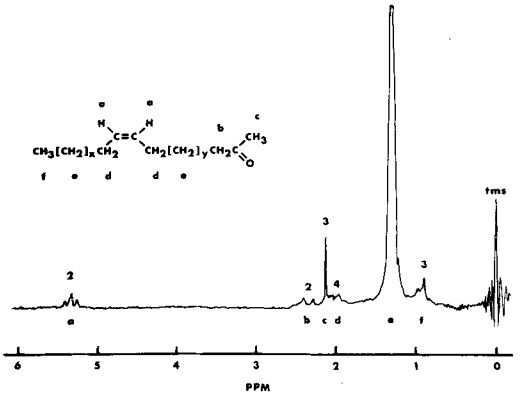

FIG. 3. NMR spectrum of the methyl ketones from the Florida indigo snake skins. Numbers above the trace represent relative peak areas obtained by integration. Letters below the trace relate to proton positional assignments in the structural formula.

bonyl function in this fraction first was indicated by the yellow coloration produced when TLC were sprayed with 2,4-dinitrophenylhydrazine. The IR spectrum showed strong carbonyl absorption at 5.8μm and moderate absorption at 8.55μm, together with peaks at 3.4, 6.8, 7.3, and 13.88μm, characteristic of hydrocarbon chains.

The NMR spectrum (Fig. 3) of the methyl ketones in deuterochloroform was obtained with a Varian A60 instrument and showed absorption peaks at 0.88, 2.0, 2.1 (singlet), 2.35, and 5.35 (triplet) ppm (δ) having relative areas of 3, 4, 3, 2, and 2, respectively, in addition to the large (methylene) absorption at 12.5ppm. This spectrum confirmed the presence of methyl ketones having an average of one isolated ethylenic bond/molecule.

Chain structure: Gas chromatography on a silicone stationary phase indicated that the methyl ketones consisted of a homologous series of long chain components (Fig. 4A). As

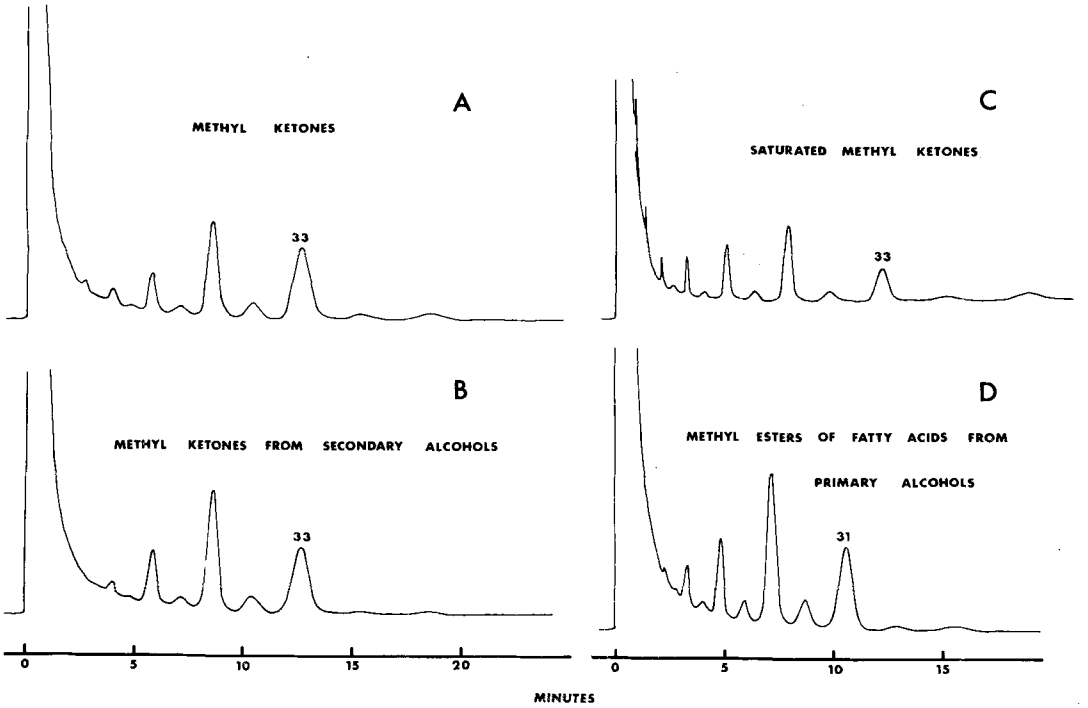

FIG. 4. Gas chromatograms of homologous series derived from skin lipids of the Florida indigo snake. A. Total mixture of the naturally-occurring methyl ketones. B. Methyl ketones obtained by mild chromic acid oxidation of the secondary alcohols. C. Saturated methyl ketones separated from the naturally occurring mixture. D. Methyl esters of the fatty acids produced by mild chromic acid oxidation of the primary alcohols. The chromatograms were obtained on a 4 ft x 1/8 in inside diameter column of 3% SE-30 silicone gum at 290 C.

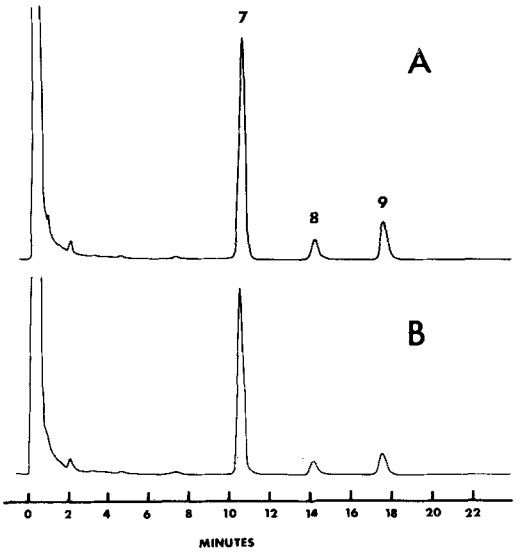

FIG. 5. Methyl esters of the short chain fatty acids produced by periodate/permanganate oxidation. A. Products from the methyl ketones. B. Products from the primary alcohols. The chromatograms were produced by pyrolysis of the tetramethylammonium salts of the fatty acids to methyl esters at 300 C in the injection port of the gas chromatograph, followed by resolution of the esters on a 3 ft x 1/8 in. outside diameter column consisting of a mixture of 9% SE-30 silicone gum and 1% diethylene glycol succinate on Diatoport S, programed from 25-200 C at 3 C/min.

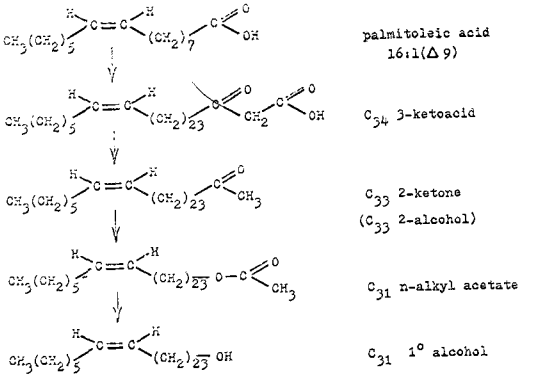

FIG. 6. Outline of a hypothetical pathway for biosynthesis of the major lipid classes found in the Florida indigo snake skin.

outlined in Figure 2, a sample of the methyl ketones was converted into the corresponding mixture of saturated hydrocarbons. Gas chromatography of this mixture and of a series of authentic normal hydrocarbons indicated that the reduction products of the methyl ketones consisted of a homologous series of straight chain hydrocarbons with the C_{29}, C_{31}, and C_{33} constituents predominating.

Unsaturation: Samples of the original mixtures of methyl ketones were separated according to degree of unsaturation by preparative TLC on 1 mm layers of silica gel containing 10% silver nitrate, with benzene as the developing solvent. Of the methyl ketones from five different skins, only one sample contained dienoic compounds, and these were not examined further. All samples showed a minor proportion of saturated ketones, ca. 5% mixture, the remainder being monounsaturated.

A sample of the monoenoic ketones was oxidized with periodate/permanganate, and the low mol wt acidic products were recovered as their tetramethylammonium salts and analyzed by pyrolysis to methyl esters in the gas chromatograph (7). These products consisted predominantly of fatty acids with 7, 8, and 9 carbon atoms (Fig. 5A). The higher mol wt oxidation fragments bearing the carbonyl group were not accessible by this procedure. To study these fragments a sample of the monoenoic ketones was reduced to the corresponding mixture of monoenoic hydrocarbons, as outlined in Figure 2, and these were oxidized with periodate/permanganate by the method of von Rudloff (8). The recovered fatty acids were methylated with BF_3/methanol and analyzed by gas chromatography on a silicone stationary phase. The chromatograms of the longer chain methyl esters obtained showed a distribution of peaks similar to that of the original methyl ketones but with seven fewer carbon atoms.

Saturated methyl ketones: Gas chromatography of the saturated methyl ketones revealed a chain length distribution similar to the monoenoic ketones and corresponding exactly with saturated ketones produced by hydrogenation of the unsaturated compounds (Fig. 4B).

Mass spectrum: To obtain a specimen of a methyl ketone with a single chain length for mass spectrometry a sample of the monoenoic ketones was converted to the dinitrophenylhydrazones and fractionated by reverse phase TLC on silica gel/10% paraffin oil developed with dioxane:water (5:2). The major discrete band was eluted with ether, and the recovered material was rechromatographed on silica gel to remove the paraffin oil. The recovered dinitrophenylhydrazone was treated with BF_3/acetone to recover the free ketone (9), which was repurified by TLC. Gas chromatographic analysis indicated that the ketone consisted of a single chain length (>98%), corresponding to the component previously designated as having 33 carbon atoms. Mass spectrometry, using a Perkin Elmer/Hitachi Model 6E instrument, was achieved by probe injection into the ionization chamber and gave a parent ion of mol wt 476,

TABLE III

Aliphatic Constituents of the Florida Indigo Snake Skin Lipids (wt %)

Chain length	Methyl ketones		Alcohols	
	Saturated	Monoenoic	1° Monoenoic	2° Monoenoic
21	1.5			
22	—			
23	2.2		0.5	
24	0.2		0.3	
25	3.9	0.8	4.6	0.2
26	0.9	0.2	1.2	0.1
27	7.2	3.1	13.8	2.5
28	1.4	0.4	3.8	0.2
29	15.2	9.2	36.0	13.2
30	3.2	2.2	7.8	2.5
31	30.1	33.4	27.4	40.3
32	4.6	9.5	1.7	6.4
33	20.8	34.9	2.8	30.0
34	2.2	1.9		1.2
35	6.5	4.3		3.3
Total	99.9	99.9	99.9	99.9

corresponding with the empirical formula $C_{33}H_{64}O$. The fragmentation pattern was virtually identical with that of heptadecan-2-one, showing in particular, ions of M/e = 58, 458 (M-15), and 461 (M-18).

Fatty Alcohols (Peaks 3 and 4, Fig. 1)

The chromatographic properties and IR spectra of these materials indicated a mixture of primary and secondary alcohols, which were not fully resolved chromatographically. The mixture was, therefore, subjected to a mild chromic acid oxidation (10), producing a mixture of free fatty acids and ketones.

Primary alcohols: The fatty acids produced by oxidation of the mixed alcohols were separated from the oxidation products by passage of the mixture through a column of silica gel previously treated with potassium hydroxide (6). The neutral material was eluted with ether, and the acids were eluted with 1% formic acid in ether.

The acids thus obtained from the primary alcohols were converted to their methyl esters with BF_3/methanol. Argentation TLC of the methyl esters indicated that they consisted almost exclusively of monounsaturated compounds. Gas chromatography revealed a pattern almost identical with that of the methyl ketones but with chain lengths of C_{27}, C_{29}, and C_{31} predominating, as shown in Figure 4C and Table III. After reduction of the ethylenic bonds with hydrogen and Adam's catalyst, the chain length assignments were substantiated by gas chromatography, using authentic straight chain saturated methyl esters as reference compounds. The hydrogenated methyl esters from the primary alcohols also were subjected to combined gas chromatography/mass spectrometry, by means of which each component was shown to have the appropriate mol wt for the assigned structure and chain length.

Oxidation with periodate/permanganate (7) of the unsaturated methyl esters derived from the primary alcohols produced a series of straight chain fatty acid methyl esters having 7, 8, and 9 carbon atoms, similar in relative amounts to those obtained by oxidative fission of the methyl ketones (Fig. 5B). The dicarboxylic acid fragments produced by oxidation were too long in chain length to be accessible in the gas chromatography system used.

Secondary alcohols: The neutral products from the chromic acid oxidation of the mixture of fatty alcohols showed chemical, chromatographic, and spectroscopic properties identical with those of the methyl ketones. Gas chromatography revealed that the chain length distribution also was similar to that of the naturally occurring ketones (Fig. 4D). Argentation TLC showed that the ketones from chromic acid oxidation of the secondary alcohols were almost exclusively monoenoic and periodate/permanganate oxidation produced a mixture of C_7-C_9 fatty acids similar to that obtained from the natural ketones.

Cholesterol (Peak 2, Fig. 1)

On TLC and argentation TLC, as well as on gas chromatography, the sterol fraction showed a single component which was indistinguishable from cholesterol in migratory properties and in the color produced during charring of the TLC plates.

Peaks 7 and 8, Fig. 1

These minor constituents of the original lipid mixture were not investigated extensively. However, neither produced color during charring of TLC plates and, therefore, contained neither sterols nor squalene. The NMR spectrum of peak 8 indicated a wax ester structure which was not an acetate. The R_f value for peak 7 was the same as that of aldehydes produced by partial oxidation of the primary alcohols.

Hydrocarbons (Peak 9, Fig. 1)

Gas chromatograms of the hydrocarbon fractions revealed a broad hump extending over the retention times expected for C_{15}-C_{35} hydrocarbons, with numerous small superimposed peaks, typical of petroleum hydrocarbons.

RESULTS AND DISCUSSION

As shown in Table I, the lipid recovered from skins of the Florida indigo snake consisted of a complex mixture of lipid classes, ca. proportions of which were determined by quantitative TLC.

The free fatty acids (Table II) were not unusual, consisting principally of palmitic, stearic, oleic, and (presumably) linoleic acids, with small proportions of palmitoleic and branched chain saturated acids.

The remarkable features of the lipids of this snake skin are the presence of high proportions of long chain methyl ketones, the corresponding secondary alcohols, and primary alcohols of predominantly odd-carbon number (Table III).

Although methyl ketones of short or moderate chain length are widespread in nature (11), their occurrence has, until now, been limited to plants, insects, and especially, microorganisms. Furthermore, such long chain methyl ketones as found in the indigo snake do not appear to have been reported previously, although equally long ketones with the functional group further from the end of the chain are common in plants (12). The biosynthesis of such ketones, as have been demonstrated for nonacosan-15-one in broccoli (13), involves the chain extension of stearic acid to triacontanoic acid, which then is decarboxylated to nonacosane. The hydrocarbon then is oxidized to a mixture of 14- and 15-hydroxynonacosanes, from which the latter is preferentially oxidized to nonacosan-15-one.

In studies with microorganisms, especially fungi, it has been established that methyl ketones may be formed from fatty acids by β-oxidation followed by decarboxylation (11). The odd-carbon methyl ketones thus formed then can be reduced to 2-alkanols or oxidized by a biological Bayer-Villiger reaction to the acetates of odd-carbon primary alcohols, in which the hydrocarbon chain has been shortened by two carbon atoms (14). An analogous series of reactions can be invoked to explain the formation of each of the three major classes of aliphatic compounds identified in the present investigation (Fig. 6). It is apparent that, in the present series of compounds, an additional clue to the pathway of biosynthesis is provided by the location of the ethylenic bonds. Their ω-7 position suggests a common origin from palmitoleic acid (16:1Δ9). Although it may seem unlikely that this acid, which is a minor constituent of the free fatty acid fraction, should be selected as a precursor over the much more abundant oleic acid, this situation is not without precedent. In human skin surface lipids (15) and in the skin surface lipids of the rat (16), longer chain unsaturated acids often appear to be derived from C_{16} precursors, rather than the more abundant C_{18} homologs.

There is no indication in this study whether the methyl ketones might be formed directly by decarboxylation of β-ketoacids or by subterminal oxidation of hydrocarbons produced by decarboxylation of unsubstituted fatty acids. Additional support for the biosynthetic pathway suggested might lie in the unidentified compounds, one of which appears to be a wax ester, perhaps analogous to undecyl undecanoate produced by *Pseudomonas* species from tridecan-2-one (14).

The complex and unusual nature of the skin lipids of the Florida indigo snake seem to deny the proposal that epidermal lipids might have a simple composition which would not differ markedly between species. However, we intend to investigate the possibility that the lipids observed may have originated in the specialized anal glands of the snake, which apparently serve as a source of trail-marking and sexual pheromones.

ACKNOWLEDGMENTS

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Immunochemical Quantification of Human Plasma Lp(a) Lipoprotein

JOHN J. ALBERS[1] and **WILLIAM R. HAZZARD**, The Northwest Lipid Research Clinic, Departments of Medicine and Biochemistry, University of Washington School of Medicine, Seattle, Washington 98104

ABSTRACT

The Lp(a) lipoprotein was purified from human plasma by ultracentrifugation and gel filtration on 6% agarose. It contained 27% protein, 65% lipid, and 8% carbohydrate. Quantification of the Lp(a) lipoprotein was performed by radial immunodiffusion. Both within-assay and between-assay coefficients of variation were inversely concentration dependent, decreasing from 20% and 27%, respectively, at 3 mg/100 ml to 7% and 12%, respectively, at concentrations above 8 mg/100 ml. The lower limit of sensitivity of the assay was 1.5 mg/100 ml. Of 340 unrelated fasting subjects tested, 81% had levels of the Lp(a) lipoprotein exceeding this lower limit. The distribution of Lp(a) concentrations in this population was skewed with a mean of 14 mg/100 ml and a median of 8 mg/100 ml. Lp(a) lipoprotein was not significantly correlated with age, sex, or cholesterol or glyceride concentrations.

INTRODUCTION

In recent years the focus of attention in plasma lipid research has shifted from the lipids themselves to their protein carriers and the lipoprotein complexes. Of the lipoproteins identified to date, the Lp(a) lipoprotein has remained especially enigmatic. This lipoprotein, which closely resembles the low density lipoprotein (LDL, *d* 1.019-1.063 g/ml) in its lipid composition (1), shares its major antigenic determinants with LDL (2); and it has been reported that 65% of its apoprotein is identical to the B or LDL apoprotein, 20% of its apoprotein is the "Lp(a) protein," and that albumin is a minor (<15%) but integral part of the Lp(a) apoprotein (3). Its density (*d* 1.050-1.12 g/ml) overlaps that of LDL and high density lipoprotein (HDL) (1,2), but its electrophoretic mobility on paper (4) or agarose (1,2,4) is pre-beta in contrast with the beta-mobility of LDL. In early reports Lp(a) was considered to be a qualitative genetic marker (5,6), but more recently it has been suggested to be a quantitative trait present in all individuals (7). Its physiological and genetic control, relationship to normal and abnormal states of lipid metabolism, and possible role in the atherosclerotic process remain unknown.

Definitive answers to these questions await the development of a highly sensitive, precise, and specific assay for the Lp(a) lipoprotein in physiological media. In the present study this problem has been approached by the development of an immunochemical assay utilizing radial immunodiffusion. The assay sensitivity and precision have been quantified and the technique applied to the measurement of the Lp(a) lipoprotein in an epidemiological survey of plasma lipid and lipoprotein concentrations in a population of 340 free-living adult employee volunteers.

MATERIALS AND METHODS

Blood Samples

For isolation of Lp(a) lipoprotein for immunization and preparation of standards, blood samples were drawn from healthy, fasting adults on ad libitum diets. In all instances, blood was drawn into tubes or bottles containing disodium ethylenediaminetetraacetate (EDTA) to give a final concentration of 1 mg/ml and the plasma promptly separated and stored at 4 C until analysis or ultracentrifugation. For determination of the distribution of Lp(a) lipoprotein, cholesterol, and glyceride levels among healthy, fasting adults, all employees of the Pacific Northwest Bell Telephone Company in Renton, Wa., were asked to volunteer for an epidemiological survey of lipid and lipoprotein levels. Ninety-two per cent (340) of this population from age 20-65 was sampled after an overnight (12-14 hr) fast.

Preparation of Lipoproteins

Lipoproteins were isolated from sera of individual donors by sequential preparative ultracentrifugation (8). Specifically, the nonprotein solvent density of plasma from a normolipidemic fasting adult was adjusted to 1.060 g/ml with solid NaCl. Ultracentrifugation then was carried out ina 50 Ti rotor at 40,000 rpm at 10 C for 24 hr. The top 3 ml of each tube was removed with a tube slicer and the bottom fraction readjusted to 1.090 g/ml with

[1] Author to whom reprint requests should be addressed.

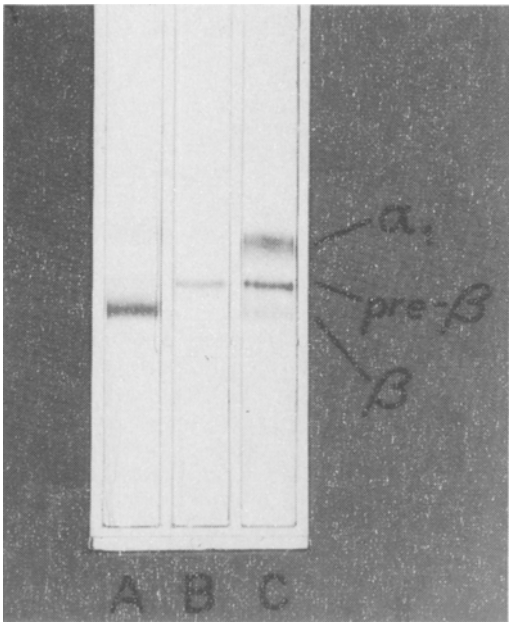

FIG. 1. Agarose electrophoresis of plasma and fractions thereof: (A) Whole plasma, (B) First major fraction or Lp(a) lipoprotein obtained from chromatography of 1.060-1.090 g/ml fraction on Bio-Gel A5m (see Fig. 2), and (C) 1.060-1.090 g/ml lipoprotein fraction.

solid NaCl and recentrifuged in a 50 Ti rotor at 45,000 rpm for 26 hr. The *d* 1.060-1.090 g/ml lipoproteins contained in the top 2.5 ml were subfractionated by ascending gel chromatography on 6% agarose gel (Bio-Gel A-5m, 200-400 mesh, Bio-Rad Laboratories, Richmond, Calif.) at 18 C utilizing a 2.6 x 90 cm column and a flow rate of 12 ml/hr (2). The sample volume was 4-6 ml and the eluting buffer 0.1 M Tris-HCl-0.15 M NaCl-0.001 M EDTA (pH 8.2).

Fractions were collected at 3 ml/tube. The absorbance of the eluates was measured with a Beckman model DU-2 ultraviolet spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) at 280 nm and the appropriate fractions pooled and dialyzed against 0.01 M Tris-HCl-0.001 M EDTA (pH 8.2) and then concentrated with Aquacide II (Calbiochem, La Jolla, Calif.). The lipoproteins subsequently were dialyzed against 0.15 M NaCl-0.001 M EDTA (pH 7.4) prior to use as standard or for preparation of antigen.

Electrophoresis

Polyacrylamide gel electrophoresis of whole lipoproteins was performed according to Narayan, et al., (9). The lipoproteins were prestained for lipid with Sudan Black B or, alternatively, poststained for protein with Amido-Schwartz in 7% acetic acid. Agarose electrophoresis was performed using the Bio-Gram A kit (Bio-Rad Laboratories).

Antigen and Antisera Preparation

The first fraction from the agarose column (see Fig. 1), constituting the Lp(a) lipoprotein, was pooled and concentrated with Aquacide II. Ca. 100 μg Lp(a) protein was placed on each 10-12 polyacryamide gel columns. The single, slowly migrating sudan positive band was cut out from each gel. The purified Lp(a) lipoprotein (ca. 1 mg) obtained from polyacrylamide gel electrophoresis along with the polyacrylamide gel was emulsified with an equal volume of Freund's complete adjuvant. Rabbits were immunized intramuscularly, subcutaneously, and intradermally, as described (10). A booster dose of ca. 1 mg Lp(a) protein in incomplete adjuvant was given at 1 month intervals. The rabbits were bled on the seventh day following the booster injection. Immunization of two rabbits with Lp(a) lipoprotein led to the production of precipitating antisera. These antisera were absorbed with LDL (*d* 1.030-1.040 g/ml) as follows: 0.5 mg LDL was added to ca. 20 ml each antiserum, incubated at 37 C for 30 min, then overnight at 4 C. This absorption procedure was repeated four-six times, until a precipitate no longer formed upon addition of LDL. This adsorbed antiserum was designated anti-Lp(a). Antiserum from one of the two rabbits was used in the present study.

Antisera against the high density lipoprotein polypeptides were those prepared and used previously (11).

Chemical Analysis

Lipids were extracted from the Lp(a) lipoproteins by the method of Folch, et al., (12). Cholesterol was determined by the method of Searcy, et al., (13); triglyceride by a modified procedure of Carlson (14) with triolein (Applied Science Laboratories, Inc., State College, Pa.) as standard; and phospholipid by the procedure of Bartlett (15). The factor 25 was used to convert phosphorus to phospholipid. It was assumed that 71.5% sterol in Lp(a) lipoprotein was sterol ester (1); the factor of 1.677, based upon cholesterol linoleate, was utilized to convert sterol ester to sterol mass.

For lipid analysis of whole plasma, samples were extracted with zeolite and isopropanol and analyzed for cholesterol and triglyceride with the Technicon Auto-Analyzer I, as outlined (16,17). During the course of this study the Northwest Lipid Research Clinic was in Phast III (Surveillance) of the Glyceride and Cholesterol Standardization Program of the

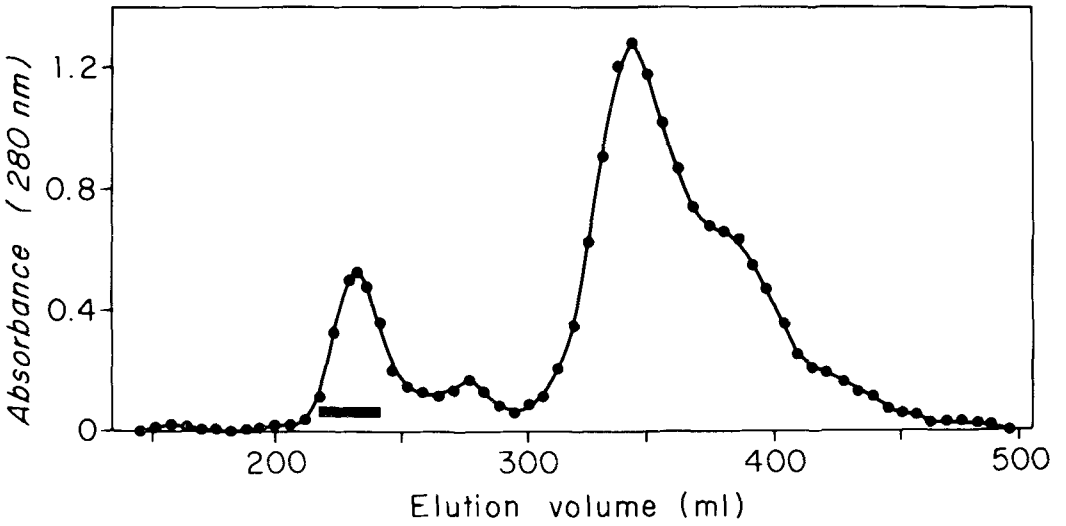

FIG. 2. Elution profile from Bio-Gel A-5m of a representative 1.060-1.090 g/ml lipoprotein fraction. ▬ = fraction constituting the Lp(a) lipoprotein.

Lipid Standardization Laboratory at the U.S. Center for Disease Control, Atlanta, Ga., and continuously met the standards for precision and accuracy specified by that program. Specifically, there was a coefficient of variation of less than 4% and accuracy within 3% of true value for cholesterol analysis and a coefficient of variation of less than 6% and accuracy within 3% for the glyceride analysis.

Protein was determined according to Lowry, et al., (18) with human serum albumin (HSA, Pentex) as standard. Protein nitrogen was determined by Kjeldahl digestion and Nesslerization (19); the factor 6.25 was used to convert protein nitrogen to protein. The Lp(a) lipoprotein preparations were diluted with 0.1% (w/v) sodium dodecyl sulfate prior to protein analysis. On the basis of Nessler nitrogen analyses, the Lp(a) lipoprotein protein, as determined by the Lowry method, was multiplied by 0.7 to convert to HSA protein equivalents. Hexose was determined by the anthrone method (20) with glucose as the standard.

Gel Diffusion

Immunodiffusion was carried out in 1% (w/v) agarose (Bio-Rad) in 0.15 M NaCl, 0.001 M EDTA, 0.05% (w/v) sodium azide, 0.02 M tris-HCl (pH 8.0), henceforth called RID buffer. An example of the micro Ouchterlony plate used for routine testing of human plasma has been shown previously (21). Anti-Lp(a) serum was diluted 1:5 (v/v) with RID buffer prior to use.

Quantitative Immunochemical Analysis

Quantitation of the Lp(a) lipoprotein was performed by the single radial immunodiffusion method of Mancini (22). To eliminate nonspecific precipitation reactions, only those plasmas which gave a positive reaction in double gel diffusion were considered positive in the quantitative method. Anti-Lp(a) serum was diluted 1:100 (v/v) with RID buffer containing 1% (w/v) bovine serum albumin (BSA) prior to use. Five ml of diluted anti-Lp(a) serum, heated to 55 C, was added to an equal volume of 2% (w/v) melted agarose solution cooled to 55 C. This antiserum-agarose solution was mixed thoroughly, avoiding bubbling, and immediately poured into prewarmed 100 x 100 x 15 mm phage typing dishes precoated with 1% silicone. The mixture was left to solidify on a level surface for 20-30 min. Thirty-six antigen wells of 2.2 mm diameter were punched out over the center of each grid (36 grids/dish) using a brass needle with a 2.2 mm bore. Three microliters of standard or plasma samples were added to each well. Unless otherwise indicated, each sample was added once to two different plates. Standards were added in duplicate wells chosen in different quadrants on each respective plate. The plates were placed in a humid chamber in a level position at 37 C. After 72-120 hr, the ring-shaped immunoprecipitates were measured in tenths of a millimeter using a micrometer lens.

The Lp(a) lipoprotein fraction, obtained from the 6% agarose column and constituting the Lp(a) lipoprotein standard, was diluted with RID buffer containing 3% (w/v) bovine serum albumin and kept at 4 C. Under these conditions the Lp(a) standard remained stable for at least two weeks, as judged by the lack of

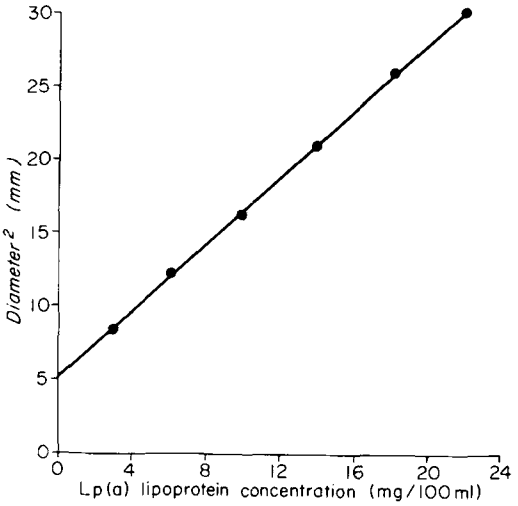

FIG. 3. A typical standard line showing the relationship between the square of the diameter of the immunoprecipitate and the Lp(a) lipoprotein concentration in mg/100 ml. Each point represents the mean of duplicate standards.

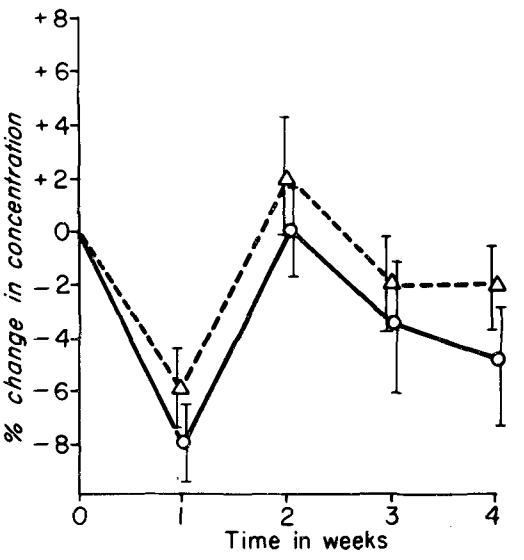

FIG. 4. Mean per cent change in concentration of Lp(a) lipoprotein at weekly intervals for samples stored unfrozen △ or frozen ○. Each point represents the average per cent change of six different samples. ⊢⊣ = standard error of the mean for each point.

change in the ring diameter of the immunoprecipitates. The Lp(a) lipoprotein standard ranged in concentration from 3-25 mg/100 ml.

RESULTS

Characterization of Lp(a) Lipoprotein Standards

The Lp(a) lipoprotein was isolated by separation of the *d* 1.060-1.090 g/ml lipoprotein fraction on Bio-Gel A-5m (6% agarose). The 1.060-1.090 g/ml lipoprotein fraction contained principally pre-beta and alpha$_1$ lipoproteins as shown in Figure 1C. This fraction was chosen, because it contains a small quantity of low density lipoproteins (see Figs. 1 and 5, ref [2]) and 77±8% of the total plasma Lp(a) lipoprotein, as determined by radial immunodiffusion Lp(a) quantification of five 1.060-1..090 g/ml lipoprotein preparations and their respective whole plasma. As indicated in Figure 2, the first major fraction obtained by agarose gel chromatography had an elution volume of ca. 227 ml and constituted the Lp(a) lipoprotein. It gave a single band with pre-beta mobility on agarose gel electrophoresis, as shown in Figure 1B. It also gave a single, slowly migrating, Sudan-positive band upon polyacrylamide gel electrophoresis (see Fig. 6, ref [2]) and reacted with anti-Lp(a) and anti-LDL sera, as shown previously (2). This Lp(a) lipoprotein fraction did not react with antihuman serum albumin or with antibodies against high density lipoprotein polypeptides R-Gln I (A-I) or R-Gln II (A-II).

The results of chemical analysis of Lp(a) are shown in Table I. Assuming that the sum of cholesterol, phospholipid, and glyceride represents total lipids and the sum of hexose, hexoseamine, and sialic acid represents total carbohydrate, this lipoprotein contained ca. 27% protein, 65% lipid, and 8% carbohydrate. The neutral lipids comprised 70% of the total lipids and the phospholipids 30%. Ca. 52% carbohydrate was hexose, the remainder consisting of hexoseamine and sialic acid (3).

Specificity of Antisera

Immunization of a rabbit with Lp(a) lipoprotein produced an antiserum which, when absorbed with low density lipoprotein, reacted specifically with the Lp(a) lipoprotein. This antiserum, anti-Lp(a) serum, and anti-a-1, previously described (2), all gave identical precipitation reactions when tested against a panel of 50 human plasmas: 39 of these plasmas gave visible precipitates when tested against each of these three antisera. Each of these antisera gave a single precipitation band when tested against whole human plasma. Furthermore, these antisera did not react against a number of antigens when tested in double gel diffusion over a concentration range of 0.1-15 mg/ml. These antigens included high density lipoprotein-3 (HDL_3, *d* 1.125-1.21 g/ml); delipidated HDL and its constituent polypeptides R-Gln-I or R-Gln-II, isolated as described (11); very low density lipoprotein (VLDL) ($d<1.006$ g/ml); delipidated VLDL and its constituent polypep-

tides, isolated as described (23); low density lipoproteins of d 1.030-1.040 g/ml; human serum albumin; and the plasma protein fraction of d>1.21 g/ml.

Assay Standard

The Lp(a) lipoprotein standards were calibrated by performing a Lowry protein. They were converted to HSA protein equivalents by multiplying by 0.7, then converted to total lipoprotein by multiplying by 3.7 (Lp(a) lipoprotein is assumed to contain 27% protein). The relationship between the square of the diameter of the precipitate ring and the amount of Lp(a) antigen was linear over the concentration range of 3-22 mg/100 ml, as shown in Figure 3. The diameter of the precipitates ranged from a minimum of 2.9 mm for the 3 mg/100 ml standard to a maximum of ca. 5.5 mm for the 22 mg/100 ml standard. The standards remained stable for at least two weeks while stored at 4 C as indicated by the lack of change in the ring diameter. Generally, most standards could be stored for four weeks or longer before a change of greater than 10% in the slope of the standard line or the diameter of precipitate was detected.

Stability of the Lp(a) Lipoprotein

An experiment was designed to test the effect of storage of both frozen and unfrozen plasma on Lp(a) concentration by immunoassay. Five 1 ml aliquots were taken from each of six different fresh plasmas. Four of the five aliquots from each plasma were frozen and stored at -20 C whereas the remaining aliquot was kept at 4 C. All samples contained .05% sodium azide. The Lp(a) lipoprotein concentration was determined on each of the fresh plasmas. At weekly intervals one of the frozen aliquots was thawed and the Lp(a) concentration determined on the thawed sample and on the original unfrozen sample. A comparison of samples stored at -20 C vs. those stored at 4 C is shown in Figure 4. The Lp(a) concentration for samples stored under either condition did not change significantly (less than 10%) over the 4 week period. Those samples stored at -20 C were consistently slightly lower on the average than those samples stored at 4 C. The test sample concnetrations ranged from 5-25 mg%. No attempt was made to assess the effects of storage as a function of initial concentration.

Assay Precision

An analysis of within-assay precision was performed, i.e. precision within the same radial immunodiffusion plate using the standards in duplicate. Twelve samples, whose concentra-

TABLE I

Chemical Composition of the Lp(a) Lipoprotein

	Protein	Free Cholesterol plus cholesteryl ester	Phospholipid[a]	Triglyceride[a]	Hexose[b]	Hexoseamine[c]	Sialic acid[c]
μg	1000	1542 ± 87	709 ± 32	138 ± 30	161 ± 12	84	66
1000 μg	27.0	41.7	19.2	3.7	4.4	2.3	1.8
Percent							

[a]Four Lp(a) preparations were analyzed with each preparation extracted in duplicate and each extract analyzed in triplicate. Free cholesterol = 0.285 x total cholesterol; cholesteryl ester = 0.715 x 1.677 x total cholesterol.

[b]Two Lp(a) preparations were analyzed with each preparation analyzed in duplicate.

[c]See ref. 3.

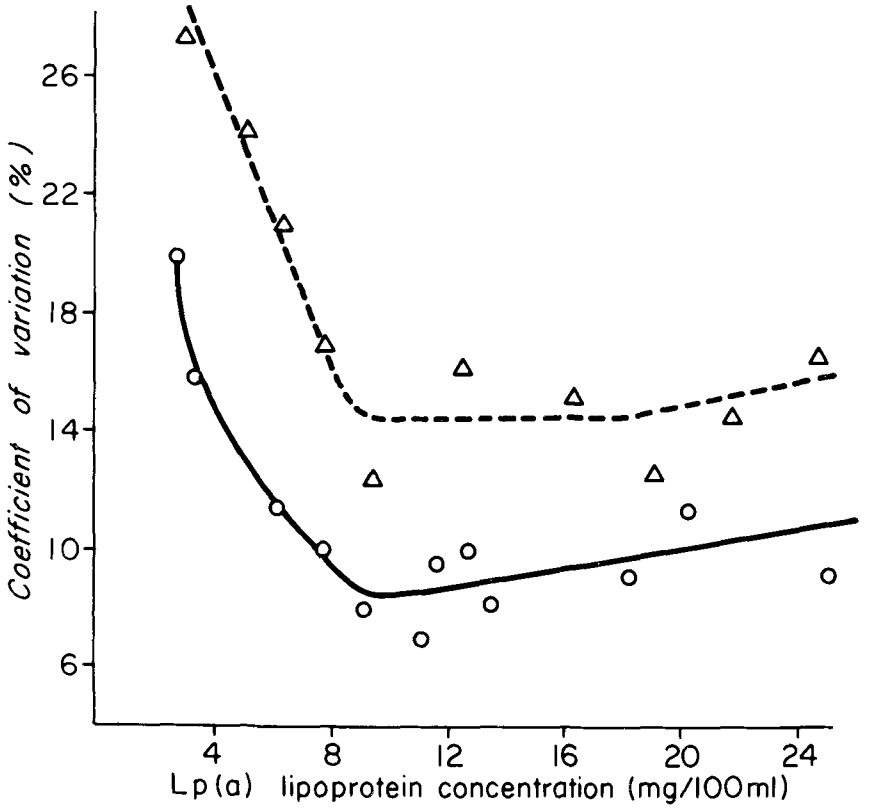

FIG. 5. Coefficient of variation as a function of Lp(a) lipoprotein concentration. o——o = within-plate variations, △----△ = between-assay variations.

tions of Lp(a) were pre-estimated and therefore known to span the entire standard range of 3-22 mg/100 ml, were analyzed on separate plates. Each sample was added to 12 wells randomly chosen within a plate. The standard deviation (S.D.) and the coefficient of variation (SD/mean x 100%) were calculated for each sample. Similarly, an analysis of between-assay reproducibility was performed, i.e. precision computed from the mean of six separate individual assays on 10 different samples performed at 2-4 week intervals utilizing different sets of standards. Each individual sample on a given day was added once to two different plates. As usual, each of the two plates contained the same set of standards in duplicate. As shown in Figure 5, the within-plate and between-assay variations were concentration dependent. The within-plate variation rose sharply at Lp(a) concentrations below 8 mg/100 ml to a maximum of 20% for sample concentrations of ca. 3 mg/100 ml. Similarly, the between-assay coefficient of variation rose steeply at Lp(a) concentrations below 8 mg/100 ml to a maximum of 27% for concentrations of ca. 3 mg/100 ml. The within-plate variation for samples between 8-25 mg/100 ml was nearly linear with an average variation of 9%, whereas the between-assay variation for this concentration range was ca. 15%.

Sensitivity of Assay

The double gel diffusion method could detect purified Lp(a) lipoprotein at a minimum concentration of 1.5 mg/100 ml (15 μg/ml). Of 340 unrelated fasting subjects tested, 275 or 81% had levels of the Lp(a) lipoprotein exceeding this lower limit. In the single radial diffusion method, the relationship between the square of the diameter of the precipitate and the amount of antigen was linear over the concentration range of 3-22 mg/100 ml, as shown in Figure 3. The quantitative immunodiffusion method, therefore, approaches the sensitivity of the qualitative gel diffusion method. As has been shown, however, the precision of the assay decreases sharply when analyzing samples with concentrations below 8 mg/100 ml. Furthermore, the immunoprecipitation rings with diameters of 2.9 mm or less, representing concentrations less than 3 mg/100 ml, were usually faint and difficult to read with

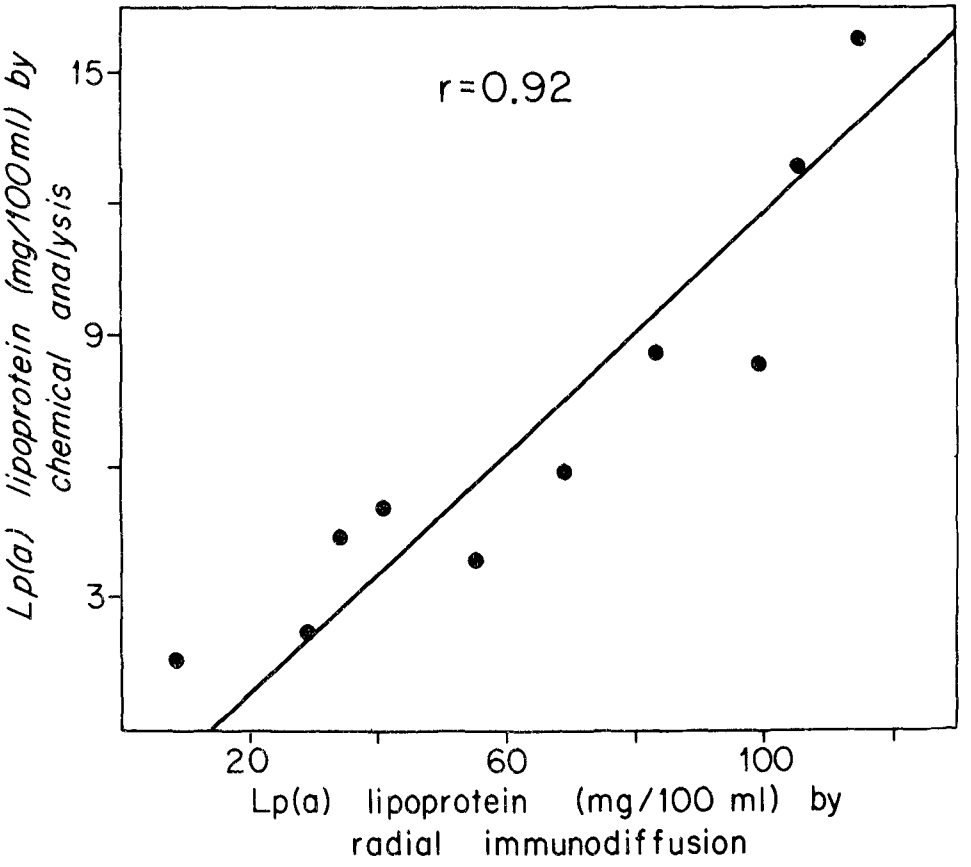

FIG. 6. Lp(a) lipoprotein levels: relationship of chemical analysis of first peak of 1.060-1.090 g/ml lipoprotein fraction from gel filtration column to the Lp(a) immunoassay of whole plasma.

a micrometer lens. Those samples which produced doubtful precipitin rings, but which were weakly positive by gel diffusion, were considered to have an Lp(a) concentration of 1.5 mg/100 ml. On the other hand, samples with concentrations of greater than 25 mg/100 ml and which gave strong positive precipitation reactions in double diffusion frequently give either faint or negative reactions upon radial immunodiffusion. These samples, therefore, were diluted until the plasma had a concentration within the standard range. When these plasmas with high concentrations were diluted serially, i.e. 1:2, 1:4, 1:8, etc., then the square of the immunoprecipitin diameter, plotted as a function of concentration, had the same slope as that produced by the purified standards.

Sixty-five individuals or 19% had undetectable Lp(a) lipoprotein concentrations in whole plasma. To determine if those plasmas lacked Lp(a) lipoprotein or merely contained levels below the threshold of detection by the gel diffusion method employed, the Lp(a) negative plasmas were concentrated ca. four-fold with Aquacide II and retested for Lp(a) lipoprotein after this concentration procedure. Thirty-nine of 65 negative plasmas had detectable Lp(a) lipoprotein after this concentration procedure. Thus, as many as 92% of the total population sampled were shown to have detectable levels of this lipoprotein.

Lp(a) Lipoprotein Levels: Relationship of Chemical Analysis to Immunoassay

Lp(a) lipoprotein was isolated from a series of 10 plasmas by agarose gel chromatography of the 1.060-1.090 g/ml lipoprotein fraction. Chemical analysis was performed on the first major peaks with an elution volume of ca. 227 ml by doing a Lowry protein analysis, correcting to HSA equivalents, and assuming 27% protein composition. The amount of Lp(a) lipoprotein obtained by this analysis was compared to the level of Lp(a) lipoprotein by immunoassay of whole plasmas. As indicated in Figure 6, Lp(a) lipoprotein by chemical analysis was highly correlated with the level of plasma Lp(a) lipoprotein by immunoassay (r = 0.92).

TABLE II

Age Distribution by Decades

Age	Males		Females		Total	
	Number	Per cent	Number	Per cent	Number	Per cent
20-29	32	20	83	47	115	34
30-39	43	26	46	26	89	26
40-49	57	35	32	18	89	26
50-59	30	18	14	8	44	13
≥60	2	1	1	1	3	1
Total	164	48	176	52	340	100

The yield of Lp(a) lipoprotein from whole plasma utilizing the above isolation procedure was 11.1 ± 3.4%.

Lp(a) Lipoprotein Concentrations: Relationship with Age, Sex, and Cholesterol and Glyceride Concentrations

Distribution of the population by age and sex is shown in Table II. The population contained ca. equal number of males and females with females having the younger age distribution. The distribution of Lp(a) concentrations for the 340 free-living, unrelated adult fasting subjects is shown in Figure 7. This distribution was highly skewed with a mean of 14.0 mg/100 ml and a median of 8.0 mg/100 ml. Males and females had similar distributions (Fig.8). The median test showed no sex difference in either mean or median Lp(a) levels, males having a mean of 14.1 mg/100 ml and females a mean of 13.9 mg/100 ml with a maximum value for both sexes of 76 mg/100 ml.

Figure 9 indicates that at the 5% level there was no statistically significant correlation of Lp(a) with either cholesterol and/or triglyceride concentrations. Analysis of the data using Spearman's nonparametric correlation in the significance test corroborated this conclusion.

As indicated in Figure 10, Lp(a) levels were found by Pearson's coefficient to be independent of age ($r = 0.052$; $0.5 < p < 0.6$). This conclusion was corroborated by Spearman's nonparametric coefficient. On the other hand, both cholesterol and triglyceride increased with age (Table III).

DISCUSSION

This article describes the quantification of the Lp(a) lipoprotein by a single radial immunodiffusion assay. The Lp(a) lipoprotein, called LDL-a-1 in a previous publication (2) and elsewhere referred to as the "sinking pre-beta" lipoprotein (4), shares the major antigenic determinants of LDL (2) and is, therefore, assumed to share the major LDL protein. In the described technique, immunization of rabbits with purified human Lp(a) lipoprotein produces antisera which reacts with both LDL and Lp(a) lipoproteins. All precipitating antibodies reacting with LDL subsequently are removed by absorption, leaving antibodies which react with the Lp(a) lipoprotein but not with any other known plasma lipoprotein or lipoprotein polypeptide from VLDL, LDL, or HDL. The Lp(a) lipoprotein, found in the density range of 1.050-1.090 g/ml (and isolated from the *d* 1.060-1.090 g/ml range in this study), therefore, differs from the other low and high density lipoproteins present in this hydrated density class by the nature of the antigenic determinant(s) unique to this lipoprotein. Whether this determinant(s) is due to a unique polypeptide in the Lp(a) molecule or, altenatively, the carbohydrate moiety has not been ascertained; nor has the possibility that the lipid moiety

TABLE III

Correlation Coefficients for the Normal Population:Age vs. Plasma Lp(a) Lipoprotein and Lipid Levels

Statistical test	Age vs. Lp(a)	Age vs. Cholesterol	Age vs. Glyceride
Pearson's correlation coefficient	0.052	0.444[a]	0.162[a]
Spearman's rank coefficient	0.074	0.441[a]	0.273[a]

[a]Significant at the 0.002 level.

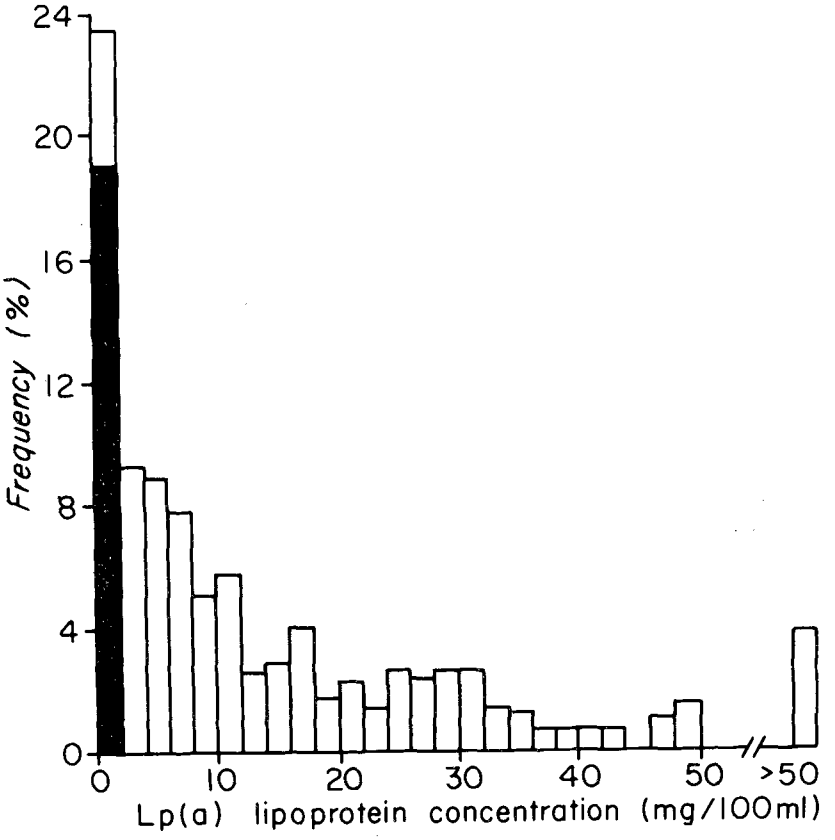

FIG. 7. Frequency distributions of plasma Lp(a) lipoprotein concentrations in 340 fasting adult subjects. ▬ = percentage of subjects with undetectable Lp(a) lipoprotein concentration in whole plasma.

could contribute to the formation or stability of this antigen been excluded.

The Lp(a) lipoprotein differs from low and high density lipoprotein, not only in immunological properties, but also in numerous chemical and physical-chemical properties. One of the distinguishing chemical characteristics of the Lp(a) lipoprotein is its high carbohydrate content; the hexoses and heoseamines are ca. three times and the sialic acid content six times as high in Lp(a) lipoprotein as in LDL. The lipid/protein ratio of Lp(a) was shown to be 2.4, considerably lesser than the value of 3.5 reported for LDL (24). On the other hand, the Lp(a) lipoprotein lipid composition was similar to that reported for LDL of *d* 1.010-1.050 g/ml (24). Other characteristics which distinguish the Lp(a) lipoprotein from LDL are its mol wt, ca. 5.4 x 10^6, compared to 2.3 x 10^6 for LDL; sedimentation coefficient at *d* 1.002 of 13.4 compared to 8.4 for LDL; and pre-beta mobility on agarose, compared to beta mobility for LDL (2).

The immunochemical quantification of lipo-

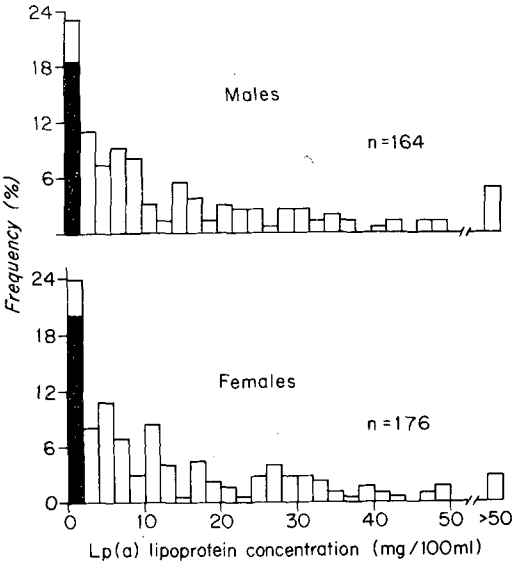

FIG. 8. Frequency distributions of plasma Lp(a) lipoprotein concentrations of male and female adult subjects.

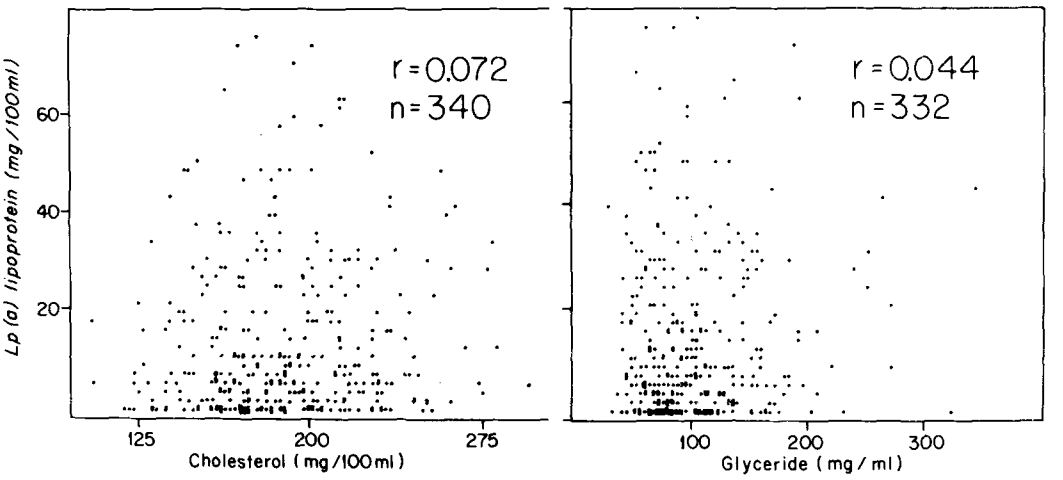

FIG. 9. The relationship between plasma Lp(a) lipoprotein and cholesterol on the left and plasma Lp(a) lipoprotein and glyceride on the right in a group of adult employee volunteers.

proteins has not been employed extensively, due to the lack of specificity and reproducibility in previous assays. Difficulties in producing monospecific antisera, failure to define precisely the specificity of each antiserum, the sharing of polypeptides among lipoproteins of different hydrated density classes, the masking of antigenic determinants by lipids, the instability of lipoprotein standards, and the lack of precision in the assay methods have all contributed to the lack of general acceptance and use of quantitative immunochemical methods among workers in the lipoprotein field. In the present study, the unique antigenic determinant(s) of the Lp(a) lipoprotein was exploited to develop a specific, reproducible, and sensitive method for the immunochemical quantification of the Lp(a) lipoprotein in human plasma.

The quantification of the Lp(a) lipoprotein by radial immunodiffusion was reasonably precise with a 15% between-assay variation for the concentration range of 8-25 mg/100 ml and somewhat less precise for the concentrations under 8 mg/100 ml.

Plasma samples with concentrations above 25 mg/100 ml were diluted and then assayed at 1:100 antiserum dilution. Alternatively the plasma could have been assayed undiluted with the antiserum at a lower dilution. On the other hand, for samples with low Lp(a) concentrations a higher dilution of antiserum would have not improved the sensitivity or the precision, because at antibody dilutions greater than 1:100 the precipitin rings were too faint to be resolved. It has been observed, however, that repeated filling of the wells with the test and standard samples increases sensitivity and precision for the samples with low Lp(a) concentrations.

The population of 340 free-living adult volunteers, representing 92% of the Pacific Northwest Bell Company employees in Renton, Wa., were predominantly white (327 of 340), middle-class residents of the suburban Seattle area. A fairly broad spectrum of socioeconomic and occupational groups was represented in the population. Additional epidemiological characteristics of this population will be reported subsequently in the description of the Northwest Lipid Research Clinic Pacific Northwest Bell Prevalence Survey. The Lp(a) lipoprotein distribution in this population was skewed dramatically (mean, 14 mg/100 ml; median 8 mg/100 ml), yielding a curve similar to a negative expoential function. Normal levels of the Lp(a) lipoprotein have not, as yet, been published; and no precise criteria have been established for the upper limits of normal for this plasma fraction. For plasma cholesterol and glyceride, the upper 5th percentile can be considered abnormally high. A similar 95th percentile upper cut-off for the normal Lp(a) concentration would be 48 mg/100 ml. However, since Lp(a) levels have yet to be correlated with any disease condition, such an arbitrary definition of an abnormal Lp(a) level currently would have no clinical utility.

The Lp(a) lipoprotein was detected in unconcentrated plasma in ca. 81% of all individuals tested, whereas in previous studies the Lp(a) lipoprotein was detected in only 30-45% plasmas tested (6). The explanation for this large difference in Lp(a)-positive individuals most likely lies in the increased sensitivity of the gel diffusion method used in the present

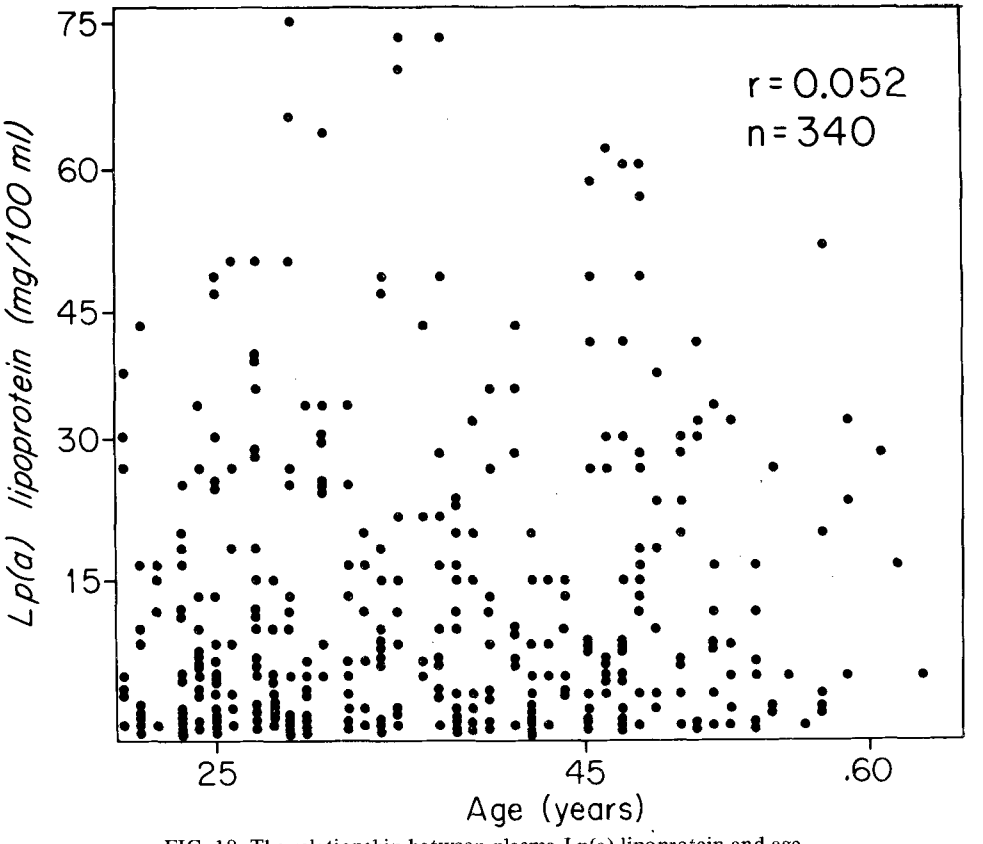

FIG. 10. The relationship between plasma Lp(a) lipoprotein and age.

studies rather than differences in the population studied. Even the presently described method is less than ideal, because of the common circumstance in which the concentration of Lp(a) in human plasma lies below its lower limit of sensitivity. Nevertheless, it appears to be the most sensitive quantitative procedure for the Lp(a) lipoprotein described thus far. Furthermore, when used in conjunction with gel diffusion studies, this assay procedure is ideal for screening of plasma samples for high Lp(a) levels.

The demonstration of detectable Lp(a) levels by only a fourfold concentration of plasma in an additional 11% of the population (92% therefore being Lp(a) positive) supports the concept that Lp(a) lipoprotein exists in all individuals. Previous investigations which suggested that Lp(a) may be a qualitative trait present in a minority of the population appear to have been based upon methods which were insufficiently sensitive to detect the lower levels measured by the present technique.

Even though 65% of the mass of the Lp(a) lipoprotein is lipid (of which 65% is cholesterol by wt), Lp(a) lipoprotein concentrations were not significantly correlated with total cholesterol levels in the population studied. This lack of correlation is not surprising, since the Lp(a) lipoprotein cholesterol generally represents only a small percentage (3%) of the total plasma cholesterol concentration, e.g. given a mean Lp(a) concentration of 14 mg% the Lp(a) lipoprotein would contribute only an average of 5.9 mg cholesterol/100 ml plasma.

Furthermore, the Lp(a) lipoprotein concentration was independent of age. This contrasted with the fact that total cholesterol significantly increased with age, and the fact that total cholesterol is highly correlated with LDL cholesterol (25) tends to suggest that the Lp(a) lipoprotein may be controlled metabolically independent of LDL despite the sharing of a common protein moiety.

ACKNOWLEDGMENTS

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Fatty Acid Distribution in Lipids and ^{32}P Incorporation into Phospholipids during Early Amphibian Development

C.A. BARASSI[1] and N.G. BAZÁN,[2]
Instituto de Investigaciones Bioquímicas,
Universidad Nacional del Sur, Bahía Blanca, Argentina

ABSTRACT

Several aspects of lipid composition and ^{32}P incorporation were studied during early embryogenesis of the toad, *Bufo arenarum*, Hensel. The surveyed stages ranged from unfertilized oocyte to neural tube formation. The fatty acid distribution in polar and neutral lipids, as well as in acetone eluate from Unisil columns was similar in unfertilized oocyte and late blastula stage. There was no significant effect of cell cleavage on the fatty acid composition of these lipid fractions. Neutral lipids represent ca. 67% of the total lipids. The main components of the phospholipids were phosphatides of choline and ethanolamine. The total lipid and phospholipid content does not change through the studied stage of neurula. However a large increment in the phospholipid's specific radioactivity occurs when ^{32}P is injected along with the hormone to induce ovulation. It is suggested that this may reflect changes in turnover rates rather than net biosynthesis. Since a large amount of cell membranes is being formed during the early development and because the level of phospholipids remains constant, an explanation is offered regarding membranogenesis. Active phospholipid biosynthesis may take place during oogenesis. These lipids may be stored in the yolk platelet, and fertilization may regulate the functioning of a transport mechanism to corresponding membrane sites. The increased incorporation of ^{32}P may reflect changes in the activity of new membranes.

INTRODUCTION

Biochemical studies on the structure and metabolism of lipids during the very early stages of vertebrate embryonic development are scarce. Several authors have reported data on the lipids of hen egg yolk (1-3), and a report exists on the phospholipid composition at one stage of amphibian development (4). However several aspects of lipid biochemistry were studied during the development of invertebrate eggs, such as those of insects (5) and nematodes (6-8).

The aim of the present study was three-fold: (a) to examine the effect of cleavage from oocyte until late blastula stage on the fatty acid distribution in lipid fractions; (b) to study the level of total, neutral and polar lipids from unfertilized oocytes until neurula; and (c) to determine the phospholipid turnover during early embryogenesis by following the ^{32}P incorporation.

MATERIALS AND METHODS

Materials

All solvents used were analytical grade and were distillated before use. Chemicals and standards used were purchased as follows: thioglycolic acid from Merck; Unisil from Clarkson Chemical Co.; lipid standards and fatty acid methyl esters from Supelco, Inc., Applied Science Labs. and The Hormel Institute, University of Minnesota; 6% diethylene glycol succinate on diatoport S, from Hewlett Packard. Human chorionic gonadotropin was obtained from E.L.E.A., Argentina, and sterile radioactive orthophosphate ($Na_2H^{32}PO_4$, 69 Ci/g of P) from the Comisión Nacional de Energía Atómica, Argentina.

Oocytes and Embryos

Adult *Bufo arenarum*, Hensel toads captured in the surrroundings of the cities of Bahía Blanca and Tucumán were used. They were kept in a humidified container without feeding for 3-6 weeks prior to the experiments.

To induce ovulation adult females were injected in the dorsal lymphatic sacs with 1000-1500 IU chorionic gonadotropin or with a freshly prepared suspension of one or two toad pituitary glands in amphibian Ringer solution. The toads began to eliminate oocytes through the cloaca 14-18 hr later. At this point they were demedulated, and the abdominal cavity was quickly opened; from the ovisacs the oocytes were collected in a petri dish containing amphibian Ringer solution. Next the ova were artificially fertilized with a homogenate of

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[2]Reprint requests should be directed to N.G. Bazán.

toad testes. The medium employed for these and all subsequent procedures was amphibian Ringer solution (NaCl 0.65 g, KCl 0.01 g and $CaCl_2$ 0.003 g/liter). Part of the unfertilized ova and the different stages of development was sampled after removal of the jelly coat by brief periods of contact with neutralized 2% thioglycolic acid. Development was allowed to proceed at 20-25 C and was followed by means of a stereoscopic microscope microscope (50x), using as a reference the morphological characteristics described by Del Conte and Sirlin (9).

Preparation of Lipid Extract

Samples were homogenized with chloroform-methanol 2:1 v/v by means of a Potter-Elvehjem type homogenizer with a motor driven teflon pestle (10). In order to assure completeness in the lipid extraction, including tightly bound polyphosphoinositides, the remaining residue was reextracted three times with 4 volumes of 0.25% HCl in chloroform-methanol 2:1 v/v (11) and centrifuged. Acidified chloroform extraction was not applied in the experiments reported in Tables III and IV. Then the supernatant was filtered through glass wool, mixed with 0.2 volumes of 1 N HCl, and centrifuged. Afterwards the lower phase was combined with the lipid extract obtained by Folch's procedure. The extracts were stored under nitrogen at -20 C until processing.

Column Chromatography

The combined neutral and acidified chloroform-methanol extracts were taken to a small volume under a nitrogen stream and applied in 1-2 ml to a Unisil column (ID 2.5 cm, height 9 cm) from which three fractions were eluted: chloroform, 100 ml; acetone, 200 ml; and methanol, 200 ml (12). The flow rate was 3 ml/min. Each fraction was taken to dryness in a rotary vacuum evaporator and resuspended in a small volume. The behavior of the column was monitored by applying pure neutral and polar lipids, and then following their distribution in the eluted fractions by means of thin layer chromatography (TLC). A similar test was conducted with several embryo extracts, determining in addition the lipid phosphorus content in each fraction. The two dimensional TLC procedure of Rouser et al. (13) was employed for the separation of the phospholipids.

Methanolysis and Purification of Methyl Esters by TLC

The volume of the eluted fractions was reduced under a nitrogen stream at 45 C and then was placed in Teflon-lined screw cap tubes where evaporation to dryness was accomplished. Then methanolysis was carried out in a boiling water bath with 14% BF_3 in methanol (14,15) for 90, 30 and 90 min for the acetone eluate, neutral lipids and total phospholipids, respectively. After partitioning, the methyl esters of fatty acids contained in the lower phase were separated on a 0.1 mm thick layer of Silica Gel G using toluene as developing solvent. The spots corresponding to the fatty acid methyl esters were eluted with ether and hexane.

Gas Liquid Chromatography

A Varian Aerograph gas chromatograph, model 1700, equipped with two hydrogen flame ionization detectors was employed. For identification purposes, a polar (diethylene glycol succinate) and a nonpolar (OV-1) column were used. Identification was completed by the use of pure standards and catalytic hydrogenation. Most of the runs were carried out in a stainless steel column coated with 6% diethylene glycol succinate on Diatoport S, 80-100 mesh (ID 2.3 mm, length 2.20 m) with nitrogen as carrier gas (flow rate 20 ml/min) and at an oven temperature of 200 C (injector port 230 C and detector 210 C).

Experimental Design for in Vivo Incorporation of ^{32}P into Phospholipids

In vivo labeling of oocytes was accomplished by the procedure described elsewhere (17). In brief, 200 μCi of ^{32}P per 100 g of body weight were injected into the dorsal lymphatic sac along with the gonadotrophin or with pituitary gland extract in order to incorporate the radioisotope into the maturating oocytes during the action of the hormone. Thus oocytes with their phosphorylated molecules and their inorganic phosphate pool labeled with ^{32}P were obtained. After fertilization development was followed by maintaining the embryos in amphibian Ringer solution without the further addition of ^{32}P.

Determination of Specific Activity

Aliquots from a washed total lipid extract made up to a known volume were taken to dryness; one of them, usually one-tenth of the final volume, was placed on an aluminium planchet and the other in a test tube. Aliquots from the latter were digested with 70% perchloric acid, and phosphorus was determined colorimetrically (18). In all instances duplicates were run. Radioactivity was measured in the planchets using a model RM gas flow counter spectrometer (Alfanuclear, S.A.I.C., Argentina). The efficiency of the system was 73% for $Na_2H^{32}P$, and data was not corrected for efficiency.

TABLE I

Quantitative Fatty Acid Distribution in Lipid Fractions from Unfertilized Oocytes and from Late Blastula Stage[a]

Fatty acid	Oocytes						Late blastula stage		
	Neutral lipids		Acetone eluate		Phospholipids		Neutral lipids	Acetone eluate	Phospholipids
	A	B	A	B	A	B			
14:0	16.3 ± 1.4	7.8 ± 1.9	0.1 ± 0.1	0.1 ± 0.04	1.3 ± 0.1	0.9 ± 0.3	6.2 ± 1.9	0.04 ± 0.02	1.3 ± 0.4
15:0	7.9 ± 1.5	3.7 ± 0.7	0.1 ± 0.1	0.1 ± 0.04	1.2 ± 0.1	0.8 ± 0.2	2.5 ± 0.8	0.03 ± 0.01	1.2 ± 0.3
16:0	222.7 ± 9.0	114.3 ± 27.5	2.2 ± 1.6	5.2 ± 3.1	59.8 ± 2.0	49.6 ± 16.5	75.0 ± 12.2	1.61 ± 0.6	69.9 ± 24.8
16:1	221.5 ± 16.3	99.9 ± 25.9	0.8 ± 0.5	0.9 ± 0.5	17.1 ± 0.8	10.8 ± 6.6	74.6 ± 16.3	0.5 ± 0.3	10.2 ± 2.9
17:0	0	6.9 ± 1.8	0.1 ± 0.1	0.3 ± 0.2	2.4 ± 0.6	1.0 ± 0.1	6.1 ± 1.1	0.1 ± 0.04	1.7 ± 0.4
18:0	19.9 ± 1.9	23.5 ± 3.7	0.8 ± 0.2	5.5 ± 3.6	14.4 ± 0.5	12.4 ± 4.4	13.0 ± 1.7	2.1 ± 0.9	20.5 ± 6.3
18:1	434.8 ± 26.4	247.6 ± 75.7	1.3 ± 0.9	3.6 ± 2.8	102.5 ± 11.2	64.9 ± 38.5	160.4 ± 35.6	1.6 ± 0.8	17.1 ± 25.7
19:0	0	0	0.1 ± 0.03	0.5 ± 0.1	0	0.5 ± 0.02	0	0.4 ± 0.1	1.3 ± 0.01
18:2	36.8 ± 2.7	24.7 ± 6.1	0.03 ± 0.01	0.9 ± 0.01	9.2 ± 1.5	6.7 ± 4.9	14.4 ± 3.9	0.02 ± 0.01	4.7 ± 0.02
20:0	1.0 ± 0.1	1.0 ± 0.1	0.06 ± 0.02	1.1 ± 0.02	0.3 ± 0.1	0.2 ± 0.02	0.7 ± 0.3	0.04 ± 0.01	0.2 ± 0.01
18:3	8.0 ± 0.8	5.2 ± 0.3	0	0	1.1 ± 0.4	1.1 ± 0.6	3.5 ± 1.1	0.04 ± 0.01	1.1 ± 0.1
21:0	4.4 ± 0.3	2.4 ± 0.8	0	0.1 ± 0.04	0.8 ± 0.2	0.5 ± 0.2	1.7 ± 0.6	0.03 ± 0.00	0.8 ± 0.2
22:0	1.8 ± 0.4	1.1 ± 0.1	0.2 ± 0.05	0.3 ± 0.1	1.4 ± 0.5	1.8 ± 0.8	0.7 ± 0.4	0.2 ± 0.1	1.1 ± 0.2
20:4	9.7 ± 1.0	10.8 ± 3.3	0	0	9.6 ± 3.3	3.7 ± 2.8	4.3 ± 1.5	0	2.3 ± 1.1
23:0	0.7 ± 0.1	3.2 ± 0.3	0.04 ± 0.01	0.1 ± 0.1	0	0.8 ± 0.1	1.2 ± 0.6	0.1 ± 0.04	1.2 ± 0.3
20:5	2.7 ± 0.6	1.9 ± 0.1	0	0	2.7 ± 0.7	2.2 ± 0.5	2.0 ± 0.1	0.1 ± 0.01	0.6 ± 0.2
24:0	0	0	0.6 ± 0.5	0.4 ± 0.1	0.5 ± 0.1	0.9 ± 0.1	0	0.2 ± 0.1	0.8 ± 0.3
24:1	0	2.2 ± 0.7	0	0	0.8 ± 0.3	0.8 ± 0.5	1.9 ± 0.1	0	0
22:6	2.9 ± 1.2	3.0 ± 2.0	0.1 ± 0.01	0.2 ± 0.1	2.8 ± 1.2	2.2 ± 1.4	1.2 ± 0.3	0.3 ± 0.1	0.5 ± 0.2

[a]Figures represent micrograms fatty acid methyl ester per 10 mg cell dry weight and were obtained by gas liquid chromatography using nonadecanoate methyl ester as internal standard. A and B indicate samples taken from two different ovulations. A and B were composed of two and three samples containing 1000 oocytes each. Three blastulas samples containing 1000 embryos each were also analyzed. At least two injections were done of each sample.

TABLE II

Ratios of Acyl Content of Neutral Lipids to Phospholipids and of Saturated to Unsaturated Fatty Acids from Oocytes and Blastula[a]

Samples	Phospholipids[b]	Saturated to unsaturated fatty acids		
	Neutral lipid	Neutral lipids	Acetone eluate	Phospholipids
Oocytes				
A	0.22	0.37	1.86	0.56
B	0.29	0.41	2.35	0.75
Blastula	0.53	0.41	1.95	1.04

[a]Ratios were obtained by dividing total peak areas of saturated by unsaturated methyl esters of fatty acids. Further details as in Table I.

[b]These ratios represent total acyl content of phospholipids to total acyl content of neutral lipids obtained by gas liquid chromatography as described in Table II.

RESULTS

Lipid Fractions and Their Methyl Esters in Unfertilized Oocytes and Embryos

Purified lipid extracts of unfertilized oocytes and embryos were fractionated on silicic acid columns. The eluted fractions were analyzed by TLC and compared with pure reference compounds. In addition the total P content was determined in each fraction. Only the runs that yielded chloroform fractions devoid of P were used. The chloroform eluate was composed of neutral lipids and of pigment (unpublished observations), and the methanol eluate was composed of phospholipids, phosphatidyl choline and phosphatidyl ethanolamine being the major components. A detailed study on the composition of phospholipids during early development is being prepared for publication in this laboratory. The fraction eluted with acetone has an unknown composition. The P content per 1000 embryos in the acetone eluate was 2.89 ± 2.13 μmol and in the methanol eluate 36.93 ± 1.61 μmol, thus ca. 7% of the total lipid P was recovered in the acetone fraction.

Fatty Acid Distribution in Lipid Fractions of Unfertilized Oocytes and Late Blastula Stage

In the oocyte, palmitate, palmitoleate and oleate comprises ca. 80% of the fatty acids of neutral lipids and ca. 75% of phospholipids. A similar composition was found in the fatty acids derived from the acetone eluate with the exception of a component that behaves in the gas chromatograph as lignocerate. This componente represented between 3 and 8% of the total fatty acids in oocytes and blastula. The level of each acyl component was quantified by gas liquid chromatography using internal standards, and the data is presented as micrograms fatty acid per 10 mg dry weight (Table I). It is interesting that in oocytes the total unsaturation is slightly higher in polar lipids, while in the acetone eluate a predominance of saturated components can be seen (Table II). A similar distribution of total ethylenic fatty acids was observed at blastula stage. The acyl total

TABLE III

Level of Phospholipids, Neutral and Total Lipids in Developing Toad Embryos[a]

Stage	mg/1000 Oocytes or embryos		
	Total lipid	Phospholipids	Nonpolar lipids
Unfertilized oocytes	182	59	123
Fifth cleavage (16 cells)	184	60	124
Late blastula	173	56	117
First invagination of dorsal lip	174	57	117
Neural fold	172	57	115
Neural tube	181	59	122

[a]Three samples of 250 oocytes or embryos of each stage were used. Figures represent average values, and results agreed within 6% for determinations in different samples. Total lipids were determined by drying aliquots of washed chloroform-methanol extracts to constant weight at 110 C. Phospholipid levels were obtained from colorimetric measurements of lipid P (16) using a conversion factor of 25. Nonpolar lipid values were obtained by difference. Other details are given in Materials and Methods.

TABLE IV

Incorporation of ^{32}P and Specific Activity of Phospholipids during Early Stages of Toad Development[a]

Stage	Specific activity, cpm/μmol of P	Incorporation, cpm/1000 oocytes or embryos
Unfertilized oocytes	559	42,492
Fifth cleavage (16 cells)	729	56,168
Blastula	915	67,748
Late blastula	998	71,912
First invagination of dorsal lip	1075	75,580
Neural fold	2210	163,140
Neural tube	2405	182,848

[a]In vivo incorporation of ^{32}P was performed as described elsewhere (15) and in Materials and Methods. The samples were three of 250 oocytes or embryos as in Table III, with the exception of blastula that were two samples of 500 embryos each. The figures represent mean values of three determinations and in all cases they agreed within 7%. Other details as in Material and Methods.

content of neutral lipids slightly decreased from fertilization until blastula (Table II).

Lipids during Early Development and ^{32}P Incorporation into Total Phospholipids

No significant changes were detected in the level of total phospholipids and lipids from unfertilized oocytes until the stage of neural tube (Table III).

Phosphatidyl choline and phosphatidyl ethanolamine were the most abundant constituents of the phospholipids in unfertilized oocyte and throughout the early stages of development until the surveyed stage of dorsal lip. Phosphatidyl choline and phosphatidyl ethanolamine comprise 44.9 ± 5.6 and 18.0 ± 1.4% of the total phospholipids, respectively, and their level is not modified during the studied period of embryogenesis. The average level from oocyte to neural tube was 57.7 ± 1.3 mg phospholipid per 1000 oocytes or embryos. A small decrease was observed in neutral lipids at the stage of blastula, dorsal lip and neural fold (Table III). Until the neural tube stage, ca. 67% of the total lipids was nonpolar.

^{32}P injected into the dorsal lymphatic sac along with the hormone to induce ovulation was used to label the phospholipids. In Table IV data on the incorporation of ^{32}P and specific activity from oocyte to the stage of neural tube are shown. A steady rise in incorporation can be seen. Since the total phospholipid content remains constant, there is an increase in specific activity. The experimental design utilized by us for in vivo radiophosphorus labeling of the cells enabled assessment of phospholipid turnover.

Since it is known that some tightly bound lipids, mainly polyphosphoinositides (11), remain in tissue residues after the Folch extraction, a further experiment (Table V) was devised to assure the completeness of the phospholipid extraction. Most of the lipid P was found to be recovered in the neutral chloroform-methanol extracts of unfertilized oocytes, blastula, dorsal lip and tail bud. Only a small amount of lipid P was found when extracts were made with acidified solvents, representing ca. 2% of the total lipid P until the stage of first invagination of dorsal lip. The most striking finding about the acidified chloroform-methanol extract is the high specific activity of the incorporated ^{32}P. Fertilization promotes an increase in the specific activity in this fraction, while during the early development changes were not seen (Table V). The lipids that comprise the fraction extracted by acidified chloroform-methanol (Table V) are not well known as yet.

The results presented in Tables IV and V show different incorporation of ^{32}P into the phospholipids extracted with neutral solvents; this is mainly due to differences in the amounts of ^{32}P injected in the two sets of experiments (Table V: 200 μCi and 400 μCi/100 g). However, in spite of the large differences in incorporation, the rise in specific activity was similar in both experiments. The per cent increases in specific activities, as compared to unfertilized oocytes found at the blastula stages, were 50 and 65% and at the first invagination of dorsal lip, 87 and 94%.

DISCUSSION

The present work provides evidence that the total lipid and phospholipid level remains unchanged from unfertilized oocyte through the early stages of amphibian embryogenesis until neurula formation. These findings are surprising, because at that stage a very large number of cells comprises the embryo. In fact, late neurulas of other amphibians, *Rana pipiens* and *Xenopus laevis* contain 172,000 and 180,000

TABLE V

Distribution of Total Phospholipids and of Radioactivity in Lipid Extracts Made Sequentially with Neutral and Acidified Chloroform-Methanol from Developing Embryos[a]

Stage	Sample no.	Lipid extraction					
		Neutral chloroform-methanol			Acidified chloroform-methanol		
		μmol/1000 units	CPM/1000 units	CPM/μmol of P	μmol/1000 units	CPM/1000 units	CPM/μmol of P
Oocyte	1	70	9920	141	1.55	1290	832
	2	74	9880	133	1.58	1934	1224
Blastula	1	69	14,880	215	1.49	3030	2033
	2	75	14,980	199	1.39	2392	1720
First Invagination of dorsal lip	1	84	20,480	243	1.45	2992	2063
	2	74	20,560	277	1.39	2712	1951
Tail bud	1	72	51,500	715	1.10	1832	1665
	2	79	48,100	608	0.97	2166	2232

[a]In vivo incorporation of ^{32}P was accomplished by injecting 41 μCi of ^{32}P/100 g of toad body weight. Each sample contained 500 eggs or embryos. Lipid extraction with neutral solvents was performed following the procedure of Folch et al. (10), and extraction with acidified solvents was carried out as described by Dawson and Eichberg (11). Units mean number of oocytes or embryos.

cells (19). Consequently a great amount of cell membranes must be formed and a large requirement of phospholipids must be met. In addition our data show that cell cleavage up to the blastula stage, 2500 to 3100 cells (19), does not alter the fatty acid composition in polar and neutral lipids. These findings are of interest, because the pathways involved in membrane biosynthesis during early embryogenesis are not known. Moreover many important functions are related to membranes, such as control of growth (20), initiation of DNA biosynthesis and cell division (21). Although little is known of the biochemistry of cell membranes during early embryogenesis, important processes are related to it. In the sea urchin egg, ultrastructural studies have shown that the surface coat is complex and that fertilization causes very rapid and extensive changes. These events are the splitting of the surface membrane into the vitelline and plasma membrane, promoting the release of cortical granules and leaving a highly convoluted plasma membrane coated by a mucoprotein-like hyaline layer. When cleavage starts, tubular formation and a dense layer develop beneath the outer cell membrane (22). Moreover, at early cleavage stages in a wide variety of embryos, begins the development of tight junctions that lead to electrical coupling between cells (23). Trelstad et al. (24) have proposed that these specialized cell contacts may participate in cell adhesion and also in cell movements. Close membrane appositions constitute areas of low electrical resistence, allowing relatively free movement of ions (23). Also, these electrical pathways may be involved in the intercellular transmission of substances related to the coordination of growth and differentiation. Besides, membranes form transitory syncytia allowing cytoplasmic connections between blastomeres of early developing embryos (25).

It is evident from all the functions of membranes during early embryogenesis that the events underlying their biosynthesis may be very precisely regulated. The polar lipids required for membrane biogenesis may evolve through one of the following paths. First, yolk platelets of amphibians are known to store molecules to be used during early development. In *Rana pipiens* 14% of yolk platelet dry weight is lipids (26). Phospholipid biosynthesis may take place concurrently with yolk platelet formation. Consequently most polar lipids of the unfertilized oocyte may be compartmentalized in the yolk platelet. Fertilization may trigger a transport mechanism for carrying the phospholipids from the storage location, yolk platelet, to corresponding membrane sites. These processes may be efficient and under genetic control. Second, another possible mechanism may be that yolk platelet, as well as other organelles, contributes to maintain lipid precursors for the biosynthesis of phospholipids. Results presented herein support the former path, because the structure and level of phospholipids do not change during cell cleavage. Furthermore the phospholipid classes, as well as the molecular species of their major classes—phosphatidyl choline and phosphatidyl ethanolamine—during early cleavage are also unchanged (unpublished observations); although the presented data shows a steady increase in specific radioactivity when ^{32}P is injected along with the hormone to induce ovulation. At late blastula stage an 80% rise of specific radioactivity was observed, while at neural fold and neural tube stages the increment amounted to 336%.

In other systems where increased cell division occurs, phospholipids vary rapidly, such as in cultured 3T3 mouse cells (27), regenerating rat liver (28,29) and rat hepatoma cells grown in suspension culture (30,31), and in neoplastic mast cells (32).

The present results show that during the early embryogenesis of the toad an increased incorporation of ^{32}P into phospholipids takes place. This may reflect metabolic changes associated with the activity of the newly formed membranes or rapid exchange reactions of the polar moiety or both. Rapid exchange reactions may be taking place upon the arrival of the phospholipid carrier complex to the membrane vicinity in order to unload the polar molecule or to make the appropriate assemblage of the phospholipid into the membrane. These processes may generate a transitory diacylglycerol and may depend on the presence of an active phosphoryl choline and phosphory ethanolamine pool. The increased specific radioactivity in phospholipids may also reflect, in part, breakdown and synthesis. If such metabolic changes also contribute to the rise in incorporation they may be very precisely regulated, because the resulting level and composition of the phospholipids do not change. In so far as the origin of the membrane protein is concerned, our hyphothesis does not exclude the possibility that the carrier phospholipid complex is comprised of membrane subunits. Current work in our laboratory is testing the proposed ideas.

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Intestinal Versus Hepatic Contribution to Circulating Triglyceride Levels

RICHARD J. CENEDELLA, Department of Pharmacology, **WILLIAM G. CROUTHAMEL,** School of Pharmacy, and **HENRY F. MENGOLI,** Department of Pathology, West Virginia University Medical Center, Morgantown, West Virginia 26506

ABSTRACT

Using intravenous injected [2-^{3}H] glycerol, measurements were made of the kinetics of appearance and disappearance of circulating [^{3}H] labeled triglyceride in rats fed a fat free diet containing either no orotic acid (controls) or 2% orotic acid. Following injection of [^{3}H] glycerol, more time is required for the initial appearance of [^{3}H] triglyceride in the circulation of orotic acid treated rats than controls. The sustained entry of triglyceride into the circulation of orotic acid fed rats was only one-half times as rapid as that seen for control rats. Ca. 10% as much [^{3}H] triglyceride entered the circulation of the orotic acid treated rats as compared with controls. However, the clearance of [^{3}H] triglyceride from the circulation of the orotic acid fed rat was only ca. one-half times as rapid as that of the control rat. This apparently is due to differences between the lipoproteins produced by the intestines and liver, rather than to changes in the ability of the orotic acid fed animal to clear lipoprotein-triglyceride from the circulation. Labeled lipoproteins taken from controls and injected into orotic acid treated rats were cleared from the circulation more than twice as rapidly as those taken from orotic acid fed rats and injected into controls. Considering the measured levels of plasma triglyceride synthesis and the slower turnover of the triglycerides produced by the orotic acid fed rat, the findings of this study indicate that the intestines supply 20% or more of the total plasma triglyceride in the absence of dietary fat.

INTRODUCTION

Recent studies employing electron microscopic techniques clearly establish that the intestines of both rat (1,2) and man (2,3) produce triglyceride-rich lipoprotein particles in addition to chylomicrons. Intestinal lipoproteins, together with those produced by the liver, are considered to be the only sources of plasma triglycerides. However, the relative importance of the intestines, as compared to the liver, as a source of plasma triglyceride in the post-absorptive state is unclear. The intestines have been estimated to account for 10-40% (4,5) of the plasma triglyceride in the fasted rat. The present studies are an attempt to resolve this important discrepancy. Using the approach of Nikkila and Kekki (6) for studying plasma triglyceride kinetics in man, the kinetics of circulating triglyceride formation and turnover were investigated in control rats and in rats treated with orotic acid. The orotic acid fed rat provides a model for evaluating the intestinal contribution to plasma triglyceride levels.

MATERIALS AND METHODS

Animals

Sprague-Dawley male rats (300 g) were maintained on a fat free diet containing either 0% or 2% orotic acid for a minimum of 7 days (4). Earlier studies demonstrated that orotic acid in the diet of the rat completely prevents hepatic release of triglyceride to the circulation (7-9). The rats were permitted free access to the diet until ca. 1 hr prior to experimental use. The rats were anesthetized with chloralose (55 mg/kg, intraperitoneal), and the right external jugular vein and the left femoral vein were cannulated with PE-50 tubing. Chloralose was used mainly to minimize anesthetic interference with gastrointestinal physiology. A plasma sample, collected prior to injection of [^{3}H] glycerol, was saved for measurement of triglyceride content. Triglycerides were measured, as described before (10).

There were no obvious differences in total food consumption by the two groups of rats. This is reinforced by the virtual identical body wt of the control (296.8 ± 11.7 g) and the orotic acid fed rats (292.3 ± 5.9 g) at the time of experimental use. All rats were of essentially the same body wt when placed on the experimental diets.

Measurement of Serum Triglyceride Formation and Turnover

Serum triglyceride formation and turnover were measured in control and orotic acid fed rats basically as described by Nikkila and Kekki for the human (6). Rats were injected via the femoral vein with 75 μCi [2-^{3}H] glycerol (specific activity 200 mCi/mM, New England Nu-

clear Corp., Boston, Mass.) in sterile saline (0.35-0.45 ml)/kg body wt. At times from 10-210 min after injection, samples of whole blood (0.3-0.4 ml) were collected from the jugular vein, immediately taken-up into microhematocrit tubes (no anticoagulant), and the serum separated by centrifugation. The tubes were broken at the serum-cell interface and the serum transferred to preweighed screw-capped centrifuge tubes (12 ml) and subsequently re-weighed. Serum volume was taken as serum wt divided by 1.022. Serum triglycerides were extracted into chloroform-methanol (2:1) and recovered according to Nikkila and Kekki (6). Phospholipids were removed from the resulting chloroform extracts with silicic acid; the chloroform was evaporated and the lipid residue dissolved in a standard toluene counting solution and counted for a minimum of 100 min in a Packard Tri-Carb scintillation counter. Background counts averaged 26.1 ± 0.5 cpm. Counting efficiency was determined by addition of a known number of dpm of [^{3}H] toluene and was generally found to be 36%. The maximum counting error encountered was with 20 min samples from the orotic acid treated rats and was found to be only 4.2%. Specifically, the average 20 min sample cpm (uncorrected for efficiency and serum sample size) was 45.7 with a standard deviation of 1.9 cpm. Thus, although the kinetic analyses conducted with the data from the orotic acid fed rats were in part based upon low radioactivity values, these values were accurate measurements. Counting errors were calculated (11). The radioactivity content of the serum then was expressed as the dpm of [^{3}H] triglyceride/ml serum at each of the times investigated. Thin layer chromatography (12) of aliquots of the silicic acid-treated chloroform extracts revealed that 98% [^{3}H] radioactivity was in triglycerides; none was recovered with phospholipids. Since all rats were fed a fat free diet, the measurements of triglyceride synthesis represent formation of endogenous serum triglycerides.

The rats appeared to tolerate the repeated blood samplings well. At 3 hr after injection of the labeled glycerol, the hematocrits fell by only 10-15% from that determined at the start of the experiment.

Measurements of Plasma Clearance of Reciprocally Transfused Serum Lipoproteins

The clearance of lipoprotein-triglyceride synthesized in control rats from the circulation of orotic acid fed rats, and the reverse was investigated by reciprocal transfusion of isolated [^{3}H] triglyceride labeled-lipoproteins. Serum (3-4 ml) recovered at 40-50 min after injection of 100 μCi [^{3}H] glycerol/kg body wt into control and orotic acid fed rats was adjusted to density 1.21 with a KCL-NaCl salt solution and the lipoproteins separated by ultracentrifugation at 100,500 x g for 22 hr in a 40.0 rotor with a Beckman Model L ultracentrifuge (13). The supernatant layer (upper 1 ml tube) containing the lipoproteins (d $<$1.21) was recovered, and the salt was removed by dialysis overnight at 10 C against 2 liters 0.8% saline. Thin layer chromatography of aliquots of extracted lipoprotein lipid showed that ca. 90% or more [^{3}H] radioactivity was present in triglyceride. One ml dialyzed lipoprotein solution from control rats (ca. 60,000 dpm [^{3}H] triglyceride) was injected into orotic acid fed rats and 1 ml from orotic acid fed rats (ca. 4000 dpm [^{3}H] triglyceride) into controls. As before, the animals were anesthetized with chloralose. Lipoproteins were injected into the femoral vein and blood samples (ca. 1 ml) were collected from the jugular vein 5-60 min after injection. The blood samples were added to 0.1 ml citrate anticoagulant, a microhematocrit was done, and the plasma separated by centrifugation. Plasma triglycerides were extracted, recovered, and the [^{3}H] content determined, as described above, for serum triglycerides. In all cases the proper correction was made for the dilution of the plasma by the citrate anticoagulant. Counting errors averaged ca. 6% or less.

Ultracentrifugational Separation of Serum Lipoproteins

In several experiments the distribution of [^{3}H] triglyceride activity among specific serum lipoprotein fractions (d $<$1.006, 1.006-1.019, 1.019-1.063, 1.063-1.21, and d $>$1.21) from control and orotic acid fed rats was determined. At 40-50 min after injection of [^{3}H] glycerol, the rats were exsanguinated, the blood allowed to clot and the serum recovered by centrifugation. The lipoprotein from 3-4 ml serum were separated by ultracentrifugation and recovered, as described by Havel and Fredrickson (14). With each lipoprotein fraction the triglycerides were extracted and assayed for [^{3}H] content using the methods already described.

Electrophoretic Separation of Serum Lipoproteins

Samples of whole serum recovered 40-50 min after injection of [^{3}H] glycerol into control and orotic acid fed rats were subjected to electrophoresis. Separation of serum lipoproteins was performed using vertical agarose gel electrophoresis. Electrophoretic conditions were as follows: 0.8% agarose (Seakem) was prepared in triethylamine-acetic acid buffer (TEA), pH-9.2 (15), and cast as a 6 mm thick

gel in a vertical slab electrophoresis cell (16); the upper and lower buffer chambers were filled with TEA buffer. Rat sera were added in 200 μl aliquots. Reference samples (2 volumes) were prestained with saturated lipid crimson in diethylene glycol (1 volume). Electrophoresis was performed without buffer recirculation at 10 C and 200 volts for 3 hr. Duration of electrophoresis was dependent upon achieving optimal lipoprotein separation. The migration of the stained lipoproteins was used to locate the [^{3}H] labeled lipoproteins in the unstained samples. The agarose zones containing the various lipoprotein fractions (chylomicrons at origin, then beta, and pre-beta) were recovered from the slab, minced, and added to 20 ml chloroform-methanol (2:1)/g agarose. The mixture was extracted overnight at room temperature with shaking. Total lipids were recovered, the phospholipids removed with silicic acid, and the triglycerides assayed for [^{3}H] content. The percentage of distribution of radioactivity among the lipoprotein fractions then was calculated. The electrophoresis was conducted primarily to determine the radioactivity content of chylomicrons separated from other lipoproteins.

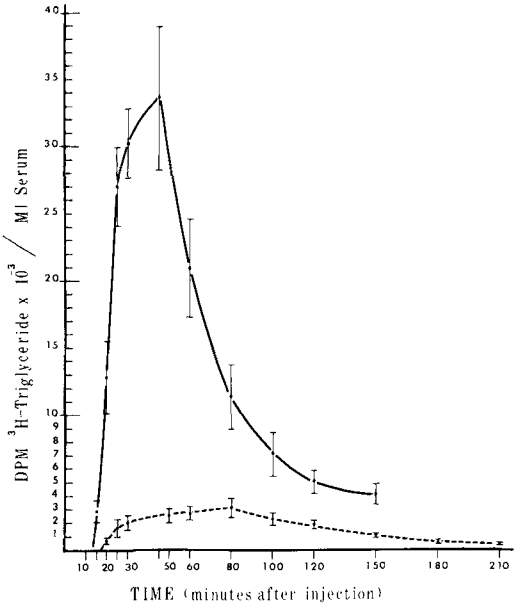

FIG. 1. Serum triglyceride radioactivity curves after intravenous injection of 75 μCi [2-^{3}H] glycerol/kg body wt at time zero. Data points are the average values for seven control rats and eight orotic acid treated rats. Bars are one standard error. ——— = Control rats and ----- = Orotic acid treated rats.

Kinetic Analyses

Serum triglyceride data for individual animals were analyzed by the method of residuals for the rate constant for appearance, (K_a), the rate constant for net disappearance (fractional turnover rate) (K_{net}), and the lag time and estimated total synthesis (DPM_{max}) (17). Straight lines were fitted to the data points by the method of least squares.

The kinetic model used in the analysis of this data is the one pool open model, which is described by Solomon (17), Portmann (18), and Shipley and Clark (19). This method assumes a single pool open model with apparent first order entry and elimination. The assumption of apparent first order kinetics was supported by linearity over several half-lives of the entry and clearance rates in individual animals (Figs. 2 and 3). The general equation describing the [^{3}H] triglyceride serum levels using this model is:

$$C_S = A\,(e^{-K_{net} \times T} - e^{-Ka \times T})$$

where C_s is the serum concentration of [^{3}H] triglyceride in DPM/ml, A is a constant related to the extrapolated concentration at the lag time (see below), K_{net} is the rate constant for net disappearance of [^{3}H] triglyceride from the circulation, K_a is the rate constant for appearance of [^{3}H] triglyceride in the circulation, and T is time. Since the serum [^{3}H] triglyceride concentration is dependent upon the difference between these two exponentials, this model predicts that the slope of the plot of the logarithms of [^{3}H] triglyceride serum concentration vs. time will be both positive and negative, but at different times. Initially the slope will be positive and the serum levels increasing, due to the large exponent containing the appearance rate constant. At later times the contribution of this exponent becomes insignificant, and the exponent containing the elimination rate constant predominates, resulting in a decreasing terminal tail.

The lag time is the time between injection of the [^{3}H] glycerol and the appearance of [^{3}H] triglyceride in the serum. Lag time can be estimated either by a visual inspection of the data or by a kinetic method. Although both methods may give ca. the same results, the visual method suffers from several deficiencies, namely that it involves the extrapolation of a nonlinear line to zero concentration, it is related to the sensitivity of the assay, it requires numerous samples be collected near the estimated lag time for accurate determination, and it places undue pressure on a single data point. The kinetic method minimizes these problems by determining the estimated lag time from a series of linear points well above the assay

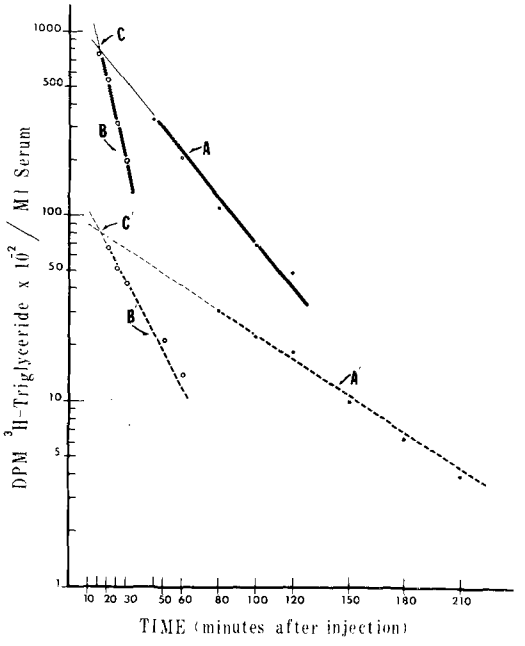

FIG. 2. Semilogarithmic plot of serum triglyceride radioactivity of control (average seven) rats and orotic acid treated (average eight) rats injected intravenously with 75 μCi [2-^{3}H] glycerol/kg body wt at time zero. • = mean data points, ○ = calculated by the method of residuals. The residual calculation involves subtraction of the rising portion of the curve (data points in Fig. 1) from the extrapolated portion of the down curve (lines A and A′) to yield lines B and B′. For example, the 30 min point (bottom point) on line B (control) is 19,985 dpm/ml. This was determined by subtracting 30,251 dpm/ml (30 min data point shown in Fig. 1) from 50,200 dpm/ml (extrapolated value from line A at 30 min). K_{net} and $T_{1/2net}$, the rate constant and half-life values for clearance of [^{3}H] triglyceride from the circulation, are calculated from the disappearance curves A and A′. K_a and $T_{1/2a}$, the rate constant and half-life value for entry of [^{3}H] triglyceride into the circulation, are calculated from the derived entry curves B and B′. Lag T and DPM_{max} were estimated from the intersection (C and C′) of the extrapolated elimination curves (A and A′) and the calculated entry curves (B and B′). Lag T is the time between injection of [^{3}H] glycerol and appearance of [^{3}H] triglyceride in the serum. DPM_{max}, dpm of [^{3}H] triglyceride/ml of serum, is a measure of total [^{3}H] triglyceride synthesis. The results of these measurements are presented in Table I.——— = Control rats and --- = Orotic acid treated rats.

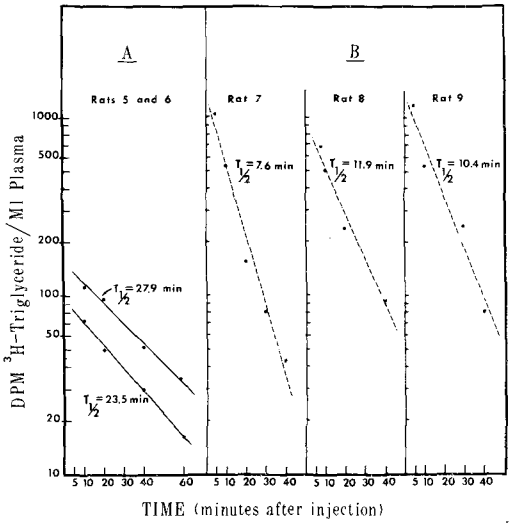

FIG. 3. Plasma clearance of reciprocally transfused [^{3}H] triglyceride labeled serum lipoproteins. A Control rats injected intravenously with [^{3}H] labeled serum lipoproteins (d <1.21) recovered from orotic acid treated rats given [^{3}H] glycerol. B Orotic acid treated rats injected intravenously with [^{3}H] labeled serum lipoproteins (d <1.21) recovered from control rats given [^{3}H] glycerol.

sensitivity point. An examination of the data in Figure 1 indicates that the results obtained by visual estimation agree with those calculated by the kinetic method. A detailed description of this method is presented by Portmann (18) and Shipley and Clark (19).

RESULTS

Synthesis of Serum Triglycerides from [^{3}H] Glycerol

Treatment of the rat with orotic acid resulted in pronounced quantitative and qualitative changes in the synthesis and metabolism of serum triglycerides. Inspection of Figure 1 clearly shows that formation of serum [^{3}H] triglyceride from intravenously-injected [^{3}H] glycerol was reduced greatly in the orotic acid treated rat. The observed maximum serum concentration of [^{3}H] triglyceride was an average 33,680 ± 5470 (standard error) dpm/ml for control rats (n=7) and only 3100 ± 700 dpm/ml for orotic acid fed rats (n=8). These concentrations were reached at ca. 45 min after injection for controls and at ca. 80 min after injection for orotic acid treated rats. Both the entry of labeled triglyceride into and clearance from the circulation was slow in orotic acid fed rats relative to that in controls (Fig. 1). A more quantitative description of these relationships is presented in Figure 2, a semilogarithmic plot of the data, and in Table I.

The time course for entry of labeled triglyceride into the circulation is a function of the rate constant for entry (K_a) and the lag time (lag T). Lag time, the time between injection of [^{3}H] glycerol and the appearance of [^{3}H] triglyceride in the serum, averaged 14.7 min in the control rats and ca. 3 min longer in orotic acid fed rats (Table I). This difference is statistically significant at ca. 0.05% level. Labeled triglyceride entered the circulation of control rats twice as rapidly as for the orotic

TABLE I

Comparison of Serum Triglyceride Formation in Control and Orotic Acid Treated Rats

Parameter	Controls[a] Average (7) rats	Orotic acid treated[a] Average (8) rats	P(t) Student's *t* test
Lag T[b]	14.7 ± 0.9 min	17.6 ± 1.0 min	= 0.05
K_a[c]	0.0907 ± 0.0114 min^{-1}	0.0479 ± 0.0063 min^{-1}	<0.01
$T_{1/2a}$[c]	7.64 min	14.47 min	<0.01
K_{net}[d]	0.0292 ± 0.0025 min^{-1}	0.0150 ± 0.0014 min^{-1}	<0.001
$T_{1/2\ net}$[d]	23.73 min	46.20 min	<0.001
DPM_{max}[e]	76,640 ± 12,800 dpm/ml	7,540 ± 2,060 dpm/ml	<0.001

[a]Rats maintained on a fat free diet containing no orotic acid (controls) or 2% orotic acid for a minimum of 7 days were injected intravenously with [2-^{3}H] glycerol at 75 μCi/kg body wt. Plasma triglyceride radioactivity content (dpm/ml serum) was determined at times from 10-210 min after injection. The values presented are means ± one standard error or harmonic means ($T_{1/2a}$ and $T_{1/2net}$) of the values determined for individual animals.

[b]Lag T = time between injection of the [^{3}H] glycerol and appearance of [^{3}H] labeled triglyceride in the circulation.

[c]K_a and $T_{1/2a}$ = rate constant and half-life values, respectively, for the entry of [^{3}H] triglyceride in the circulation.

[d]K_{net} and $T_{1/2net}$ = rate constant and half-life values, respectively for the disappearance of [^{3}H] triglyceride from the circulation.

[e]DPM_{max} = calculated maximum dpm of [^{3}H] triglyceride/ml serum. This was determined by extrapolation of the elimination curve to the lag time (lag T).

acid fed animals (Fig. 2 and Table I). This difference is also statistically significant.

The clearance of [^{3}H] triglyceride from the circulation is described by K_{net} or $T_{1/2\ net}$. The half-life for serum clearance of labeled triglyceride was ca. 24 min in control animals and 46 min for orotic acid fed rats (Fig. 2 and Table I), a highly significant difference. This difference could be due to either changes in the ability of the orotic acid treated rat to clear lipoprotein triglyceride from the circulation or to differences in the nature of the lipoproteins manufactured by the control and orotic acid fed rats. These possibilities are explored in experiments reported below.

The quantitative measure of total triglyceride synthesis is described by DPM_{max} (maximum dpm of [^{3}H] triglyceride/ml of serum). DPM_{max} is calculated for each animal by extrapolating the disappearance curve back to the time of initial entry of [^{3}H] triglyceride into the circulation, i.e., back to lag T. This value represents the maximum dpm of [^{3}H] triglyceride/ml serum which would have been attained if the removal rate (K_{net}) had been zero. DPM_{max} is representative of total serum [^{3}H] triglyceride synthesis, since the size and thus the serum volume of control and orotic acid fed rats were essentially identical. Treatment with orotic acid reduced [^{3}H] triglyceride synthesis from intravenous injected [^{3}H] glycerol to 10% of that measured for the control rat (Table I).

Clearance of Reciprocally Transfused [^{3}H] Triglyceride Labeled Lipoproteins

When the [^{3}H] triglyceride labeled lipoproteins synthesized in orotic acid fed rats were injected into control rats, [^{3}H] triglyceride disappeared from the circulation with an average half-life of ca. 26 min. (Fig. 3). In the opposite experiment, i.e., labeled lipoproteins from controls injected into orotic acid treated, the [^{3}H] triglyceride disappeared with an average half-life of only 10 min (Fig. 3). Thus, as seen before, the lipoproteins of orotic acid treated rats were cleared from the circulation much slower than those produced in the untreated controls.

In general the [^{3}H] lipoproteins from both control and orotic acid treated rats seemed to be cleared from the circulation more rapidly when transfused than when cleared endogenously. Whether the handling of the lipoproteins could have produced these changes is unclear. Presumably any changes in [^{3}H] labeled lipoproteins as a consequence of the isolation procedures would have been proportionately similar for those from control and treated animals.

Identification of [^{3}H] Triglyceride Labeled Lipoproteins

Ultracentrifugational and electrophoretic

TABLE II

Distribution of [^{3}H] Triglyceride Among Various Serum Lipoprotein Fractions from Control and Orotic Acid Treated Rats

Lipoprotein fraction (density)	Percentage distribution[a]					
	Controls			Orotic acid fed		
	Rat 1	Rat 2	Average	Rat 3	Rat 4	Average
<1.006 (Chylomicron)	0.3	0.7	0.5	15.8	10.7	13.2
<1.006 (Nonchylomicron)	78.5	92.6	85.6	63.2	55.6	59.4
1.006-1.019	17.8	4.8	11.3	14.8	25.4	20.1
1.019-1.063	1.6	1.2	1.4	2.8	5.7	4.3
1.063-1.21	1.0	0.5	0.7	2.0	2.1	2.0
>1.21	0.8	0.2	0.5	1.4	0.5	1.0

[a]Samples of serum recovered 40-50 min after intravenous injection of [^{3}H] glycerol into control and orotic acid fed rats were subjected to ultracentrifugation and electrophoresis. The [^{3}H] triglyceride content of the various lipoprotein separated fractions was determined and the percentage distribution calculated. The chylomicron relative content of [^{3}H] triglyceride was determined on chylomicrons separated from other lipoproteins by electrophoresis in agarose.

separation of the labeled lipoproteins synthesized by the orotic acid fed and control rats revealed some interesting differences between the distribution of [^{3}H] label among various lipoprotein fractions from these two sources. Lipoproteins of density <1.006, excluding chylomicrons, contained 80-90% of the [^{3}H] triglyceride in control rats but contained only ca. 60% total [^{3}H] lipoprotein radioactivity in orotic acid fed animals (Table II). Chylomicrons, separated by electrophoresis, from control rats contained only trace amounts of radioactivity but constituted ca. 10-15% total [^{3}H] labeled lipoproteins in the orotic acid fed animals. The higher density lipoproteins from the treated rats also contained relatively more radioactivity than those from the controls. Lipoproteins of density 1.019-1.21 contained ca. 6% total [^{3}H] triglyceride in the orotic acid rats but only ca. 2.5% in the controls. In treated rats ca. 20% of the total [^{3}H] radioactivity was recovered with lipoproteins of density 1.006-1.019 vs. an average of ca. 11% total for controls.

Plasma Triglyceride Levels in Control and Orotic Acid Treated Rats

Lastly the plasma of rats treated with orotic acid at 2% of diet for 7-9 days contained an average of 40.8 ± 4.4 (standard error) mg % triglyceride (n=17), as compared with an average plasma value of 120.2 ± 18.2 mg % for rats (n=14) maintained solely on the basic fat free diet.

DISCUSSION

The studies of Windmueller (7), Windmueller and Spaeth (8), and Windmueller and Levy (9) clearly demonstrate that adding orotic acid to the fat free diet of the rat completely blocks the production of beta-lipoproteins by the liver and the release of triglyceride into the circulation. After 7 or more days on a fat free diet, including 2% orotic acid, the livers of the rats used for the present studies were exceedingly fatty, appearing creamy white in color. Since the liver and intestines are taken as the only source of plasma triglycerides, the triglyceride present in the circulation of the orotic acid treated animals are considered to be derived from the intestines. This idea is further supported below. Based upon the quantitative measurements of [^{3}H] glycerol incorporation into plasma triglyceride, the plasma triglycerides of control animals are primarily of hepatic origin.

Tritium-labeled glycerol, rather than labeled free fatty acid (FFA), was chosen for these studies for basically two reasons. First plasma recycling of glycerol relative to FFA is minimal (20) and second only a small fraction of the fatty acid required for formation of lymph triglyceride in the rat appears to be derived from circulating FFA (21). It is recognized that plasma glycerol may not be totally descriptive of the glycerol pool utilized by the gut for triglyceride synthesis and thus the quantitative measurements presented for intestinal contribution to circulating triglycerides could be low.

Several interesting differences were noted between the parameters of triglyceride metabolism measured in control and orotic acid fed rats. First several additional min were required for [^{3}H] triglyceride to appear in the circula-

tion after injection of [^{3}H] glycerol into the treated rats, as compared with controls. This observation is compatible with the idea that triglycerides released from the liver gain almost immediate access to the circulation but those released from the intestines must course through the lymphatics to gain access to the systemic circulation at the thoracic duct. The average 14.7 min lag time measured for entry of [^{3}H] labeled triglyceride into the circulation of control rats is similar to that observed by Laurell (22) for plasma appearance of [^{14}C] labeled triglycerides after intravenous injection of 1-[^{14}C] palmitic acid into the rat. A second difference is that the sustained entry of triglyceride into the circulation of the orotic acid fed rat was slow relative to that in the control. The half-life for entry of [^{3}H] triglyceride into the circulation of control rats was ca. 7.6 min but 14.5 min for orotic acid treated animals. The source of the circulating lipoproteins in the orotic acid fed rat is, of course, a point of central importance to this work. The differences just cited between control and treated rats support the idea that the triglycerides entering the circulation of the orotic acid fed rat largely represent intestinal triglycerides rather than, for example, a residual triglyceride lipoprotein secretion by the liver. Although one might argue that the serum high density lipoproteins could be of hepatic origin (orotic acid apparently only partially blocks hepatic secretion of high density lipoproteins), the great bulk of the labeled serum triglycerides circulating in the orotic acid fed rat must have originated in the intestines, since they circulate as very low density lipoproteins. There is little doubt that orotic acid completely blocks hepatic secretion of very low density lipoprotein triglyceride (7-9).

The [^{3}H] triglyceride synthesized in the control fed rats was cleared from the circulation ca. twice as rapidly as that formed in the orotic acid fed animals. This difference probably is not due to an impaired ability of the orotic acid treated rat to clear lipoproteins from the circulation, since [^{3}H] triglyceride labeled lipoproteins taken from control rats and injected into orotic acid fed rats were cleared from the blood more than twice as rapidly as labeled lipoproteins taken from orotic acid fed rats and injected into control animals. Rather, these observations suggest that lipoprotein-triglyceride manufactured by the intestines is removed from the circulation more slowly than that produced by the liver. Theoretically, differences in the relative composition of the circulating lipoproteins formed by orotic acid treated and control rats could account for this situation. Lipoproteins of different sizes are cleared from the circulation at different rates (23,24). However, since most of the [^{3}H] triglyceride formed by both the orotic acid treated and control rats was present in lipoproteins of density less than 1.006, it is unlikely that such changes accounted for the pronounced differences in triglyceride turnover between the two groups.

Orotic acid treated rats formed only 10% as much circulating [^{3}H] triglyceride from intravenous injected [^{3}H] glycerol as controls. Standing alone, this observation would support the results presented by Windmueller and Levy (4) and by Roheim, et al., (25) which indicate that the intestines furnish a maximum of only 5-10% of the circulating triglyceride. Windmueller and Levy's conclusion was based, in part, upon measurements of lymph triglyceride output in control and orotic acid fed rats. Roheim, et al., showed with the dog that hepatectomy reduced labeling of serum lipoproteins from intravenously injected [^{14}C] lysine by 90-95% (25). If one now considers that the circulating lipoproteins produced by the orotic acid fed rat (largely the intestines) appear to turnover much less rapidly than those from the controls (mainly the liver), the conclusion changes. Synthesis of 10% of the new lipoprotein-triglyceride molecules which enter the circulation but which turnover one-half or less times as rapidly as the other 90% means that the intestines could maintain at least 20% of the plasma triglyceride level if the liver was removed. Supporting this view, the plasma triglyceride levels of the orotic acid treated animals in the current studies fell to only 30-35% of that measured in untreated controls. Thus, in agreement with the conclusions of Ockner and coworkers (5), the results of the present investigation support the concept that the intestines furnish a larger fraction of the plasma triglyceride than previously believed, perhaps as much as one-third of the total.

Windmueller (7,26) and Windmueller and Levy (4) observed decreases of 85-95% in the concentration of plasma triglycerides of rats fed orotic acid, as compared to ca. 70% decrease seen in the present study. This apparent difference may partially reflect that the rats used in these earlier reports possessed much lower initial plasma triglyceride concentrations (ca. 0.7 μM/ml or 60 mg%) than those used in the current studies (ca. 120 mg%). Perhaps the importance of the intestine's contribution to plasma triglyceride levels could be strain dependent, being more important in rats with the higher triglyceride levels. Also the accuracy of determining reductions in plasma triglyceride levels decreases as the initial plasma triglyceride

level decreases.

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In Vitro Incorporation of ^{14}C-Acetate into Lipids of Hamster Flank Organ (Costovertebral Organ) Sebaceous Glands and Epidermis

BARRY N. LUTSKY, CHARLES CASMER, and **PATRICIA KOZIOL**, Department of Physiology, Biological Research, Schering Corporation, Bloomfield, New Jersey 07003

ABSTRACT

Following in vitro incubation of flank organs from male golden Syrian hamsters with sodium [1-^{14}C] acetate, sebaceous glands and appendage-freed epidermis were obtained by treating the flank organ tissue with calcium chloride. This method permitted the study of incorporation of carbon-14 into the lipids of these skin components. Extracted lipids were identified by thin layer chromatography and autoradiography and were quantitated by liquid scintillation counting. Mono-, di-, and triglycerides, free sterols, fatty acids, wax monoesters, and squalene were identified as products of sebaceous gland metabolism of labeled acetate. In marked contrast, little incorporation of ^{14}C into triglycerides by the epidermal preparations was noted, although the epidermal lipids showed higher relative proportions of free sterols and polar lipids (including phospholipids). Accumulation of sterol esters did not occur. In both preparations phosphatidylcholine represented the major labeled phospholipid.

INTRODUCTION

The difficulty in isolating sebaceous glands free of contamination by other dermal and epidermal components generally has limited the study of sebaceous gland lipogenesis in species other than human to analysis of hair or skin surface sebum. Although predominantly a product of sebaceous gland metabolism, surface sebum necessarily is contaminated by lipids of the keratinizing epidermis, and its chemical definition may be hindered further by epithelial (or bacterial) lipase activity. On occasion, examination of lipogenesis by specialized sebaceous structures, such as the preputial gland of rodents (1), has provided additional information regarding sebaceous gland metabolism.

Recently, Kellum (2) described a method for obtaining morphologically intact sebaceous glands and overlying epidermis from human scalp specimens; employing these methods, biochemical analysis of human scalp sebaceous gland lipids was reported (3). Further, Summerly, et al., described studies on isotopic incorporation into the lipids of human sebaceous glands and epidermis (4-6) and into rat tail sebaceous glands (6) isolated by the Kellum method.

The flank organs (or costovertebral organs) of golden Syrian hamsters are paired, pigmented areas on either flank. In mature males these areas are heavily pigmented and are histologically distinguished by groups of large sebaceous glands forming pilosebaceous complexes with coarse hairs (7). The observed morphological changes caused by castration and subsequent administration of androgens are similar to those of ordinary sebaceous glands (7). Our interest in the flank organs of hamsters as potentially useful tools for study of sebaceous gland function prompted us to employ Kellum's method to examine hamster sebaceous gland (and epidermal) lipogenesis in greater detail than previously possible. We now wish to report preliminary studies on the in vitro incorporation of the label of sodium [1-^{14}C]-acetate into the lipids of isolated sebaceous glands and appendage-freed epidermis from mature, intact male hamsters.

MATERIALS AND METHODS

Mature intact male golden Syrian hamsters (Lakeview Hamster Colony, Newfield, N.J.) weighing 110-130 g were housed singly and allowed access to food ad libitum. Constant temperature and 12 hr light and dark periods were maintained throughout. At sacrifice, hair was removed by shaving and depilation, both flank organs were removed, and the remaining skin and subcutaneous fat were trimmed. The glands were blotted, then immediately added to incubation vessels.

Incubation of flank organs was carried out at 37 C in 2 ml Krebs-Ringer phosphate buffer (pH 7.4) containing 5 μc (50 μM) of sodium [1-^{14}C]-acetate (New England Nuclear, Boston, Mass.) (specific activity 59 mC/mmole), 5.0 mM unlabeled glucose, and 0.1 mg gentamicin sulfate (Schering Corporation, Bloomfield, N.J.). For convenience incubation was carried out for 4 hr in 10 ml Erlenmeyer flasks and an air atmosphere, in an Aquatherm water bath shaker

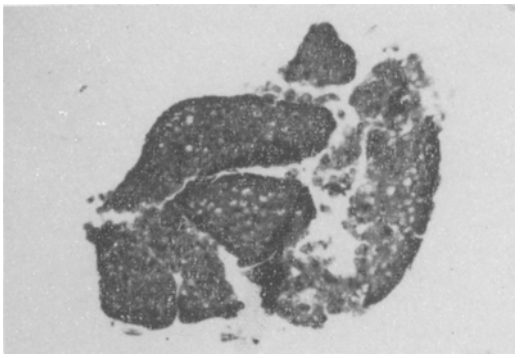

FIG. 1. Male golden Syrian hamster flank organ sebaceous glands after epidermal-dermal separation with 2 M calcium chloride. Low magnification of a group of sebaceous glands shows freedom of contamination by hair, dermal, and epidermal components (hematoxylin and eosin, x 100).

(New Brunswick Scientific Co., New Brunswick, N.J.).

Following incubation, the gland specimens were placed in 2 M calcium chloride solution for 2 hr, according to the method of Kellum (2), after which time the epidermis could be peeled from the underlying dermis as a continuous sheet containing rows of large pale yellow sebaceous glands. Using watchmaker's forceps, teasing needle, and stereoscopic dissecting microscope, the sebaceous glands adhering to the epidermis were harvested and the remaining epidermis processed separately. A further yield of smaller, white sebaceous glands was obtained from the dermis by gentle downward pressure with forceps, resulting in extrusion of massed sebaceous cells with minor contamination by hair roots and melanocytes. These contaminants were clearly distinguishable from the sebaceous glands and discarded.

For histological examinations, sebaceous glands, obtained following calcium chloride immersion, were fixed in neutral buffered formalin, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin.

For analysis of lipids, sebaceous gland and epidermis were homogenized separately in small Potter glass tissue homogenizers with 5 ml chloroform-methanol (2:1, v/v). The subsequent extraction was that of Folch (8). Tissue debris and precipitated proteins were removed by filtration on sintered glass funnels, and the recovered extracts were evaporated under nitrogen. To remove unincorporated labeled acetate and nonlipid contaminants, the residues were dissolved in minimal chloroform-methanol-water (19:1:0.1, v/v) and passed through small (1 x 20 cm) columns of G-25 Sephadex (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) suspended in the same solvent (9). Elution volume was 75 ml. The eluates were dried under vacuum and redissolved in chloroform for thin layer chromatography (TLC) or for separation of polar lipids from neutral lipids.

Conventional techniques of TLC were used for separation of total lipids. Plates (20 x 20 cm x 0.25 mm) of Silica Gel G containing a fluorescent indicator (E. Merck AG, Darmstadt, W. Germany) were activated at 110 C for 30 min after which time the radioactive lipids were applied, along with appropriate reference standards. Mixtures of neutral lipids were obtained from Supelco (Bellefonte, Pa.) and Analabs (North Haven, Conn.). The plates were developed in one dimension using each of three solvent systems: (A) isopropyl ether-acetic acid (96:4, v/v) to 13 cm followed by hexane-diethyl ether-acetic acid (90:10:1, v/v) to 18 cm (10); (B) hexane to 18 cm, benzene to 18 cm, and hexane-diethyl ether-acetic acid (70:30:2, v/v) to 9 cm; (C) hexane-benzene (1:1, v/v) to 18 cm, hexane to 18 cm, and hexane-diethyl ether-acetic acid (80:20:1, v/v), to 18 cm. Each of 12 samples was examined by the three systems described above. Results are expressed as percentage of total incorporation present in each spot. Monoglycerides were quantitated on the basis of chromatography in the system of Skipski, et al., (10).

Phospholipids were separated from neutral lipids by elution of the lipid extracts on columns (1 x 20 cm) of silicic acid (BioRad Laboratories, Richmond, Calif.) suspended in chloroform-methanol (99:1, v/v). Nonpolar lipids were eluted with 40 ml same solvent, while polar lipids were recovered by elution with 40 ml chloroform-methanol (85:15, v/v) followed by 30 ml methanol-water (98:2, v/v) (11). Separations of recovered polar lipids and of applied authentic phospholipids (Supelco) were achieved by two dimensional TLC on activated plates (20 x 20 cm x 0.4 mm) of silica gel-magnesium acetate (Supelco), using the solvent system of Turner and Rouser (12): chloroform-methanol-28% ammonium hydroxide (65:25:5) in the first dimension followed by chloroform-acetone-methanol-acetic acid-water (3:4:1:1:0.5) in the second. Plates were developed in both directions to 16 cm.

Autoradiographs were prepared by exposing the developed thin layer plates to Kodak Royal Blue Medical X-ray film for a period of 1-2 weeks. Visualization of the unlabeled lipid bands on the thin layer plates was achieved by iodine vapor or by spraying with 3% cupric acetate in 20% aqueous phosphoric acid followed by charring at 120 C. Realignment of the thin layer plate with the autoradiograph estab-

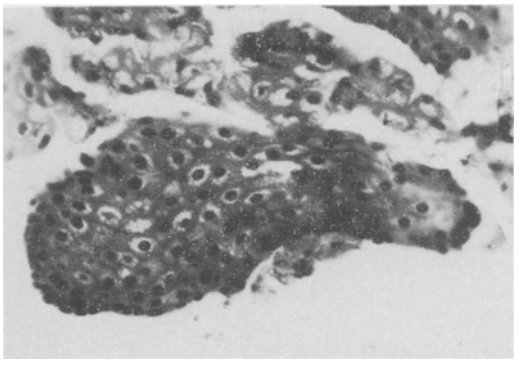

FIG. 2. High magnification of individual sebaceous gland following calcium chloride treatment shows preservation of sebaceous gland structure. Occasionally, glands show some vacuolation of peripheral sebaceous gland cells (hematoxylin and eosin, x 400).

lished zones of radioactivity to be scraped from the plates. Radioactive lipid bands were identified by comparing their mobilities to those of the authentic reference lipids.

Radioactivity was measured with an Intertechnique SL 30 Liquid Scintillation Counter, using a toluene phosphor containing 4 g 2,5-diphenyloxazole (PPO) and 50 mg 1,4-bis-(2-[5-diphenyloxazolyl])-benzene (POPOP)/liter for neutral lipids, and Aquasol (New England Nuclear) for phospholipids. Radioactive neutral lipids on thin layer plates were scraped directly into scintillation vials and counted. Phospholipids were eluted from thin layer plates with chloroform-methanol-28% ammonium hydroxide (56:42:2) according to Kritchevsky and Malhatra (13) and counted in Aquasol (New England Nuclear). Quenching was corrected by means of an external standard.

RESULTS

Sebaceous glands obtained from calcium chloride-treated male hamster flank organs appeared to be histologically intact and free of epidermal or dermal contamination (Fig. 1 and 2). Our experience with this method suggests, however, that, while calcium chloride immersion does provide a convenient route to isolation of sebaceous glands from hamster flank organs, some lipid displacement may occur. Histological examination showed some mature sebaceous gland cells with nuclear displacement and cytoplasmic swelling. These cells also lacked cytoplasmic structures usually associated with lipid body formation. In conjunction with these observations, free lipid masses which lacked membranes occasionally were observed peripheral to the glands. The effects of this treatment, if any, upon patterns of lipids

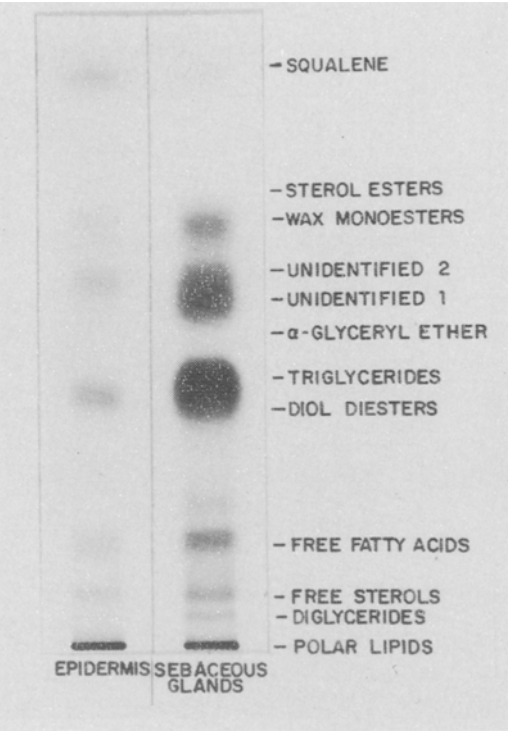

FIG. 3. Thin layer chromatogram of endogenous unlabeled total lipids from sebaceous glands and appendage-freed epidermis of male golden Syrian hamster flank organs. Lipid (200 μg) from each tissue preparation was spotted on Silica Gel G; developed in hexane-benzene (1:1) to 18 cm, hexane to 18 cm, and hexane-diethyl ether-acetic acid (80:20:1) to 18 cm; then sprayed with cupric acetate-aqueous phosphoric acid and charred. Lipid class designations refer to position of authentic compounds, visualized by charring.

isolated from these glands is presently under investigation. In accord with previously reported studies with human sebaceous glands (3,4), sebaceous lipogenic activity was lost following calcium chloride treatment (B.N. Lutsky, unpublished observations).

Extraction of unlabeled lipid from isolated sebaceous glands and epidermis, followed by TLC analysis, showed bands corresponding to polar lipids (including phospholipids), diglycerides, free sterols and unesterified fatty acids, and squalene (Fig. 3). Although the presence of long chain monoesters of wax alcohols was evident, sterol esters were not seen. Because of their proximity to triglycerides in this chromatogram, wax esters corresponding to authentic ethylene glycol distearate could not be differentiated from triglycerides. The prominent spots migrating between wax monoesters and triglycerides were not identified. Faint bands corresponding in mobility to squalene

TABLE I

Pattern of Incorporation of ^{14}C-acetate into the Lipid Fractions from Isolated Sebaceous Glands and Epidermis of Golden Syrian Hamster Costovertebral Organs[a]

Lipid class	Appendage-freed epidermis (12)[b]	Isolated sebaceous glands (12)[b]
Polar lipids (including phospholipids)	38.9 ± 0.6[c]	27.9 ± 0.6
Monoglycerides	2.5 ± 0.2[c]	1.0 ± 0.1
Diglycerides	5.5 ± 0.3[c]	12.9 ± 0.3
Free sterols	18.4 ± 0.1[c]	5.1 ± 0.2
Free fatty acids	11.1 ± 0.7[c]	9.3 ± 0.3
Ethylene glycol diesters	4.2 ± 0.3[c]	5.7 ± 0.3
Triglycerides	1.0 ± 0.1[c]	24.5 ± 0.3
α-Glyceryl ether diesters	Not detected	0.5 ± 0.1
Wax ester (monoesters)	5.8 ± 0.7[c]	2.5 ± 0.2
Sterol esters	1.4 ± 0.3[c]	0.4 ± 0.1
Squalene	2.9 ± 0.2[c]	3.9 ± 0.2
Unidentified	8.3	6.3

[a]Expressed as percent of total incorporation present in each lipid class ± standard error.
[b]Number of samples.
[c]Significantly different from sebaceous glands, $p < 0.01$.

also were seen.

Table I summarizes the patterns of incorporation of carbon-14 from sodium acetate into the lipid classes of the sebaceous glands and epidermis. The percentages represent data pooled from analysis of 12 samples each of sebaceous gland and epidermal lipids. The patterns of incorporation for all preparations were remarkably consistent, with the greatest percentages of radioactivity present in the polar lipids and in sebaceous gland triglycerides. Significant sebaceous gland synthesis of diglycerides, free sterols and unesterified fatty acids, wax esters, and squalene also were observed. Sterol esters were not visualized by autoradiography within the exposure period, and only low levels of radioactivity were present in this area in both epidermal and sebaceous gland preparations. However, the former showed significantly higher relative incorporation into both the sterol ester and the wax monoester fractions, when compared to sebaceous gland synthesis.

The general pattern of epidermal lipids was significantly different from that of sebaceous glands, the greatest differences being lower diglycerides, little evidence of labeled triglyceride, and higher percentages of free sterols. Free fatty acids were evident in both the epidermal and sebaceous gland preparations.

The distribution of radioactivity incorporated into sebaceous gland and epidermal phospholipids, summarized in Table II, shows that, in the sebaceous gland preparations, phosphatidylcholine was by far the major labeled phospholipid. All other identified phospholipids were present in much lower amounts. Because of the low levels of radioactivity associated with the phospholipids, insufficient samples for statistical analysis were examined. The epidermal preparations, although containing phosphatidylcholine as the major component, also showed significant amounts of sphingomyelin and phosphaditic acid. Fully one-third of the recovered radioactivity was associated with unidentified components.

DISCUSSION

Literature concerning the chemical analysis of lipids of isolated sebaceous glands, either by isotopic incorporation or by measurement of endogenous lipids, is sparse. To our knowledge the only previously published material specifically related to hamster sebaceous glands is the histochemical study reported by Montagna and Hamilton (14) in 1949. A direct comparison of their work to that reported here is not possible since theirs was a qualitative study; nevertheless, it is interesting that Montagna and Hamilton reported the presence of glycerides (including triglycerides), cholesterol-containing species, phospholipids, plasmalogens, and free fatty acids in sebaceous glands of male hamster flank organs (14).

The demonstration of hamster sebaceous gland triglycerides by histochemical (14) and isotopic techniques reported here contrasts to previously reported studies on hamster surface sebum, in which little, if any, triglyceride could be detected (15). Montagna and Hamilton showed abundant lipase activity in degenerating flank organ cells, sebum, and stratum corneum (14). It is conceivable, therefore, that triglyc-

TABLE II

Pattern of Incorporation of ^{14}C-acetate into the Phospholipids of Isolated Sebaceous Glands and Epidermis from Golden Syrian Hamster Costovertebral Organs[a]

Lipid class	Appendage-freed epidermis (3)[b]	Isolated sebaceous glands (3)[b]
Diphosphatidyl glycerol	1.5	0.8
Phosphatidyl ethanolamine	5.1	6.9
Phosphatidylcholine	39.1	81.9
Sphingomyelin	6.5	1.4
Lysophosphatidylcholine	2.0	1.2
Phosphatidyl inositol	4.1	4.6
Phosphatidyl serine	1.7	0.4
Phosphatidic acid	7.1	0.7
Unidentified	32.9	2.1

[a]Expressed as percent of total incorporation into phospholipids present in each component.

[b]Number of samples insufficient for meaningful statistical analysis.

erides from the sebaceous glands are hydrolyzed on their way to the surface and, as a consequence, are not detected in the surface fat. Although de novo synthesis by adipocytes and panniculus (followed by transport to the sebaceous glands) has not been definitely established yet, preliminary results of in vitro incubations of previously untreated, isolated hamster sebaceous glands suggest that sebaceous gland synthesis of triglycerides does occur readily (B.N. Lutsky, unpublished observations).

Although patterns of incorporation of carbon-14 during a short in vitro incubation are assumed to be a reflection of in vivo sebaceous gland metabolism, the patterns may not correlate with the actual amounts of lipid present. One obvious indication of the differences in isotopic incorporation and endogenous lipid patterns is the rather low incorporation of ^{14}C into the components with migration characteristics similar to those previously reported for nonpolar diester waxes (15,16). Chromatography of endogenous sebaceous gland and epidermal lipids showed prominent spots with chromatographic mobilities similar to diester waxes; the presence of these compounds in hamster skin surface lipids has already been suggested (15,16). The low proportion of radioactivity incorporated into these fractions may indicate that incubation conditions did not permit accumulation of these labeled lipids or that acetate is not a "physiological" substrate under the in vitro incubation conditions used here.

Summerly, et al., recently published results of studies involving isotopic incorporation of the label of sodium [1-^{14}C]-acetate into lipids of rat tail sebaceous glands (6) and of human sebaceous glands (4,6), isolated by the method of Kellum (2). Despite differences in incubation and chromatography conditions the qualitative similarities in methodology invite comparison. Hamster flank organ sebaceous glands appear to differ from both rats and humans in their markedly greater accumulation of labeled polar lipids, diglycerides, free sterols, and free fatty acids. Conversely, both rats and humans show 5-10 fold greater incorporation of label into the wax-sterol ester and squalene fractions. The percentage incorporation into hamster triglyceride was greater than rat but less than human sebaceous glands. Whether the differences found between the hamster preparations and those reported for rats and humans represent entirely species differences or reflect differences in incubation conditions and tissue location remains to be established.

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Removal of Lipid from Intact Erythrocytes and Ghosts by Aqueous Solutions and Its Relevance to Membrane Structure

JOHN D. TURNER, Division of Metabolic Diseases, Department of Medicine, University of California, La Jolla, California 92037 and **GEORGE ROUSER,** Section of Lipid Research, Division of Neurosciences, City of Hope National Medical Center, Duarte, California 91010

ABSTRACT

Molar values for cholesterol, total phospholipid, and individual phospholipid classes of intact erythrocytes and their membranes (ghosts) washed with various aqueous solutions are presented. The data show that lipid can be washed from erythrocyte ghosts prepared rapidly from freshly drawn blood but that lipid is not removed from intact erythrocytes under the same conditions. Thus, it appears that the polar groups of lipids of intact cells are not exposed as they are in ghosts. In the preparation of hemoglobin-free ghosts, up to 25% cholesterol and phospholipid can be removed, while loss of ca. 50% cholesterol and phospholipid from ghosts can be achieved with aqueous solutions containing ethylenediamine tetraacetate. No significant loss of membrane protein was encountered even when almost half of the lipid had been removed from the ghosts. Phospholipid classes were removed to different extents with different wash solutions. Lipid loss from ghosts can be prevented, in part, by adding 0.5% albumin or calcium to wash solutions containing ethylenediamine tetraacetate. These findings contrast a report where insignificant lipid loss was noted in the preparation of hemoglobin-free human erythrocyte membranes, but agree with results reported for bovine red cell ghosts.

INTRODUCTION

The method of Dodge, et al., (1) commonly is used to prepare hemoglobin-free human erythrocyte ghosts for membrane structure studies. The method was reported to give insignificant lipid loss (1); however, during the course of our work, we observed substantial lipid loss. A study of lipid removal from intact red cells and ghosts by washing with aqueous solutions then was undertaken because of its practical importance for red cell membrane structure studies and its possible relevance to lipid loss during the isolation of subcellular particulates in general. The data obtained are presented in this report.

MATERIALS AND METHODS

Composition and Preparation of Aqueous Wash Solutions

Wash 1: Sodium phosphate buffer (isosmotic with human blood plasma) 0.132 M, pH 7.4, 286 mOsm, prepared by mixing 5 vol solution A (35.92 g $Na_2HPO_4 \cdot 7H_2O$/liter) with 1 vol solution B (17.80 g $NaH_2PO_4 \cdot H_2O$/liter) and adjusting the pH to 7.4 with 1.0 N HCl.

Wash 2: Sodium phosphate buffer, 0.107 M, pH 7.4, 242 mOsm, prepared by mixing 7.14 vol solution A (27.61 g $Na_2HPO_4 \cdot 7H_2O$/liter) with 1 vol solution B (21.61 g $NaH_2PO_4 \cdot H_2O$/liter) and adjusting the pH to 7.4 with 1.0 N HCl.

Wash 3: Disodium ethylenediamine tetraacetate (EDTA) (1.00×10^{-5} M) in 0.150 M NaCl, pH 7.4, 275 mOsm, prepared by dissolving 8.77 g NaCl and 3.72 mg Na_2EDTA/liter and adjusting the pH to 7.4 with 1.0 M $NaHCO_3$.

Wash 4: Disodium EDTA (1.34×10^{-2} M) in 0.123 M NaCl, pH 4.5, 255 mOsm prepared by mixing 1 vol solution A (20 g Na_2EDTA + 3.3 g NaCl/liter) with 3 vol solution B (8.5 g NaCl/liter).

Wash 5: Solution 4 (903 ml) diluted with water (ca. 97 ml) to 232 mOsm (final pH 4.5).

Wash 6: Disodium EDTA (5.37×10^{-2} M) in 0.111 M sodium phosphate buffer, pH 7.4, 370 mOsm prepared by dissolving 23.67 g $Na_2HPO_4 \cdot 7H_2O$, 3.06 g $NaH_2PO_4 \cdot H_2O$, and 20 g Na_2EDTA/liter and adjusting the pH to 7.4 with 1.0 N NaOH.

Wash 7: Hypotonic sodium phosphate buffer (9.18×10^{-3} M, pH 7.4, 25 mOsm prepared by mixing 5 vol solution A (2.48 g $Na_2HPO_4 \cdot 7H_2O$/liter) with 1 vol solution B (1.23 g $NaH_2PO_4 \cdot H_2O$/liter) and adjusting the pH to 7.4 with 1.0 N HCl.

Wash 8: Solution 4 containing 0.50% human serum albumin prepared by mixing 98 vol solution 4 with 2 vol 25% by wt human serum albumin (salt poor, Cutter Labs, Berkeley, Calif.), final pH 5.0, and 262 mOsm.

Wash 9: Solution 7 containing 0.50% human serum albumin prepared by mixing 98 vol solution 7 with 2 vol 25% human serum

TABLE I

Lipid Composition of Human Erythrocytes Washed with Different Aqueous Solutions[a,b]

Subject	Aliquot number	Wash solution[c]	Number washes	MCV μ^3	Chol	TPL	PE[d]	PC[d]	Sph	PS	PA	PI	Other
1	1	1	3	90	3.20	3.84	1.02	1.10	0.95	0.53	0.072	0.031	0.146
2	1	1	3	88	3.64	4.26	1.09	1.23	1.07	0.57	0.081	0.052	0.168
3	1	1	3	85	3.41	3.79	0.98	1.12	0.92	0.51	0.083	0.053	0.129
4	1	1	3	78	3.63	3.89	0.98	1.10	0.94	0.54	0.068	0.045	0.179
5	1	1	3	85	3.51	3.95	1.00	1.18	0.98	0.52	0.086	0.054	0.137
6	1	1	3	85	3.42	3.86	0.98	1.18	0.95	0.51	0.081	0.051	0.132
7	1	1	3	79	3.47	3.92	1.04	1.17	1.03	0.48	0.073	0.054	0.092
8	1	1	3	89	3.41	4.10	1.06	1.17	1.01	0.58	0.080	0.066	0.178
Mean				85	3.46	3.94	1.02	1.16	0.98	0.53	0.078	0.051	0.145
±S.D.				±4	±0.14	±0.15	±0.04	±0.05	±0.05	±0.03	±0.006	±0.010	±0.029
9	1	2	3	97	3.30	4.09	1.08	1.16	1.01	0.59	0.081	0.024	0.145
	2	3	3	101	3.41	4.11	1.09	1.18	0.99	0.58	0.092	0.023	0.150
	3	4	1	120	---	3.85	0.99	1.09	0.97	0.53	0.064	0.025	0.170
	4	4	3	127	3.27	3.87	1.08	1.07	0.92	0.56	0.078	0.024	0.137
10	1	2	3	85	3.30	3.79	1.00	1.12	0.95	0.46	0.088	0.042	0.148
	2	3	3	89	3.33	3.95	1.03	1.16	0.95	0.52	0.071	0.043	0.181
	3	4	1	111	3.31	4.05	1.09	1.20	0.98	0.53	0.082	0.045	0.129
	4	4	3	115	3.49	3.82	1.06	1.15	0.95	0.48	0.064	0.037	0.088
11	1	2	3	90	3.69	4.14	1.09	1.22	1.05	0.57	0.088	0.070	0.138
	2	4	15	132	3.75	4.17	1.15	1.24	1.08	0.49	0.072	0.050	0.077
	3	5	15	143	3.81	4.28	1.07	1.28	1.13	0.51	0.075	0.053	0.067
	4	5	25	138	3.69	4.20	---	---	---	---	---	---	---
12-20[e]		4	3	121	---	3.79	1.02	1.11	0.98	0.48	0.075	0.038	0.064
21	1	1	3	82	3.11	4.00	---	---	---	---	---	---	---
	2	6	15	70	3.30	4.13	---	---	---	---	---	---	---

[a]Values as μmoles/ml packed cells; mean corpuscular vol of each subject's cells was measured in quadruplicate after washing with each solution, and lipid values were corrected for cell volume differences to permit direct comparison with solution 1.

[b]MCV = mean corpuscular vol in μ^3, Chol = cholesterol, TPL = total phospholipid, PE = phosphatidyl ethanolamine, PC = phosphatidyl choline, Sph = sphingomyelin, PS = phosphatidyl serine, PA = phosphatidic acid, PI = phosphatidyl inositol, Other = sum of all trace acidic phospholipid components, and ±S.D. = standard deviation.

[c]See text for composition of eash solutions and for details of washing intact erythrocytes.

[d]In some cases, conversion of up to 5% PE to LPE and 1-2% PC to LPC took place when EDTA was present; PE and PC values, thus, are the sums of the parent and lyso compounds.

[e]Mean values for nine subjects.

albumin (salt poor), final pH 7.4, and 30 mOsm.

Wash 10: Disodium EDTA (9.54 x 10^{-3} M), $CaCl_2$ (4.77 x 10^{-3} M) in 0.110 M NaCl-sodium phosphate buffer (7.52 x 10^{-3} M) prepared by dissolving 9.0 g NaCl + 4.99 g Na_2EDTA and 0.74 g $CaCl_2$/liter of solution 7, diluting with distilled water to 287 mOsm, and adjusting the pH to 7.4 with 0.5 N NaOH.

Wash 11: Solution 7 containing 10^{-3} M magnesium chloride.

Osmolality of the wash solutions was measured with an osmometer just prior to washing the cells and ghosts.

Healthy human subjects, of both sexes (23 subjects age 21-25 and one 39 year old) were sampled, some on several occasions. Blood was drawn after an overnight fast with disodium EDTA (1 mg/ml blood) as anticoagulant. All centrifugations and solutions were at 4 C. The blood was centrifuged immediately at 80 x g for 15 min. After removal of the platelet-rich plasma, the cells were centrifuged again at 1500 x g for 10 min. The supernatant plasma was removed and the upper 1-2 mm containing white cells was aspirated and discarded. The cells from each subject, after gentle but thorough mixing, were divided into several aliquots. Each aliquot was mixed with an equal volume of one of the solutions given above and centrifuged at 1500 x g for 10 min. The supernatant was removed and the washing procedure was repeated as rapidly as possible using equal volumes of the appropriate wash solution and cells. After the final wash, a measured volume of well mixed cells from each aliquot was mixed with an equal volume of the same solution with which it had been washed, and an aliquot was removed for determination in quadruplicate of the mean corpuscular volume. The red cell suspensions were extracted immediately. Erythrocyte ghosts were prepared essentially according to the method of Dodge, et al., (1) whereby cold hypotonic phosphate butter (solution 7) is mixed 14:1 (v/v) with cells, centrifuged at 20,000 x g for 30 min, the supernatant decanted carefully, and the membranes either extracted immediately or washed further as noted below.

Red cells and ghosts were extracted twice with chloroform-methanol 2:1 (v/v), once with chloroform-methanol 1:2, and once with chloroform-methanol 7:1 saturated with freshly prepared aqueous ammonia (2,3). Care was taken not to evaporate extracts to complete dryness to avoid alteration of lipids (4,5). Lipids were separated from gangliosides and water soluble nonlipid contaminants by Sephadex column chromatography (4,6). Cholesterol was determined after one dimensional thin layer chromatography (TLC) with hexane-ether 7:3 by the method of Hanel and Dam (7). Phospholipids were determined by phosphorus analysis after separation by two dimensional TLC (3,8,9). Protein was measured by the method of Lowry, et al. (10). Osmolality was determined with a model 2007 osmometer (Precision Systems, Framingham, Mass.).

RESULTS

Exposure of red cells to solutions of different osmolalities produces cell volume differences. Accordingly, all whole cell lipid values (as μmoles/ml packed cells) were corrected for volume differences to permit comparison with cells washed in isosmotic phosphate buffer (solution 1). Lipid values for ghosts (Tables II and III) are given as μmoles/ml cells used to prepare the ghosts.

Washing of intact erythrocytes (Table I) with slightly hypotonic phosphate buffer (solution 2), saline solutions containing Na_2EDTA in low concentration and at pH 7.4 (solution 3), or Na_2EDTA in high concentration at pH 4.5 (solutions 4 and 5), and hypertonic phosphate buffer (solution 6) did not result in any significant loss of lipid, lipid values for all subjects being similar to those for controls when corrected for volume differences. Similar findings were encountered when the volume ratio of solution 4 to cells was changed from 1:1 to 7:1 (Table I).

Ghosts prepared by hemolysis of cells in hypotonic phosphate buffer (solution 7) without subsequent washing lost in some cases 6-8% their cholesterol and 2-3% their phospholipid (Table II). Hemoglobin-free ghosts prepared by washing five times with solution 7 lost 26% cholesterol and 23% phospholipid (Table II). Two other preparations were analyzed with similar results. Ghosts washed additionally 5-15 times with solution 4 showed an average total loss of 50% cholesterol and maximal total loss of 46% phospholipid (Table II). In general, cholesterol loss was somewhat greater than phospholipid loss (Table II), and acidic and nonacidic phospholipids were removed to differing extents (Table III). Acidic phospholipids were not removed as readily as the nonacidic phospholipids with hypotonic buffer or Na_2EDTA solutions (subject 22, Table III). In contrast, washing with hypotonic phosphate buffer containing 10^{-3} M magnesium chloride preferentially removed phosphatidyl ethanolamine and acidic phospholipids (subject 24, Table III). Ca. 80% recovery of cholesterol and 90% recovery of most phospholipid classes lost

TABLE II

Removal of Cholesterol and Total Phospholipid from Human Erythrocyte Ghosts[a,b]

Subject	Type of preparation	Wash solution[c]	Number washes	MCV μ^3	μMoles/ml packed cells		Percent loss compared to control whole cells	
					Chol	TPL	Chol	TPL
22[d]	Whole cells	1	3	88	3.51	2.86	—	—
	Ghosts 1	7	5	—	2.59	2.97	26.2	23.0
	2	7 } 4 }	5 } 5 }	—	1.62	2.07	53.8	46.3
23[d,e]	Whole cells	1	3	88	3.64	4.30	—	—
	Ghosts	7 } 4 }	2 } 15 }	—	1.94	2.58	46.7	40.0
9[f]	Whole cells	2	3	97	3.25	4.09	—	—
	Ghosts 1	None	None	—	3.10	3.96	4.6	3.2
	2	8	3	—	2.79	3.82	14.2	6.6
	3	9	2	—	2.74	3.73	15.7	8.8
21[f,g]	Whole cells	2	3	82	3.11	4.00	—	—
	Ghosts 1	None	None	—	2.90	3.91	6.8	2.3
	2	10	3	—	2.87	3.89	7.7	2.8
	3	10	6	—	2.69	3.71	13.5	7.3
	4	10	9	—	2.46	3.62	20.9	9.5

[a]See Table III for phospholipid class values for subjects 22 and 23.

[b]Abbreviations and lipid values as for Table I.

[c]See text for composition of wash solutions and details of procedures used to wash intact erythrocytes and to prepare ghosts.

[d]Intact cells were first washed three times with solution 1; aliquots were removed to prepare ghosts, hemolyzed in solution 7, and washed as detailed above.

[e]In the preparation of subject 23 ghosts, the decanted supernatants after each centrifugation, were pooled, lyophilized, and the lipid composition analyzed; values in μmoles/ml packed cells were: Chol, 1.17; TPL, 1.40.

[f]A second sample was drawn from subjects 9 and 21 for these studies; in this case, intact cells were first washed three times in solution 2; aliquots were removed to prepare ghosts, hemolyzed in solution 7, and washed as detailed above; lipid values for intact cells were corrected for vol differences between solutions 1 and 2.

[g]Molar concentrations (μmoles/ml packed cells) for phospholipid classes in subject 21 ghost preparation 4 were: PE, 1.01; PC, 0.96; Sph, 0.82; PS, 0.53; PA, 0.061; PI, 0.066; and Other, 0.163.

TABLE III

Removal of Phospholipid Classes from Human Erythrocyte Ghosts[a,b]

Subject	Type of preparation[c]	TPL	PE[d]	PC[d]	Sph	PS	PA	PI	Other
22	Whole cells	3.86	1.02	1.15	0.92	0.53	0.074	0.048	0.119
	Ghosts 1	2.97	0.68	0.87	0.70	0.41	0.049	0.040	0.210
	2	2.07	0.54	0.62	0.43	0.33	0.040	0.031	0.088
23[e]	Whole cells	4.30	1.10	1.22	1.08	0.57	0.081	0.053	0.170
	Ghosts	2.58	0.71	0.74	0.59	0.39	0.052	0.029	0.071
24	Whole cells	3.48	0.92	0.98	0.88	0.52	0.089	0.052	0.097
	Ghosts	2.56	0.53	0.71	0.68	0.32	0.060	0.039	—
	Percent loss compared to control whole cells for above subjects								
22	Ghosts 1	23.1	33.3	24.3	23.9	22.6	33.8	16.7	—
	2	46.4	47.0	46.1	53.3	37.7	45.9	35.4	26.1
23	Ghosts	40.0	35.4	39.4	45.3	31.6	35.8	45.2	58.2
24	Ghosts	26.4	42.4	27.6	22.7	38.5	32.3	35.0	—

[a]Values as μmoles/ml packed cells; see Table II for choiesterol values and percent cholesterol loss on the same samples.

[b]Abbreviations as for Table I.

[c]See Table II for wash solutions used and number of washes with each for each aliquot.

[d]See footnote d in Table I; PE and PC values represent the sum of the parent and lyso compounds.

[e]In the preparation of subject 23 ghosts, the decanted supernatants from each centrifugation were pooled, lyophilized, and the lipid composition analyzed; values in μmoles/ml packed cells were: TPL, 1.40; PE, 0.34; PC, 0.44; Sph, 0.45; PS, 0.05; PA, 0.026; PI, 0.008; Other, 0.083.

from the ghosts were achieved from the large volume of pooled wash solution from the most extensively washed ghosts (Table II, subject 23). In one study, loss of protein along with lipid was not detected. The protein content of the ghost preparation having twice the lipid loss of another was not reduced (Table II, subject 22). The lipid-protein (w/w) ratios for the two preparations were 1.09 and 0.67, respectively. Compared to whole cell lipid values for subject 22, ghost preparation 1 showed a 25% loss of cholesterol and phospholipid. This suggests that the true lipid-protein ratio of the red cell membrane may be higher than 1.09 (possibly closer to 1.4).

It appears that EDTA in the concentration used in solution 4 increases lipid loss from erythrocyte ghosts. The lipid loss from ghosts is reduced when calcium is added to solution 7, i.e. solution 10, molar ratio EDTA/Ca^{++}, 2:1, subject 21—ghost preparations 2-4 compared to subject 22—preparations 1 and 2).

DISCUSSION

Our data show that there is no significant lipid loss from intact red cells washed with aqueous solutions, including those that cause cells to undergo large volume changes. In contrast, lipid can be washed from erythrocyte ghosts with relative ease. Our findings agree with those of Burger, et al., (11) who observed lipid loss during preparation of ghosts from bovine red cells but differ from those of Dodge, et al., (1) who reported insignificant lipid loss from human red cells. If Dodge, et al., used stored blood, the differences between the two studies may be explained since it has been shown that cells can lose up to 30% of their lipid when stored (12), and washing of such cells probably would not remove much additional lipid. From our data, it appears that lipid molecules of intact cells are not exposed to the external medium, whereas those of ghosts are. The data argue against the validity of recently proposed models of membrane structure (13-19) in which lipid polar groups are depicted as being exposed to the external medium.

Lipid polar groups of red cell ghosts are attacked readily by pancreatic phospholipase A and some purified snake venom phospholipases that do not attack lipids of intact cells unless the membrane is altered by the presence of a hemolytic basic protein (20,21), by a detergent (22), or by swelling induced by exposure to a hypotonic solution (23). Damage to membranes was found to be essential for phospholipase A degradation of white blood cell phospholipids (24). Some preparations of phospholipase A (25) and sphingomyelinase (26) can degrade phospholipids of intact cells without causing hemolysis. It appears that these enzyme preparations can alter the membrane and expose lipid polar groups.

Only 40% free cholesterol of human, pig, and rat red blood cells and part of the phosphatidyl choline and sphingomyelin of human and dog red cells exchange with plasma or artificial media (27,28). Exchange of phosphatidyl ethanolamine and acidic phospholipid of human and dog red blood cells was not observed (28), and it appears that none of the phospholipid of bovine erythrocytes is exchangeable, although there is cholesterol exchange (29). These observations are difficult to reconcile with models of membrane structure that depict lipid polar groups as being exposed. Since some exchange is noted, it is clear that, although lipids may be largely in a central core, some molecules may, at least transiently, occupy more external positions and thus be available for reactions of various types.

Washing with aqueous solutions appears to be a relatively mild procedure, and mobility of lipid molecules is not as great at 4 C as at 37 C, where molecules are in the liquid crystalline state (30). It is important to note that concentrations of salt solutions containing EDTA can be chosen that will solubilize over 90% red blood cell protein and yet leave the lipid bound to the remaining protein in particulate form (30) or solubilize much of the lipid with little solubilization of protein, as in our studies.

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SHORT COMMUNICATIONS

Detritylation by Silicic Acid Boric Acid Column Chromatography[1]

ABSTRACT

Chromatography on silicic acid-boric acid columns permits detritylation of ester lipids, essentially without acyl migration, and simultaneous separation of the reaction products. Described is the preparation of the column and its application for the synthesis of diacyl-*sn*-glycerols and 2-acyl-1,2-propanediols.

INTRODUCTION

Tritylation is an excellent technique for blocking specific alcohol groups prior to acylation of polyalcoholic compounds. The technique is particularly useful in the synthesis of mono- and diglycerides (1,2). However, migration of acyl groups occurs during detritylation with gaseous hydrogen chloride in petroleum ether or diethyl ether (1). This article describes the removal of the triphenylmethyl protecting group from trityl-diacyl-*sn*-glycerols and trityl-2-acyl-1,2-propanediols by silicic acid-boric acid column chromatography with petroleum ether. The detritylation, as well as the chromatographic separation, of diacyl-*sn*-glycerol or 2-acyl-1,2-propanediol from triphenylcarbinol is a continuous operation and proceeds with little or no acyl migration.

The procedure is simple and practical and is an excellent method for the synthesis of mixed acid, saturated and polyunsaturated, diacyl-*sn*-glycerols (2), as well as 2-acyl-1,2-propanediol (3).

1,3-Bis-trityl-2-linolenoyl-*sn*-glycerol, the only compound containing two trityl groups, was only half detritylated on silicic acid-boric acid column. We observed that silicic acid alone catalyzes the detritylation to completion when used with petroleum ether as detritylating solvent. The presence of boric acid appears to prevent acyl migration during detritylation and chromatographic separation (4).

[1] Presented in part at the Canadian Federation of Biological Societies Annual Meeting in Toronto, June 1971.

TABLE I

Ester Lipids Detritylated by Silicic Acid-Boric Acid Column Chromatography

	Detritylated compounds	Detritylation percent	Acyl migration percent[a]
1.	1-Stearoyl-2-linoleoyl-*sn*-glycerol (2)	95	1
2.	1-Stearoyl-2-linolenoyl-*sn*-glycerol (2)	76	2
3.	2-Linoleoyl-3-stearoyl-*sn*-glycerol (2)	94	1
4.	2-Linolenoyl-3-stearoyl-*sn*-glycerol (2)	92	1
5.	2-Linolenoyl-3-oleoyl-*sn*-glycerol (2)	70	5
6.	1-Palmitoyl-2-oleoyl-*sn*-glycerol	82	2
7.	1-Palmitoyl-2-linoleoyl-*sn*-glycerol	79	2
8.	1-Palmitoyl-2-linolenoyl-*sn*-glycerol	70	3
9.	2-Oleoyl-3-palmitoyl-*sn*-glycerol	85	1
10.	2-Linoleoyl-3-palmitoyl-*sn*-glycerol	78	2
11.	2-Linolenoyl-3-palmitoyl-*sn*-glycerol	71	3
12.	2-Stearoyl-3-benzyl-*sn*-glycerol (1)	73	0
13.	2-Oleoyl-3-benzyl-*sn*-glycerol (1)	70	0
14.	2-Stearoyl-1,2-propanediol (3)	97	0
15.	2-Oleoyl-1,2-propanediol (3)	98	0
16.	1,3-Bis(trityl)-2-linolenoyl-*sn*-glycerol	50	0

[a] Ca. percentage of acyl migration induced during the detritylation and chromatographic separation by the silicic acid-boric acid column was estimated from the size of the spots detected on thin layer chromatographic plates.

TABLE II

Chemical Analyses

Compounds	Formula and mol wt	Percent carbon		Percent hydrogen		Iodine value		Percent yield
		calculated	found	calculated	found	calculated	found	
6.	$C_{37}H_{70}O_5$(595)	74.69	74.35	12.00	11.86	42.6	42.1	82
7.	$C_{37}H_{68}O_5$(593)	74.95	75.01	11.56	11.52	84.0	83.8	79
8.	$C_{37}H_{66}O_5$(591)	75.18	75.20	11.09	11.12	128.9	128.2	70
9.	$C_{37}H_{70}O_5$(595)	74.69	74.52	12.00	12.02	42.6	42.6	85
10.	$C_{37}H_{68}O_5$(593)	74.95	74.88	11.56	11.48	84.0	84.0	78
11.	$C_{37}H_{66}O_5$(591)	75.18	75.16	11.09	11.10	128.9	128.5	71
16.	$C_{59}H_{64}O_4$(837)	84.70	84.52	7.71	7.57	91.0	89.5	50

It should be emphasized that removal of the trityl group is a relatively slow procedure and requires prolonged washing of the column with petroleum ether to bring detritylation to completion.

The compounds listed in Table I were detritylated by silicic acid-boric acid column chromatography. The analytical values and physical properties of compounds 1-5 and 12-15 are reported, respectively, (1-3). The chemical analysis, yields, and physical data of compounds 6-11 and 16 are summarized in Tables II and III, respectively.

EXPERIMENTAL PROCEDURES

Preparation of the silicic acid-boric acid column: The silicic acid (Mallinckrodt Analytical Reagent, 100 mesh) was suspended five times in distilled water, and the water was decanted each time to remove finer particles. Residual water was filtered off on a Buchner funnel; the silicic acid was mixed carefully with a hot, saturated aqueous solution of boric acid (British Drug House, Analar), 10% dry wt silicic acid; and the mixture was air dried and activiated at 115-120 C for 24 hr.

Activated silicic acid-boric acid mixture (100 g) was suspended in 400 ml petroleum ether (bp 30-60 C) by stirring, and the slurry was poured into a glass column 70 x 2.5 cm, containing 50 ml petroleum ether. The column was tapped occasionally to ensure uniform packing. Excess petroleum ether then was drained off to a level of ca. 1 cm above the impregnated absorbent. This column is suitable for the detritylation of ca. 10 g lipid material.

Detritylation and chromatographic separation: The trityl-acyl-compounds were prepared, as described previously (1-3). Trityl-diacyl-*sn*-glycerol or trityl-acyl-propanediol (10 g) dissolved in 50 ml petroleum ether was poured onto a freshly prepared silicic acid-boric acid column. The triphenylmethyl compounds turn the mixture slight yellow.

The column then was eluted with: (A) petroleum ether, (B) petroleum ether and diethyl ether mixture (95:5, v/v), and (C) with petroleum ether and diethyl ether mixture (3:1, v/v) or benzene and diethyl ether mixture (3:1, v/v). During elution with petroleum ether, detrithylation takes place. The column must be washed with petroleum ether until traces of triphenylcarbinol appear in the effluent. After ca. 24 hr and a consumption of ca. 2.5-3.0 liters petroleum ether, only traces of the original material and of triphenylcarbinol were recovered. Petroleum ether-diethyl ether (95:5,

TABLE III

Physical Properties

Compounds	Specific rotation in substance at 25 C	Density at 20 C	Physical state at 20 C
6.	+2.5° ± 0.1°	0.9385	Oil
7.	+2.5° ± 0.1°	0.9366	Oil
8.	+2.5° ± 0.1°	0.9360	Oil
9.	−2.6° ± 0.1°	0.9372	Oil
10.	−2.4° ± 0.1°	0.9361	Oil
11.	−2.4° ± 0.1°	0.9345	Oil
16.	–	–	Paste

v/v) elutes triphenylcarbinol. Triphenylcarbinol migrates slowly and crystallizes in the lower half of the column and around the outlet. The column was washed with petroleum ether-diethyl ether (95:5, v/v) until the effluent was free of triphenylcarbinol, using ca. 2.5 liters solvent mixture.

Finally, the detritylated product was recovered from column with petroleum ether-diethyl ether, (3:1, v/v) or with benzene-diethyl ether, (3:1, v/v). The elute collected with a fraction collector was checked by thin layer chromatography. The recovery of the detritylated product from the column varied from 70-98%, and the overall yield of pure diglycerides was found to range from 56-95% and 2-acyl-1,2-propanediol from 80-98%.

Use of benzene instead of petroleum ether for preparation of the column and for initial elution leads to partial detritylation ranging from 2-30%.

DMYTRO BUCHNEA
Banting and Best Department of Medical Research
University of Toronto
Toronto, Ontario, Canada

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Effect of Ethanol on Liver Triglyceride Concentration in Fed and Fasted Rats

ABSTRACT

The effect of ethanol, given by gastric tube or by intraperitoneal injection, on the liver triglyceride concentration in fasted and ad libitum fed rats was investigated. Ethanol, independent of the route of administration, increased the liver triglyceride concentration in fasted rats during an 8 hr period but caused a much smaller increase in the ad libitum fed rats. The incorporation of intravenous injected ^{3}H-oleic acid into the liver triglycerides increased significantly after ethanol feeding in fasted but not in fed rats. Based upon this and the plasma free fatty acid concentrations, it is concluded that ethanol causes a marked increase in the utilization of plasma free fatty acids for synthesis of liver triglycerides in fasted but not in fed rats. It is suggested that this is part of the explanation for the different responses to ethanol of the liver triglyceride concentrations in fasted and fed rats.

INTRODUCTION

A single large dose of ethanol given to a fasted rat leads to a reversible increase in the liver triglyceride concentration (1). The liver triglyceride accumulation probably is caused by an increase in the rate of triglyceride synthesis from free fatty acids (FFA) taken up from the plasma (for a review see ref. 2). This may be due to an increased influx into the liver of plasma FFA or an increased utilization in the liver cells of FFA taken up from the plasma for triglyceride synthesis or both. Since both these alternatives are influenced by the nutritional

TABLE III

Physical Properties

Compounds	Specific rotation in substance at 25 C	Density at 20 C	Physical state at 20 C
6.	+2.5° ± 0.1°	0.9385	Oil
7.	+2.5° ± 0.1°	0.9366	Oil
8.	+2.5° ± 0.1°	0.9360	Oil
9.	−2.6° ± 0.1°	0.9372	Oil
10.	−2.4° ± 0.1°	0.9361	Oil
11.	−2.4° ± 0.1°	0.9345	Oil
16.	–	–	Paste

v/v) elutes triphenylcarbinol. Triphenylcarbinol migrates slowly and crystallizes in the lower half of the column and around the outlet. The column was washed with petroleum ether-diethyl ether (95:5, v/v) until the effluent was free of triphenylcarbinol, using ca. 2.5 liters solvent mixture.

Finally, the detritylated product was recovered from column with petroleum ether-diethyl ether, (3:1, v/v) or with benzene-diethyl ether, (3:1, v/v). The elute collected with a fraction collector was checked by thin layer chromatography. The recovery of the detritylated product from the column varied from 70-98%, and the overall yield of pure diglycerides was found to range from 56-95% and 2-acyl-1,2-propanediol from 80-98%.

Use of benzene instead of petroleum ether for preparation of the column and for initial elution leads to partial detritylation ranging from 2-30%.

DMYTRO BUCHNEA
Banting and Best Department of Medical Research
University of Toronto
Toronto, Ontario, Canada

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INTRODUCTION

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state, it was interesting to compare the effects of ethanol on the liver triglyceride concentration in fed and in fasted rats.

EXPERIMENTAL PROCEDURES

Female Sprague-Dawley rats (Anticimex, Stockholm, Sweden) weighing 200-220 g were used. In each experiment care was taken to make a similar distribution of body wt of the animals in the different groups. They were kept in an artifically lighted room with lights on 8 a.m.-8 p.m. and fed a standard laboratory chow diet (AB EWOS, Södertälje, Sweden). In experiment one ethanol as a 38% solution (v/v) or saline (0.9% NaCl) was given by gastric tube to the unanesthetized rats. In experiment two ethanol or a corresponding volume of saline was given by intraperitoneal injection. In this experiment a 10% (w/v) ethanol solution was used, since more concentrated ethanol solutions given intraperitoneal become irritant and may cause intense inflammation (3). Fasted rats were fasted for 24 hr prior to treatment and fed rats given the chow diet ad libitum. Ethanol or saline always was given between 8 a.m. and 10 a.m. All animals were fasted after administration of ethanol or saline. In experiment one at 0 and 8 hr after treatment, the rats were anaesthetized with ether, and 0.5 ml fatty acid-albumin complex (4) containing $5 \cdot 10^6$cpm 9,10 ^{3}H-oleic acid (The Radiochemical Centre, Amersham, England) ($2 \cdot 10^{12}$cpm/mmole) was injected into a neck vein. The rats were killed 5 min later. The rats in all experiments were killed by exsanguination via the aortic bifurcation under light ether anaesthesia. The plasma FFA concentration was determined by the double extraction method of Dole and Meinertz (5). A liver lipid extract was prepared, as described by Folch, et al., (6) and lipid classes were separated by thin layer chromatography, as previously described (7). Glyceride-glycerol was determined essentially according to Eggstein and Kreutz (8) in aliquots of the liver lipid (Folch) extracts. The remains of the rat, the carcass, was placed in 30% ethanolic KOH, digested, and the fatty acids extracted from aliquots of the digest (9). The radioactivities of aliquots of liver triglycerides and carcass fatty acids were determined in a Packard, model 3320, Tri-Carb scintillation spectrometer. For statistical calculations of differences between groups of rats, Wilcoxon's Rank Sum Test (10) was used. Differences were considered significant for p-values less than 0.05.

In fasted rats tube-fed ethanol, the liver triglycerides increased markedly (Table I). In contrast, in the ad libitum fed rats, the liver triglyceride concentrations were not increased significantly over control values 8 hr after per oral ethanol administration (Table I).

When ethanol was given by intraperitoneal injection, the liver triglyceride concentration was increased significantly over the corresponding control values at 8 hr in both fed and fasted ethanol-treated rats; but the increase was much larger in fasted than in fed rats ($p < 0.005$) (Table I). Thus, the observation that in fed rats, in contrast to fasted rats, the liver triglyceride concentrations do not increase rapidly after an oral ethanol dose can only, to a minor extent, be attributed to differences in the rate of absorption of ethanol.

Intravenous injected labeled fatty acids rapidly are incorporated into liver lipids, and, 3 min after injection, only negligible amounts of radioactivity are present in FFA in the liver and the plasma (9). The radioactivity in the liver triglycerides reaches a maximum ca. 3 min after injection and remains essentially unchanged up to 20 min after injection (9,11). In these and previous experiments (4,12), the incorporation of radioactivity from 9,10-^{3}H-oleic acid into the liver triglycerides 5 min after injection was used to estimate the utilization of plasma FFA for synthesis of liver triglycerides. The incorporation of radioactivity from intravenous injected labeled oleic acid was much higher in fed than in fasted control rats both at 0 and 8 hr and was increased significantly by ethanol feeding in fasted but not in fed rats (Table I). This ethanol-induced increased utilization of plasma FFA for synthesis of liver triglycerides in fasted rats has been shown previously and is probably due to a change in the metabolism of FFA in the liver cells and not to an increase in the part of the plasma FFA flux entering the liver cells (12). This interpretation is supported by the fact that ethanol causes such a change in the FFA metabolism in isolated hepatocytes (13).

The hepatic uptake of FFA, both in vivo and in perfused livers, (14,15) has been shown to be proportional to the concentration in the external medium. Therefore, in fasted rats, due to the high plasma FFA concentration, the influx of plasma FFA into the liver cells is much larger than in fed rats. The plasma FFA concentration in the fasted rats was increased over control values at 8 hr (Table I), and this increase was statistically significant when ethanol was given intraperitoneally. Thus, the acute ethanol-induced liver triglyceride accumulation in fasted rats is probably, to a large extent, due to an increased rate of triglyceride synthesis in the liver as a consequence of an increased utiliza-

TABLE I

Liver Triglyceride Concentration, Plasma Free Fatty Acid Concentration, and Incorporation of Radioactivity from Intravenous injected ^{3}H-oleic Acid into Liver and Carcass Lipids in Rats Given Ethanol[a]

Treatment	Time after treatment hr		Liver triglyceride concentration μmole/whole liver	Plasma free fatty acid concentration μmole/ml	Incorporation of radioactivity into liver triglycerides % of injected dose	carcass fatty acids % of injected dose
	Experiment 1		Ethanol (6g/kg) given by gastric tube			
Fed	0	(6)	99.0 ± 5.9	0.19 ± 0.02	26.6 ± 2.8	40.8 ± 3.0
Fasted	0	(6)	57.6 ± 4.8	0.68 ± 0.06	18.6 ± 0.9	34.9 ± 2.8
Fed+ethanol	8	(6)	102.5 ± 7.6[b]	0.34 ± 0.01[b]	35.6 ± 2.8[b]	41.4 ± 1.6[b]
Fed+saline	8	(6)	84.7 ± 8.2	0.30 ± 0.04	29.9 ± 2.0	40.9 ± 2.9
Fasted+ethanol	8	(6)	270.2 ± 42.7[d]	0.81 ± 0.03[b]	32.7 ± 1.8[d]	35.3 ± 1.2[b]
Fasted+saline	8	(6)	69.7 ± 4.8	0.70 ± 0.05	17.4 ± 1.5	38.3 ± 0.6
	Experiment 2		Ethanol (3.75 g/kg) given by intraperitoneal injection			
Fed	0	(6)	83.0 ± 7.8	0.15 ± 0.03		
Fasted	0	(6)	88.2 ± 5.7	0.71 ± 0.06		
Fed+ethanol	8	(7)	116.3 ± 9.6[c]	0.47 ± 0.03[b]		
Fed+saline	8	(7)	83.4 ± 8.5	0.39 ± 0.04		
Fasted+ethanol	8	(6)	226.5 ± 15.2[d]	0.73 ± 0.06[c]		
Fasted+saline	8	(6)	88.4 ± 7.7	0.53 ± 0.04		

[a]Values are mean ± standard error. Number of rats in parantheses. P-values refer to differences between a group of rats given ethanol and the corresponding control group.

[b]Not significantly different.

[c]$p < 0.05$.

[d]$p < 0.005$.

tion in the liver of plasma FFA for the synthesis of triglycerides. At certain times increased plasma FFA concentrations also may contribute to the liver lipid accumulation. In agreement with this, the fatty acid composition of the accumulated lipids suggests that the fatty acids are derived from the adipose tissue via the plasma FFA (16).

In the present experiments ethanol treatment caused statistically significant increases in the liver triglyceride concentrations in fed rats and in the plasma FFA concentrations in fasted rats only when ethanol was given by intraperitoneal injection. However, ethanol given by mouth also increased the mean values of these parameters in the corresponding groups. Since the standard errors are comparatively large, the results are not conclusive as to whether the observed differences between the results in the two experiments are due to the different routes of ethanol administration or not.

In fed rats a large fraction of the plasma FFA entering the liver cells is used for triglyceride synthesis, but the hepatic uptake pf plasma FFA is smaller than in the fasted rats, due to a lower plasma FFA concentration (Table I). The rate of triglyceride synthesis from this source may well be of similar magnitude in the fed and the fasted control rats. In the fed rats ethanol did not increase significantly the fraction of plasma FFA used for liver triglyceride synthesis and did not significantly change the plasma FFA concentration (Table I). Thus, in the fed rats ethanol did not cause a large increase in the rate of triglyceride synthesis from plasma FFA and also caused a much smaller increase in the liver triglyceride concentration than in the fasted rats. This difference in the effect of ethanol on the utilization of plasma FFA for liver triglyceride synthesis in fed and fasted rats probably is not due to differences in the rate of ethanol absorption; since this effect of ethanol occurs at low ethanol concentrations (13,17), and the magnitude of it seems to be unaffected when the blood ethanol concentrations are increased (17). The observation that the increase in the liver triglyceride concentration after a single large dose of ethanol is partly inhibited by the simultaneous administration of an isocaloric dose of glucose (18) can, in part, be explained analogously, since glucose alters the utilization of plasma FFA for liver triglyceride synthesis and the plasma FFA concentration toward the situation in the fed state (19).

Our observation that ethanol does not cause a rapid rise in the liver triglyceride concentration in fed rats agrees with the fact that no rapid rise in the liver triglyceride concentration occurs when ethanol is given to rats in the diet in studies of the effect of chronic administration of ethanol (20).

It is evident that other differences not discussed here between fed and fasted rats, e.g. the presence of exogenous lipids, also may influence the observed difference in the acute effects of ethanol on the liver triglyceride concentration.

OWE JOHNSON
Department of Chemistry,
Section on Physiological Chemistry,
University of Umeå, S-901 87 Umeå, Sweden

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Synthesis of Phospholipids and Phospholipid Fatty Acids by Isolated Perfused Rat Lung[1]

M.C. WANG and **H.C. MENG**, Department of Physiology, School of Medicine, Vanderbilt University, Nashville, Tennessee 37232

ABSTRACT

Synthesis of phospholipids and phospholipid fatty acids in isolated perfused rat lung was studied. The perfusion fluid was a Krebs-Ringer bicarbonate buffer containing a ^{14}C labeled substrate. It was found that 1-^{14}C-acetate, 1-^{14}C-laurate, 1-^{14}C-palmitate, 1-^{14}C-stearate, 1-^{14}C-oleate, or U-^{14}C-D-glucose was incorporated into tissue lipids in the isolated perfused lung at a rate greater than that in incubated minced tissue. However, the patterns of the newly synthesized lipids from these two systems were similar. In 1 hr of perfusion, 6.8, 3, 14.5, 7.5, 7, and 2% of the initial ^{14}C-radioactivity of 1-^{14}C-acetate, 1-^{14}C-laurate, 1-^{14}C-palmitate, 1-^{14}C-stearate, 1-^{14}C-oleate, and U-^{14}C-D-glucose, respectively, were incorporated into phospholipids. Phospholipid fatty acids accounted for 95-96% total phospholipids-^{14}C when ^{14}C-substrates, other than glucose, were used. For glucose, only 20% phospholipids-^{14}C was in phospholipid fatty acids. More than 80% phospholipid fatty acids-^{14}C was in palmitic acid when 1-^{14}C-acetate and U-^{14}C-D-glucose were used, while 37, 61, 80, and 94% phospholipid fatty acid-^{14}C from 1-^{14}C-laurate, 1-^{14}C-stearate, 1-^{14}C-oleate, and 1-^{14}C-palmitate, respectively were recovered in the original form of the fatty acid used. The newly synthesized phospholipid fatty acid (13-24%) from 1-^{14}C-laurate, 1-^{14}C-stearate, and 1-^{14}C-oleate was palmitic, and 10% of phospholipid fatty acid from 1-^{14}C-stearate was in oleic acid. Hydrolysis by phospholipase A showed that ^{14}C from perfused substrates was esterified to both α and β positions of phospholipids. It was found that positional selectivity of phospholipid fatty acids was determined by chain length, degree of unsaturation, and source of fatty acid.

INTRODUCTION

In a previous communication (1), we demonstrated that minced rat lung was capable of oxidizing a series of ^{14}C-substrates to $^{14}CO_2$ and incorporating them into ^{14}C-lipids. Most of the lipid-^{14}C was recovered in phospholipids (PL), more specifically phosphatidyl choline (PC). With the exception of glucose, more than 90% PL-^{14}C from all other substrates was in the fatty acid (FA) moiety. The newly formed phospholipid fatty acids (PLFA) were predominantly palmitic acid, synthesized via the de novo pathway.

Present work, discussed in this article, is a continuation of previous work to investigate: (A) the rate of utilization of various ^{14}C-labeled substrates and the patterns of lipids synthesized in isolated perfused rat lung in comparison to the incubated minced lung, (B) synthesis of PLFA from various ^{14}C-substrates, and (C) the species of the newly formed PLFA and their positions of esterification.

MATERIALS AND METHODS

Animals and Perfusion Apparatus

Normal fed male rats of the Sprague-Dawley strain, weighing 250-300 g, were used. The apparatus for the perfusion of isolated rat lung was designed and constructed by Delaunois (2). Perfusion with this apparatus allows the isolated lung to be ventilated in a close artificial thorax in which a negative pressure is produced periodically at a frequency of ca. 20 cycles/min, while the lung is being perfused simultaneously with a buffered fluid through the pulmonary artery through a perfusion cannula. During perfusion, the lungs are breathing O_2-CO_2 (95:5%) through a tracheal cannula in which the respiratory gas passes freely and under normal atmospheric pressure. In addition, the respiratory gas can be saturated with water or with an aerosol. A selector stopcock is used to switch either to the normal water saturated gas or to the aerosol generator.

Preparation of Lung and Perfusion System

The cannulation and isolation of rat lung were performed according to the procedure described by Delaunois (2) with some modifications. These modifications are described briefly. The rats were anesthetized with Nembutal and bled by cutting the descending aorta in the abdominal cavity; the thorax then was opened widely. Two loose ligatures were made,

[1]Presented at the AOCS Meeting, Atlantic City, October 1971.

TABLE I

Conversion of Various ^{14}C-Substrates into Total Lipids and Distribution of ^{14}C in Various Lipid Fractions[a]

Substrate	TL		PL	NL	FFA
	Percent[b]	μmoles/g/hr[c]	Percent[d]	Percent[d]	Percent[d]
1-^{14}C-Acetate	7.4	0.86 ± 0.07	91.4	7.7	0.9
1-^{14}C-Laurate	4.8	0.57 ± 0.06	61.9	27.5	10.5
1-^{14}C-Palmitate	19.3	2.05 ± 0.18	75.2	21.4	3.4
1-^{14}C-Stearate	11.2	1.22 ± 0.10	66.5	23.2	10.3
1-^{14}C-Oleate	12.5	1.35 ± 0.09	55.8	34.7	9.5
U-^{14}C-D-Glucose	2.2	0.28 ± 0.02	92.7	6.9	0.4

[a]TL = total lipids, PL = phospholipids, NL = neutral lipids, and FFA = free fatty acids.

[b]Percentage of initial ^{14}C present in the perfusion fluid.

[c]μmoles substrate utilized/g wet lung/hr (mean ± standard error).

[d]Percentage of total lipid-^{14}C, calculated from (individual lipid fraction-^{14}C/total lipid-^{14}C) x 100. Results are averages of four experiments.

one around the pulmonary artery and the other around the trachea. An incision was made into the right ventricle, and the perfusion cannula was inserted into the pulmonary artery and securely tied with the ligature. The heart then was cut off quickly. The other cannula was inserted into the trachea through an incision in the mid-trachea and securely tied. The lung then was isolated and placed in the artificial thorax. The lung was first perfusion washed with warm (37 C) Krebs-Ringer bicarbonate (KRB) buffer for 3 min. The experimental perfusion fluid containing ^{14}C-substrate then was introduced and recirculated at 37 C for 60 min with a flow rate of 10-12 ml/min.

The perfusion fluid was a KRB buffer, pH 7.4, 0.15 M, containing 20 μmoles ^{14}C-labeled substrate with a specific activity of 0.20-0.25 μCi/μmole, 15 mg glucose, and 370 mg bovine serum albumin (BSA). The total perfusion was 15 ml. The perfusion fluid was equilibrated with a gas phase of 95% O_2-5% CO_2 before and throughout the perfusion. The FA substrates were complexed with BSA by the method of Fillerup, et al. (3).

Analysis of Lipids

At the end of the perfusion, the lungs were perfusion washed with KRB buffer for 2 min, then homogenized, and lipids extracted by the method of Folch, et al. (4). Procedures for lipid analysis and radioactivity determinations were as previously described (1). To determine the position of PLFA esterification, the PL was incubated with snake venom phospholipase A (*Crotalus admanteus* lyophilized venom, Ross Allen's Reptile Institute, Silver Springs, Fla.) according to the modified method of Robertson and Lands (5). The completeness of phospholipase A hydrolysis was monitored by sampling the incubation medium at time intervals and analyzed by thin layer chromatography (TLC) for free fatty acids (FFA) and lysophospholipids. The hydrolytic reaction was considered complete when the total PL radioactivity was distributed completely between FFA and lysophospholipid spots. The hydrolysates were extracted using the method of Folch, et al., (4) and the β position PLFA was separated from lysophospholipids by silicic acid column chromatography (6). To determine the FA pattern of the α position of PL, the lysophospholipids were saponified with 10% ethanolic potassium hydroxide, as described previously (1).

1-^{14}C-Acetate, U-^{14}C-D-glucose, 1-^{14}C-palmitic, 1-^{14}C-stearic, and 1-^{14}C-oleic acids were purchased from New England Nuclear Corp., Boston, Mass. 1-^{14}C-Lauric acid was obtained from Amersham Searle Corp., Arlington Heights, Ill. These labeled materials were tested for purity by TLC and gas liquid chromatography (GLC) and were found to be 98% pure or better. Unlabeled FA were purchased from the Hormel Institute, Austin, Minn.

RESULTS

Total Lipid Synthesis and Fractions of Lung Lipids

The isolated perfused rat lung readily incorporated ^{14}C-radioactivity from various ^{14}C-substrates into tissue lipids. Table I summarizes the results of total lipids synthesized from various ^{14}C-substrates and the distribution of ^{14}C-radioactivity in the major lipid fractions. Long chain FA substrates (palmitate, stearate, and oleate) were incorporated into lipids at a rate greater than acetate and laurate. Glucose was the poorest substrate for lipid synthesis.

Radioactivity in tissue total lipids (TL) of

TABLE II

Incorporation of ^{14}C-Labeled Substrates into Phospholipid Fatty Acids by Isolated Perfused Rat Lung

Substrate	Percent[a]	μmoles/g/hr[b] (mean ± standard error)
1-^{14}C-Acetate	6.54 ± 0.13	0.76 ± 0.06[a]
1-^{14}C-Laurate	2.85 ± 0.22	0.34 ± 0.04
1-^{14}C-Palmitate	13.89 ± 1.32	1.48 ± 0.13
1-^{14}C-Stearate	7.11 ± 0.79	0.78 ± 0.06
1-^{14}C-Oleate	6.79 ± 0.85	0.75 ± 0.05
U-^{14}C-D-Glucose	0.55 ± 0.03	0.07 ± 0.01

[a]Percentage of the initial ^{14}C-substrate present in perfusate. Results are averages of four experiments (mean ± standard error).

[b]μmoles ^{14}C-substrate/g fresh tissue/hr.

the perfused lung was found mainly in PL. In experiments using ^{14}C-acetate or ^{14}C-U-glucose as substrate, more than 90% TL-^{14}C was recovered from PL fraction, while neutral lipids (NL) accounted for the remaining TL-^{14}C. When long chain FA were used as substrates, the ^{14}C-radioactivity in PL fraction was 56-75% that in TL. In addition to NL, 3-10% TL radioactivity was found in tissue FFA.

Further fractionation of PL and NL was performed by TLC. Of the PL fractions, PC contained the major amount of ^{14}C-radioactivity ranging from 76-89% of that in the total PL. The major ^{14}C-lipid in NL was triglyceride which accounted for 58-87% total NL radioactivity. The radioactivity of PL was mainly in the FA moiety. When FA were used as substrates in the perfusion of rat lung, 95-96% PL radioactivity was in PLFA. With glucose as substrate, only 20% total PL radioactivity was recovered from the FA moiety.

PLFA Synthesis

The incorporation of ^{14}C-labeled substrates into the FA moiety of PL by the isolated perfused lung is shown in Table II. Of all the FA substrates used, palmitate was incorporated into PLFA to the greatest extent, while laurate was the poorest FA to be utilized. In 1 hr of perfusion, 6.5, 2.9, 13.9, 7.1, and 6.8% of the ^{14}C-radioactivity in the perfusate was incorporated into PLFA for ^{14}C-labeled acetate, laurate, palmitate, stearate, and oleate, respectively. Only 0.6% ^{14}C-radioactivity of glucose was incorporated into the FA moiety of PL.

Distribution of Radioactivity in PLFA

Table III shows the distribution of ^{14}C in the individual PLFA. It can be seen that acetate and glucose were converted primarily to palmitic acid amounting to 82% total ^{14}C-PLFA. Long chain FA substrates can either be esterified directly into PL or metabolized and incorporated into PL as other FA. Direct esterification accounted for 37, 94, 61, and 82% of ^{14}C-PLFA when laurate, palmitate, stearate, and oleate, respectively, were used as substrate.

TABLE III

Distribution of ^{14}C-Radioactivity in Individual Phospholipid Fatty Acids[a]

	^{14}C-Substrate					
Fatty acid	Acetate	Laurate	Palmitate	Stearate	Oleate	Glucose
12:0[b]	0.6	37.4	0.2	Trace	2.5[c]	10.6[c]
14:0	9.2	14.7	0.6	3.4		
16:0	82.4	41.5	94.4	22.4	12.7	82.4
16:1	Trace	Trace	Trace	2.5	Trace	Trace
18:0	3.9	3.9	3.5	60.7	2.4	4.5
18:1	3.0	3.7	1.1	10.5	80.1	1.8
18:2	Trace	0.6	Trace	0.6	0.9	Trace
18:3	Trace	Trace	Trace	Trace	Trace	Trace
20:4	Trace	Trace	Trace	Trace	Trace	Trace

[a]Values are expressed as percentage of total phospholipid fatty acid ^{14}C. Results are from average of two experiments.

[b]Carbon chain length and number of double bonds.

[c]Combination of 12:0 and 14:0.

TABLE IV

Distribution of Phospholipid Fatty Acid Radioactivity in α and β Positions

Substrate	α (percent)[a]	β (percent)[a]
1-^{14}C-Acetate	38.3	61.7
1-^{14}C-Laurate	31.5	68.5
1-^{14}C-Palmitate	49.2	50.8
1-^{14}C-Stearate	47.6	52.4
1-^{14}C-Oleate	33.2	66.8
U-^{14}C-D-Glucose	36.7	63.3

[a]Percentage of total phospholipid fatty acid was calculated from (α or β phospholipid fatty acid/total phospholipid fatty acid) x 100. Results are averages of three experiments.

On the other hand, 42, 22, and 13% ^{14}C-PLFA from laurate, stearate, and oleate, respectively, were incorporated as palmitic acid. In addition to palmitic, 11% ^{14}C-PLFA from stearate was in the form of oleic acid.

Position of PLFA

The distribution of the radioactivity between the α and β positions of PL is shown in Table IV. In experiments using ^{14}C-labeled palmitate or stearate as substrate, the radioactivity was distributed evenly between the α and β position when ^{14}C-labeled acetate, glucose, laurate, and oleate were perfused as substrate.

Table V shows the radioactivity of the individual FA at the α and β positions of PL. More lauric (12:0) and myristic (14:0) acids were esterified to the β position than to α position. Palmitic acid (16:0) from the perfusion fluid was incorporated evenly between these two positions; however, the newly formed palmitic acid from the other substrates was esterified preferably to the β position. Stearic (18:0) was esterified primarily to the α position and oleic acid (18:1) was esterified mostly to the β position.

DISCUSSION

The results of this study confirm the findings of other researchers that isolated perfused lung is capable of utilizing ^{14}C-labeled substrates (7-11) and of incorporating them into FA and other lipids. In addition, these investigations indicate that the isolated perfused lung is a useful system for the study of the metabolic and synthetic functions of this organ.

The conversion of various substrates to tissue lipids, especially PL, was greater in the perfusion system than that in the tissue incubation system. The increase in the rate of lipid synthesis is believed to be due to the higher efficiency of the perfusion system in transferring substrates from the medium to the cells or synthetic site.

The observations made in experiments with lung perfused with various FA indicated that there was an indiscriminate uptake of different FA from the perfusion fluid and that the rate of uptake or incorporation of these FA is variable. Results from this study revealed that palmitic (16:0), stearic (18:0), lauric (12:0), and oleic (18:1) acids were incorporated indiscriminately into TL by the isolated perfused lung, and the rate of incorporation was in the order of 16:0 > 18:1 > 18:0 > 12:0. From these observations, it is evident that the FA composition of the biosynthetically formed lung PL can vary to a great extent depending upon the availability and concentration of FA present in the perfusion medium. It is also interesting to note that the isolated perfused lung not only esterified ^{14}C-labeled laurate, stearate, or oleate directly to PL, but also converted them to palmitic acid, then incorporated it to PL. The PL palmitic acids synthesized from laurate, stearate, and oleate were apparently the products of an active β oxidation (11) and of the de novo pathway (12) present in lung tissue (1). The isolated perfused rat lung also possesses the ability to desaturate circulating stearic acid. One-tenth of PLFA from labeled stearic (18:0) was found as oleic (18:1) within 1 hr of perfusion (Table II).

It is now well established that the PLFA of mammalian tissues are arranged in a nonrandom fashion with respect to α and β positions. In most mammalian tissues, the saturated FA of PL are esterified mainly at the α position, while unsaturated FA are confined chiefly to the β position (13-16). In contrast to other tissues, lung PL contain a large percentage of saturated FA, particularly palmitic acid at the β position (17).

There is no simple generalization to predict the selective esterification of a saturated or unsaturated FA in the lung. The chain length, unsaturation, and source (endogenous or exogenous) of FA all must be considered. Present work indicates that the positional selectivity by isolated perfused rat lung for FA manifests a relative rather than an absolute specificity. A single FA can be esterified to either α or β position of PL (Table V).

With regard to FA chain length, palmitic (16:0) seems to represent a critical carbon chain length by which the relative selectivity of a FA may be predicted. FA with carbon chain length less than 16 were esterified primarily to the β position, while those with chain length

greater than 16 carbons were favored for the α position. Palmitic acid was distributed ca. evenly between α and β positions.

Unsaturation of FA is another important factor which helps to determine the position of esterification. In the PL of lung tissue, unsaturated FA of most chain length were confined mainly to the β position, while saturated acids with chain lengths longer than 16 carbons were esterified primarily to the α position. However, the source of FA also must be considered. A good example of this is palmitic acid. The palmitic acid in the perfusate (exogenous) was esterified ca. evenly between the two positions, whereas the newly formed palmitic acid (endogenous) from all other substrates in this study shows a preference for the β position. The unusual characteristics of lung tissue to confine such a large percentage of palmitic acid to the β position of PL provide evidence for the formation of dipalmityl PC (17) and for the theory that the lung itself is the source of the surface active component of pulmonary surfactant (18).

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TABLE V

Distribution of ^{14}C-Radioactivity in α and β Positions of Phospholipid Fatty Acids[a]

Fatty acid	^{14}C-Substrate											
	Acetate		Laurate		Palmitate		Stearate		Oleate		Glucose	
	α	β	α	β	α	β	α	β	α	β	α	β
12:0[b]	0.4	0.2	11.2	26.2	0.1	0.1	Trace	Trace	0.7	1.7[c]	4.3	6.3[c]
14:0	3.1	6.1	4.5	10.1	0.3	0.3	0.2	0.2	4.4	8.6	24.1	58.5
16:0	30.5	51.9	13.2	28.2	45.3	49.1	5.9	16.2	Trace	Trace	Trace	Trace
16:1	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	3.4	1.0
18:0	3.2	0.7	2.7	1.1	3.2	Trace	38.9	20.2	1.8	0.6	0.3	1.5
18:1	0.5	2.5	0.4	1.3	0.3	0.7	1.1	10.4	27.2	53.6	Trace	Trace
18:2	Trace	Trace	0.2	0.4	Trace	0.2	Trace	1.3	0.2	0.7	Trace	Trace
18:3	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
20:4	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace

[a]Values are expressed as percentage of total ^{14}C phospholipid fatty acids. Results are averages of two experiments.
[b]Carbon chain length and number of double bonds.
[c]Combination of 12:0 and 14:0.

The Effect of Peroxidized Arachidonic Acid upon Human Platelet Aggregation

HUBERT S. MICKEL, Department of Neurology, Children's Hospital Medical Center, and **JEFFREY HORBAR**, Department of Neurology, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT

Human platelet aggregation was studied in vitro following exposure to free arachidonic acid and peroxidized arachidonic acid. A slow aggregation response was caused by free arachidonic acid, whereas a rapid, marked response resulted from exposure to peroxidized free arachidonic acid. Aggregation resulting from peroxidized arachidonic acid was not counteracted by adenosine nor by prostaglandin E_1, both in high concentrations. Peroxide-induced platelet aggregation required the presence of added calcium ions in vitro. The aggregation resulting from exposure to peroxidized arachidonic acid was abolished by prior treatment of the lipid peroxide with tocopherol and butylated hydroxy toluene.

INTRODUCTION

In this study, we investigated the effect of the polyunsaturated fatty acid, arachidonic acid, on human platelet aggregation. Specifically, we determined that there is a marked effect upon platelet aggregation if the fatty acid has been peroxidized. Peroxidized lipids have been demonstrated in platelet concentrates, increasing in concentration with increasing age of the platelets (1).

Prostaglandin E_2, which is derived from arachidonic acid, enhances platelet aggregation, whereas prostaglandin E_1 does not (2). Long chain fatty acids induce the aggregation of platelets (3-6). The aggregation of platelets induced by saturated fatty acids involves adenosine diphosphate (ADP) and is inhibited by the addition of adenosine (3,7). Saturated fatty acids produce a greater degree of aggregation than do unperoxidized unsaturated or polyunsaturated fatty acids, such as linoleic or linolenic acid (3,5,8).

It is possible that the aggregation of platelets induced by peroxidized arachidonic acid contributes to intravascular hypercoagulability states. Lipids have been implicated in the hypercoagulability of blood (8,9). Hypercoagulability has been demonstrated in blood from humans taken after a meal with a high fat content (10-12). It also has been shown that the addition of certain fatty acids to plasma accelerates clotting time (13). Lipid peroxides usually are not well absorbed from the intestine (14,15); but, in experimental animals, absorption occurs with ingestion of large amounts of lipid peroxides (16), resulting in toxic effects (17).

MATERIALS AND METHODS

For each experiment, platelets were isolated from human blood collected in Fenwal packs containing 67.5 ml citric acid-dextrose (ACD)/450 ml unit blood. The whole blood was centrifuged at 2500 rpm for 4-1/2 min in a PR-6 International centrifuge. The plasma was decanted and centrifuged again at 3500 rpm for 6 min, and the platelet-poor plasma was pressed off. The platelet-rich plasma was stored at 5 C and used ca. 24 hr after the blood was withdrawn.

Arachidonic acid (Grade I, Sigma Chemical Co., St. Louis, Mo.) was oxygenated using soybean lipoxygenase (75,000 units/mg, P.L. Biochemicals, Milwaukee, Wisc.) as catalyst, to the corresponding 15-hydroperoxy eicosatetraenoic acid by the method of Hamberg and Samuelsson (18). Peroxide numbers in milliequivalents/1000 g sample were determined using the Olcott and Dolev modification (19) of AOCS method Cd 8-53 (20). For each experiment, 100 mg arachidonic acid were oxygenated and taken up in 1 ml methanol. The peroxide number of an aliquot then was taken.

Suspensions of washed platelets for use in the incubation and aggregometry studies were prepared by the method of Haslam (3). The platelet-rich plasma was centrifuged for 15 min at 750 g in polyprene test tubes. The plasma was decanted, and the platelet pellet was resuspended in washing medium composed of 0.154 M NaCl, 0.154 M Tris(hydroxymethyl)-aminomethane hydrochloride, pH 7.4, and 0.77 M ethylenediamine tetraacetic acid (EDTA) in proportions of 90:8:2 by vol. The suspension was centrifuged at 250 g for 12 min. The platelet pellet was resuspended to a final count of 2×10^8 platelets/ml in a medium composed of 0.154 M NaCl and 0.154 M Tris buffer, pH 7.4, in proportions of 9:1 by vol.

Platelet aggregation was measured on a

Chronolog aggregometer as decrease in extinction of white light by a stirred 0.5 ml sample of resuspended platelets. An aliquot of 0.020 ml 0.11 $CaCl_2$ was added to 0.5 ml washed resuspended platelets prior to each trial. The suspension was stirred automatically and the temperature equilibrated to 37 C. The effect of 0.020 ml 0.328 M arachidonic acid in methanol (peroxide number, 1450) upon the platelets was determined. The experiment was repeated using 0.328 M arachidonic acid in methanol (peroxide number, 50).

An aliquot of 0.010 ml 10^{-3} M adenosine was added to a platelet suspension as described above. After preincubation with adenosine, 0.010 ml 0.328 M arachidonic acid in methanol (peroxide number, 1260) was added and the aggregation recorded and compared to the effect of 0.010 ml peroxidized arachidonic acid in the absence of previously added adenosine. The experiment was repeated using 0.050 ml 10^{-3} M adenosine.

The effect of prostaglandin E_1 (PGE_1) also was determined (Upjohn Co., Kalamazoo, Mich.). An aliquot of 0.010 ml PGE_1 solution (10 ng/ul) was added to a platelet suspension. After equilibration, 0.010 ml 0.328 M arachidonic acid in methanol (peroxide number, 1260) was added and aggregation recorded and compared to that obtained in the absence of added prostaglandin. The experiment was repeated using 0.100 ml PGE_1 solution (10 μg/ml).

A solution of 0.328 M arachidonic acid in methanol (peroxide number, 1450) was incubated with ca. 1 M α-tocopherol and butylated hydroxy toluene (BHT) at 55 C for 15 min. The effect of 0.020 ml this solution upon platelet aggregation was determined and compared to that of the peroxidized arachidonic acid not treated.

An aliquot of 0.10 ml 0.328 M arachidonic acid in methanol (peroxide number 150), was added to 1 ml resuspended platelets (2 x 10^9 platelets/ml). The tubes were incubated with shaking for 30 min at 37 C and then centrifuged for 15 min at 10,000 rpm in a Sorvall S-24 centrifuge head. The supernatant was decanted and the tube rinsed twice with 1 ml aliquots of resuspension medium. The amount of peroxide present in the platelet pellet and supernatant rinse was determined, after extraction, by the Olcott and Dolev modification (19) of AOCS method Cd 8-53 (20).

RESULTS

Peroxidized arachidonic acid produced a marked aggregation of human platelets. The effect of 0.020 ml 0.328 M arachidonic acid (peroxide number, 1450) added in methanol to 0.5 ml human platelet suspension is shown in Figure 1A. The initial increase in turbidity, as shown by the upward deflection of the aggregometer response, is due to calcium soap formation, since the same effect is observed upon mixing fatty acid with the buffer containing calcium. In the absence of platelets, the increase in turbidity remains constant throughout the period of observation. The downward deflection is attributable predominantly to platelet aggregation. A small portion of the downward deflection might be due to uptake of the fatty acid by platelets. This uptake is not likely to be a major contribution, since the decrease in turbidity from both uptake of fatty acid and platelet aggregation with free, unperoxidized arachidonic acid is a slow response, as shown in Figure 1B.

A secondary disaggregation was seen occasionally, as shown in Figure 1A. This observation was not seen consistently with this concentration of peroxidized arachidonic acid, and it was not observed with samples using a concentration of peroxidized arachidonic acid

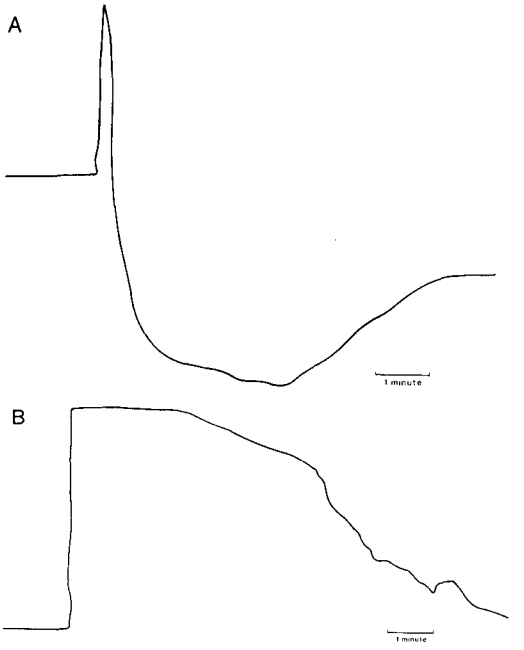

FIG. 1A. Aggregometer response to addition of 5.85 x 10^{-3} M peroxidized arachidonic acid (0.020 ml 0.328 M arachidonic acid, peroxide number, 1450) to 0.5 ml suspension of human platelets containing 2 x 10^8 platelets/ml. 1B. Aggregometer response to addition of relatively unperoxidized arachidonic acid; total peroxide concentration: 1.8 x 10^{-5} M (0.020 ml 0.328 M arachidonic acid, peroxide number, 50) to 0.5 ml suspension of human platelets containing 2 x 10^8 platelets/ml.

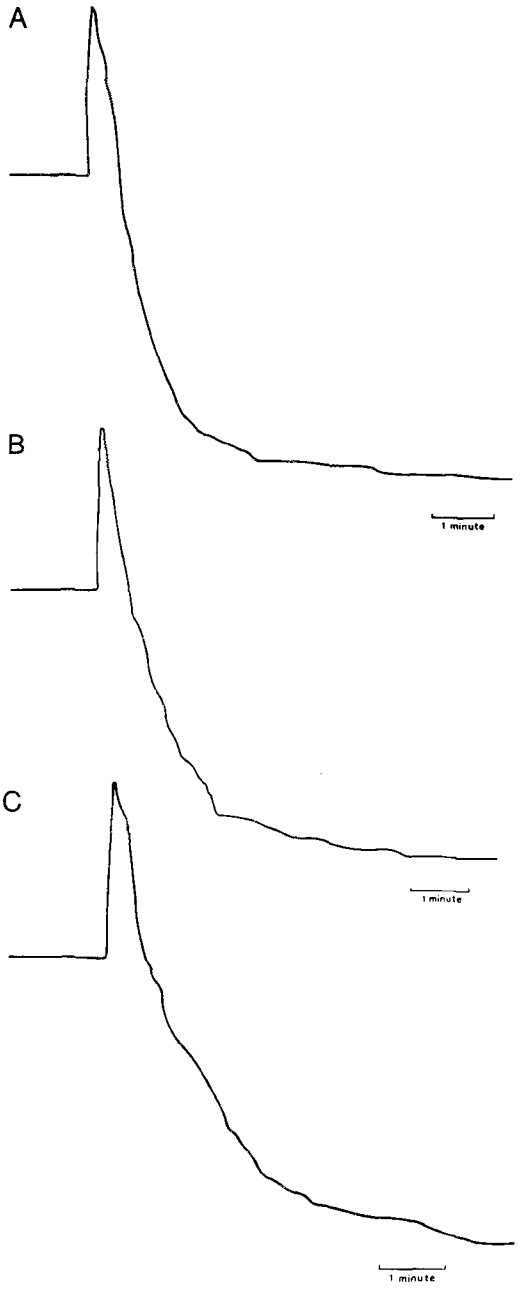

FIG. 2A. Aggregometer response to addition of 2.5 x 10^{-3} M peroxidized arachidonic acid (0.010 ml 0.328 M arachidonic acid, peroxide number, 1260) to 0.5 ml suspension of human platelets containing 2 x 10^8 platelets/ml. 2B. Aggregometer response to addition of the same amount of peroxidized arachidonic acid as in 2A to platelets preincubated with 1 x 10^{-4} M adenosine (0.050 ml 10^{-3} M adenosine to 0.5 ml suspension). 2C. Aggregometer response to addition of the same amount of peroxidized arachidonic acid as in 2B to platelets preincubated with 6 x 10^{-6} M prostaglandin E_1 (0.10 ml PGE_1 solution, 10 ng/ul, added to 0.5 ml platelet suspension).

of ca. half, as shown in Figure 2A.

The aggregation effect of the peroxidized arachidonic acid was abolished by prior incubation of the peroxidized arachidonic acid with excess α-tocopherol and BHT at 55 C for 15 min. Little, if any, peroxide was recovered from the sample of arachidonic acid treated in this manner with antioxidants (peroxide number, 0-50).

In the absence of calcium ions, no aggregation occurred, even with high concentrations of peroxidized arachidonic acid, 0.020 ml 0.328 M (peroxide number, 1450). The addition of 0.010 ml or 0.020 ml methanol alone produced no effect upon platelet suspensions.

Preincubation with adenosine or prostaglandin E_1 had no observable effect upon the aggregation induced by peroxidized arachidonic acid, as shown in Figure 2A, B, and C. In these experiments, 0.010 ml 0.328 M arachidonic acid (peroxide number, 1260) was added. Concentrations of adenosine of 2 x 10^{-5} M or 1 x 10^{-4} M produced no effect. Final concentrations of prostaglandin E_1 of 1.2 x 10^{-6} M and 6 x 10^{-6} M produced no observable difference from that of the peroxidized arachidonic acid alone.

Incubation of peroxidized arachidonic acid with human platelet suspension for 30 min at 37 C resulted in the recovery of 21% original peroxide still associated with the platelet pellet.

DISCUSSION

Peroxidized arachidonic acid has an effect upon platelet aggregation that differs from that produced by free, unperoxidized fatty acids. A slow aggregation response was observed to free relatively unperoxidized arachidonic acid, comparable to that reported with long chain saturated fatty acids and observed to require ADP (3-7). Rapid aggregation resulted when peroxidized arachidonic acid was added. The preparation of platelet suspensions in buffer solutions usually produces platelets that do not aggregate with the usual stimuli, such as ADP, collagen, or epinephrine. However, the platelets were slowly responsive to unperoxidized arachidonic acid, which is considered to be mediated through an ADP-associated mechanism. To have such a dramatic response of platelets in which their ability to aggregate might be reduced underscores the potency of the lipid peroxide studied.

It is possible that ADP is not required for the aggregation of platelets induced by peroxidized arachidonic acid. The effect is not blocked by concentrations of adenosine which should block ADP-induced aggregation (7).

Similarly, prostaglandin E_1 had no measurable effect upon aggregation in concentrations greater than that required to block the effect of prostaglandin E_2 (21). Boullin, et al., concluded that ADP induces platelet aggregation by binding to specific receptors and that PGE_1 inhibits this by interfering with the ADP binding (22).

Peroxidized arachidonic acid can be associated with platelets in vitro without being destroyed. It is possible that peroxidized arachidonic acid is adsorbed onto platelet surfaces without destruction of the labile peroxyl group. It is not determined whether the association of peroxidized arachidonic acid with platelets can occur in vivo. However, lipid peroxides have been shown to increase in concentration with increasing age of harvested platelets (1,23).

The findings of Hoak, et al., suggest that a large portion of the uptake of free, saturated fatty acids by platelets is associated with the platelet surfaces (24). Fatty acids were recoverable from platelet surfaces despite the presence of albumin in the incubating solutions. If saturated fatty acids can be adsorbed onto platelet surfaces, peroxidized fatty acids might be adsorbed as well.

The biological implications of the effect of peroxidized arachidonic acid upon platelet aggregation are varied. The hypercoagulability of blood, following a meal with a high fat content, might be attributable in part to the adsorption of lipid peroxides along with other lipids onto platelet surfaces. The altered platelet function induced by peroxidized fatty acids might contribute to thromboembolic phenomena. On the other hand, adsorption of peroxidized fatty acids onto platelet surfaces might be a mechanism for the transport of these highly labile and toxic lipids by the blood. Such a phenomenon could explain the damage to brain resulting from an intraperitoneal injection of peroxidized linoleic acid, reported in the chick by Nishida, et al. (25).

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Differential Trypsin Effect upon (1-^{14}C) Acetate Incorporation into Choline and Ethanolamine Glycerophosphatides of Rat Brain and Liver

STEVEN R. COHEN and **JOSEPH BERNSOHN**, Neuropsychiatric Research Laboratory, Veterans Administration Hospital, Hines, Illinois, and Loyola University Medical Center, Maywood, Illinois 60141

ABSTRACT

Preincubation of rat brain and liver slices in a medium (5% glucose, 5% fructose, 1% albumin, 1% trypsin, 10 mM phosphate buffer pH 6.0) used to pretreat brain tissue for the separation of cell types was found to uncouple the incorporation of (1-^{14}C) acetate into ethanolamine phosphoglycerides from that of the choline phosphoglycerides. Incorporation into ethanolamine phosphoglycerides was stimulated in both brain (330%) and liver (780%) slices, while the incorporation of (1-^{14}C) acetate into choline phosphoglycerides was reduced for both brain (71%) and liver (63%) slices, compared to control values from nonpreincubated material. With (1-^{14}C) linolenic acid as a precursor, no significant differences were found in incorporation into ethanolamine phosphoglycerides and choline phosphoglycerides.

INTRODUCTION

Studies of (1-^{14}C) acetate incorporation into brain phosphatides have demonstrated generally that the choline phosphoglycerides (CPG) have a more rapid turnover than the other major phosphatide fractions (1,2). The ethanolamine phosphoglycerides (EPG) usually incorporate less radioactivity than the CPG fraction, and this occurs whether measurements are made in vivo (3-5) or in vitro (1). Similar results were obtained in this laboratory when brain slices were incubated with (1-^{14}C) acetate, and the glia and neuron cell types were isolated subsequently (6). Under these conditions, the CPG fraction was the most active phosphatide in both cell types. We have reported also that, if neurons and glia are first isolated from developing rat brain by the method of Norton and Poduslo (7), which involves trypsinization of the cells prior to incubation, the cells readily incorporate (1-^{14}C) acetate into total lipids; but under these conditions EPG contains more radioactivity than CPG, which is relatively inactive (8). If isolated neurons and glia were incubated together, the CPG fraction remains relatively inactive. The apparent divergent results were studied extensively, and it was found that the incorporation of labeled acetate into the two major glycerophosphatides was affected differentially by the type of medium employed during the cell isolation procedure.

METHODS AND MATERIALS

The brains and livers of 10 day old Sprague-Dawley rats of both sexes were removed and sliced (0.70 mm) in a McIlwain tissue chopper. Neurons and astrocytes were isolated according to Norton and Poduslo (7). The tissue slices (ca. 200 mg) were preincubated in a medium of 0.5% trypsin (twice crystallized, salt-free from beef pancreas (Nutritional Biochemicals, Cleveland, Ohio), 1% bovine serum albumin (Cohn fraction V, Sigma Chemical, St. Louis, Mo.), 5% glucose, and 5% fructose in 10 mM phosphate buffer at pH 6.0 for 45 min (HAP-Tr medium). The tissue was washed free of trypsin and sieved successively through 150 μ nylon and 74 μ stainless steel meshes into 0.9 M sucrose, and separation of the cell types was accomplished on sucrose density gradient layers as previously described (7). When the effect of trypsin was being studied, brain or liver slices were incubated in the medium described in the absence of trypsin (HAP medium) to simulate the preincubation conditions required to separate the two brain cell types. Fifteen (linolenate experiments) brains and thirty (acetate experiments) livers were sliced, pooled, and divided into aliquots. After this preincubation period, the slices were washed free of medium by the addition of a 10 ml mixture of calf serum + 10 mM phosphate buffer pH 6.0 (9:1), cooled to 4 C and centrifuged at 120 x g for 5 min. The supernatant was discarded, and incorporation of the (1-^{14}C) acetate or (1-^{14}C) linolenate into the slices was studied by resuspension of the slices in salt medium (123 mM NaCl, 6 mM KCl, 1.5 mM $MgSO_4$, 12 mM glucose, 20 mM phosphate buffer pH 7.4) for 1 hr with 8 mg adenosine 5′-triphosphate (ATP), 2 mg nicotinamide adenine dinucleotide (NADH), 1 mg nicotinamide adenine dinucleotide phosphate (NADPH), and 1 mg coenzyme A (CoASH), (all purchased from Sigma Chemical). Ten μCi of (1-^{14}C)

TABLE I

Comparison of Slice and Cell Incubated Incorporation of (1-^{14}C) Acetate into Ethanolamine and Choline Phosphoglycerides of Isolated Neurons and Astrocytes in Rat Brain

Lipid fraction	Slice incubation		Cell incubation	
	Neurons	Astrocytes	Neurons	Astrocytes
EPG	0.39[a]	0.87	3.05	3.16
CPG	1.06	1.50	0.05	0.09

[a]Relative specific activity = $\frac{\text{specific activity phospholipid fraction}}{\text{specific activity total phospholipids}}$. The cells were prepared as described in the text according to Norton and Poduslo (7) with the use of trypsin. They were then incubated for 1 hr in the medium with (1-^{14}C) acetate, and the incorporation of the isotope into the two phosphatides was measured. The brain slides first were incubated in the medium with (1-^{14}C) acetate; and, after incorporation had occurred, the cells from the slices then were prepared as above. Details are described in the text.

sodium acetate (specific activity 62 mCi/mM) or 0.75 μCi linolenic acid (specific activity 57 mCi/mM) (all radioactive materials from Amersham/Searle, Arlington Heights, Ill.) and 5 ml of medium, plus sliced brains or livers, were used in all incorporation studies. The linolenic acid was prepared as the potassium salt and suspended in 10% albumin by sonication. The linolenate incorporation medium contained albumin at a final concentration of 0.2%. Three different conditions of incubations with brain and liver slices were evaluated: (A) Control (1 hr in salt medium + 10 μCi [1-^{14}C] Na acetate + cofactors, after 45 min in isotonic saline), (B) HAP (45 min preincubation in HAP without trypsin followed by 1 hr in salt medium + cofactors with 10 μCi [1-^{14}C] Na acetate), and (C) HAP-Tr (45 min preincubation in HAP trypsin followed by 1 hr in salt medium + cofactors + 10 μCi [1-^{14}C] Na acetate).

After the final incubation, the lipids were extracted by homogenization in $CHCl_3$-CH_3OH (2:1), and 0.9% NaCl was added to bring the aqueous volume to 20%. The organic phase was washed five times with upper phase to remove all acetate (9). In the linolenate experiments the free linolenic acid was separated from the phospholipids by chromatography on a (1.0 x 5.0 cm) silicic acid column. The neutral lipids, consisting principally of cholesterol and free fatty acids (FFA), were eluted with chloroform (150 ml) and the phospholipids with methanol (100 ml). The galactolipids remain on the column under these elution conditions. An aliquot of the total lipids (acetate experiments) or the phospholipid fraction (linolenate incubations) was taken for lipid specific activity determinations. The remainder was streaked across a 0.5 mm thick Silica Gel HR thin layer plate (Brinkman Instruments, Des Plaines, Ill.). The chromatogram was developed in $CHCl_3$-CH_3OH-acetic acid-H_2O (60:35:2:2) (10) and the bands identified by spraying one channel of the plate with ninhydrin and one channel with 50% H_2SO_4. Authentic standards (Applied Science, State College, Pa.) also were run for identification. Neutral lipid (acetate incorporation experiments only) and EPG and CPG spots were scraped from the nonsprayed part of the plate and the lipids eluted according to the method of Skipski (11). Aliquots were taken for phosphorus determination according to Bartlett (12) and radioactivity of the remainder determined in a Packard liquid scintillation counter model 3003. The scintillation fluid contained 98% diphenyl oxazole (PPO)-2% 1,4-bis-2-(5-phenyloxazoyl)-benzene (POPOP) in toluene (5 gm/liter). Counting efficiency was greater than 80%. Lipid wt were calculated by multiplying μmoles of phospholipid phosphorus by 750. Neutral lipid values were based upon the constant ethanolamine phosphatides-neutral lipid ratio in the same sample.

RESULTS AND DISCUSSION

The influence of the medium employed in the isolation of the neurons and astrocytes on the incorporation of (1-^{14}C) acetate into CPG and EPG is shown in Table I. When the isotope is incorporated into the brain slices first, and the cell types are then isolated by the trypsinization, the CPG fraction is ca. three times more active than EPG in neurons and twice as active in astrocytes. When the two cell types are isolated first with the use of trypsin and the isotope then incorporated into the phospholipids, the CPG fraction is inactive virtually in both cell types, whereas the EPG fraction is more active than if the cells are isolated after the slices are incubated. This was indicative of an effect of the trypsin medium upon the

TABLE II

Incorporation of (1-^{14}C) Acetate into Lipid Fractions of Rat Brain and Liver under Various Incubation Conditions

Lipid fraction	(A) Control	(B) HAP	(C) HAP + trypsin
Brain			
Total lipids	40.3[a]	5.4	3.7
Ethanolamine phosphatides	1.5	1.1	6.5
Choline phosphatides	2.0	0.7	0.7
Neutral lipids	125.0	15.0	6.0
Liver			
Total lipids	9.8	1.5	3.0
Ethanolamine phosphatides	1.0	1.4	8.8
Choline phosphatides	1.5	0.4	0.4
Neutral lipids	22.6	3.5	4.2

[a]Values are expressed as cpm/mg lipid x 10^{-2}. (A) Incubated in 123 mM NaCl, 6 mM KCl, 1.5 mM $MgSO_4$, 12 mM glucose, 20 mM phosphate buffer pH 7.4 and 10 μCi (1-^{14}C) Na acetate, 8 mg adenosine 5'-triphosphate, 2 mg nicotinamide adenine dinucleotide, 1 mg nicotinamide adenine dinucleotide phosphate, and 1 mg coenzyme A; total volume = 5 ml. (B) Preincubated in 5% glucose; 5% fructose; 1% serum albumin, 10 mM phosphate buffer pH 6.0; then incubated in medium A. (C) Preincubated in medium B and 1% trypsin, then incubated in medium A. The vessels contained 55-90 mg brain phospholipids or 30-50 mg liver phospholipids. Air was the gas phase for the incubation period, and preincubation was in 50 ml HAP or HAP-trypsin with O_2 as the gas phase.

uptake of acetate into the two phosphatides.

To investigate this phenomenon, the effect of the incubation medium with and without trypsin on brain slices was studied and the results are shown in Table II. Comparisons of brain and liver phosphatides are presented also. The data indicate that compared to controls (A), the cell isolation medium without trypsin (B) reduced (1-^{14}C) acetate incorporation into total lipids, neutral lipids, and the CPG fractions of brain and liver markedly. The medium reduced the specific activity of the CPG fraction from 200 to 70 in brain and from 150 to 40 in liver. Incorporation into the EPG fraction was not depressed by the HAP medium. Preincubation with HAP also decreased acetate incorporation into total brain lipids from control values of 4030 cpm/mg lipid to 540 cpm/mg lipid. The corresponding values for neutral lipids were 12,500 cpm/mg lipid for controls and 1500 for the HAP medium. However, the addition of trypsin to the HAP medium stimulated the incorporation of (1-^{14}C) acetate into brain and liver EPG above the control values, while the CPG values were unaffected by the addition of the enzyme. In brain and liver the sp. act. for this fraction was found to be 650 and 880 in the HAP-Tr (C) medium, compared to control values of 150 and 100 respectively, an increase of 330% for brain and 780% for liver. Comparable values for brain and liver CPG with trypsin are ca. 30% control values. The addition of 5 mM choline had no effect upon the incorporation of acetate into either CPG or EPG. Preincubation in salt medium for 45 min also had no effect upon acetate incorporation.

To determine whether this differential effect of the medium and of trypsin occurred in the de novo synthetic pathway or during the elongation or acylation steps, a similar study was performed using (1-^{14}C) linolenic acid as a precursor (Table III). (1-^{14}C) Linolenate incorporation did not appear to be affected by preincubation in HAP (B) in brain. Some stimulation of incorporation into phosphatides was observed in liver. In the case of brain slices, the inclusion of trypsin in the preincubation medium reduced incorporation into total phospholipids by ca. 50%. The EPG and CPG fractions were ca. equally affected, the specific activity of both being reduced by 45% (CPG) and 60% (EPG). The differences between EPG and CPG probably are not significant. No stimulatory effect of the trypsin observed above with (1-^{14}C) acetate as a precursor was noted. In fact, preincubation with the enzyme appeared to inhibit incorporation into the phosphatides. Preincubation with trypsin affected the two phosphatide fractions in liver differently. EPG incorporation was decreased slightly, while CPG incorporation was increased. The EPG changes may not be significant, while the CPG alterations are ca. twice as great as the control values. The results with (1-^{14}C) linolenate, thus, show no effect of the trypsin, as was observed with (1-^{14}C) acetate, and indicate that the de novo pathways are affected by the enzyme.

It appears that preincubation of brain and

TABLE III

Incorporation of (1-^{14}C) Linolenate into Phospholipid Fractions of Rat Brain and Liver under Various Incubation Conditions

Lipid fraction	(A) Control	(B) HAP	(C) HAP + trypsin
Brain			
Total phospholipids	66.4[a]	63.4	30.9
Ethanolamine phosphatides	43.4	57.8	19.4
Choline phosphatides	84.8	70.8	40.1
Liver			
Total phospholipids	39.6	47.6	32.5
Ethanolamine phosphatides	18.7	34.7	11.9
Choline phosphatides	22.6	37.6	39.0

[a]Values are expressed as cpm/mg lipid x 10^{-2}. Columns A, B, and C are as in Table II except that each incubation vessel contained 25-50 mg brain phospholipid or 9-17 mg liver phospholipid and 0.75 μCi (1-^{14}C) linolenic acid instead of (1-^{14}C) acetate.

liver slices with HAP-Tr uncouples the incorporation of (1-^{14}C) acetate into brain and liver EPG from that of brain and liver CPG, as well as the neutral lipid fraction. The uncoupling is demonstrable by the fact that the addition of trypsin to the preincubation medium reverses the inhibition of acetate incorporation into EPG produced by incubation in HAP but does not affect CPG labeling in both brain and liver. The inhibitory effect of HAP on CPG uptake of acetate and its irreversibility by trypsin also appears to prevail for the neutral lipid fraction.

Measurements have been made on the total EPG fraction; and, due to the diversity of molecular species in that fraction and their heterogenous metabolic activity, it is possible that not all the EPG's are affected similarly. Thus, trypsin may differentially affect the diacyl phosphatides, as compared to the plasmalogens.

The activation effect of trypsin on acetyl CoA carboxylase is well-documented (13), so that a stimulation of (^{14}C) acetate-uptake by this enzyme may not be surprising. That this effect is not observed with linolenic acid as a precursor would seem to implicate the fatty acid (FA) de novo synthetic pathway as the metabolic sequence affected by the trypsin. However, trypsin is thought not to enter viable tissue (14), and the observed effect may be due to a modification of the plasma membrane by trypsin. The results obtained do not appear explicable on the basis of a preferential degradation of one of the phosphatides, since the ratio of EPG/CPG did not change during the experiment.

There is, derived from phosphatidic acid, a mixed pool of 1,2 diacyl glycerol, the acceptor molecule for both EPG and CPG synthesis via the cytidine pathways (15). Some of the 1,2 diacyl glycerol species will be more unsaturated than others, and there is a selective utilization of the more unsaturated species by the enzyme CDP-ethanolamine:1,2-diglyceride-ethanolamine phosphotransferase (EC 2.7.8.1) (16-18). Since the major brain polyunsaturated FA found in EPG are formed by elongation, this process may proceed unaffected by the medium employed to supply these acids for ultimate EPG synthesis. The de novo FA which go into CPG are affected by the medium. Therefore, an alteration in the composition of the available pool of FA due to inhibition of de novo synthesis may reduce the pool of these FA for synthesis of CPG. The enzymes ATP:choline phosphotransferase (EC 2.7.1.32), CTP:choline phosphate cytidyl transferase (EC 2.7.7.15) and CDP choline:1,2-diglyceride choline phosphotransferase (EC 2.7.8.2) probably are not affected differentially from the anologous enzymes involved in EPG synthesis, since linolenic acid incorporation into the two phosphatides is unaffected by conditions which alter acetate incorporation.

That the medium employed may affect lipid metabolism in a specific manner is significant, since such medium is used widely in tissue culture preparations and tissue prepared in this manner may not represent normal tissue with regard to lipid metabolism. A similar line of reasoning may be advanced on the use of trypsin in the preparation of specific brain cell types.

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Inhibition of Phospholipase C by Isosteric Phosphinic Acid Analogues of Lecithin

ARTHUR F. ROSENTHAL and **SERGEI V. CHODSKY**, Department of Laboratories, The Long Island Jewish-Hillside Medical Center, New Hyde Park, New York 11040

ABSTRACT

Four synthetic diether phosphinate analogues of lecithin were studied for inhibitory activity against *Clostridium perfringens* phospholipase C. An order of inhibitory effectiveness of phosphonate and phosphinate was, thereby, developed. The inhibitions seem relatively independent of specific structural features; this is discussed with emphasis on physical effects which complicate the inhibition kinetics.

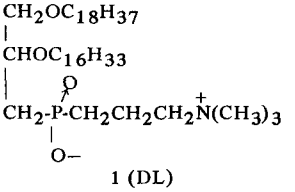
INTRODUCTION

We have reported the strongly inhibitory effect of several phosphonates (1) $\left[-C-\overset{\overset{O}{\nearrow}}{P}\begin{matrix}-O-\\ \diagdown\\ O-\end{matrix}\right]$ and a phosphinate $\left[-C-\overset{\overset{O}{\nearrow}}{P}\begin{matrix}-C-\\ \diagdown\\ O-\end{matrix}\right]$ (2) analogue of lecithin on *Cl. perfringens* phospholipase C. These substances appear to be the only specific, i.e. substrate analogue, inhibitors of this enzyme so far reported. In these studies, it was somewhat difficult to discern a pattern of inhibition as a function of structural geometry, in part because few synthetic analogues of these classes were available. A further complication in such work is the difficulty of separating purely physicochemical, e.g. particle-size, effects from those due to molecular geometry.

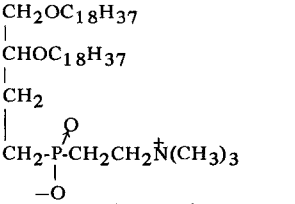
Subsequently, we reported syntheses of phosphinate analogues isosteric with natural lecithin on either (3) or both (4) sides of the phosphorus function. Optically active forms of two phosphinate analogues also were synthesized (5) in the same configuration as the natural substrate. Thus, it was of interest to determine the potency of these newer analogues as inhibitors of phospholipase C and, from the available data, to deduce, if possible, what structural features contribute to inhibitor specificity.

MATERIALS AND METHODS

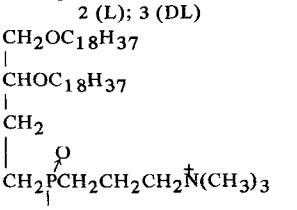
The phosphinic acid analogues of lecithin used were of the diether type and have the following structures:



1 (DL)



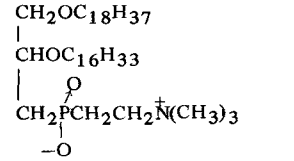
2 (L); 3 (DL)



4 (L)

(The optical nomenclature used is that of the original system for naming phospholipids. Thus, L has the same configuration around its optically active carbon as *sn*-3 in the stereochemical numbering system. However, the latter was specifically devised only for substitution products of glycerol, while the present compounds contain at best substitution derivatives of analogous alcohols rather than of glycerol itself; thus, the *sn* system could not, strictly speaking, be applicable. The newer R-S system is not yet widely employed for lipids and is, therefore, not used here.)

The synthesis of each of these substances has been described in detail (3-5). For comparison the following nonisosteric analogue, whose synthesis and effects on phospholipase C already have been reported (2), was used:



5 (DL)

TABLE I

Inhibition of Phospholipase C Catalyzed Lecithin Hydrolysis by Analogue 1[a]

Temperature (C)	Analogue, mg (μmol)	v, nmol/min	Percent of control
25	0	490	100
25	1.0 (1.35)	340	69
25	1.5 (2.1)	168	34
25	2.0 (2.7)	145	30
25	3.0 (4.1)	75	15
37	0	720	100
37	1.5 (2.1)	150	21
37	2.5 (3.4)	135	19

[a]Assay system contained the analogue (sonicated 2-3 min while cooling at 0 C) in the amounts shown; egg lecithin (chromatographically pure, hand-shaken dispersion in water), 4.0 μmol; calcium chloride, 5 μmol; enzyme, 0.2 mg; and water to a total volume of 6.0 ml. Reactions were followed at the temperature indicated (10.02 C) in a jacketed, closed vessel with magnetic stirring under a static nitrogen atmosphere by continuous automatic addition of 0.060 M KOH to maintain a pH of 7.00 ± 0.08.

The lecithin analogues were dispersed by sonication in water for 2 min, as previously described (1).

Phospholipase C was a partially purified product obtained from Sigma Chemical Co. The specific activity of the enzyme varied with different samples, but all preparations were much more active than those available for earlier studies of this enzyme (1,2).

Egg lecithin was prepared by the method of Singleton, et al., (6) followed by that of Lea, et al., (7), and dispersed as previously reported (1).

The enzymatic reaction was performed by the pH-stat procedure previously reported (1).

TABLE II

Inhibition of Phospholipase C Catalyzed Lecithin Hydrolysis by Analogues 2 and 3[a]

Temperature (C)	Analogue	mg Analogue (μmol)	v, nmol/min	Percent of control
25	None	0	420	100
25	2	1.75 (2.3)	168	40
25	3	1.75 (2.3)	230	55
37	None	0	600	100
37	2	1.50 (1.95)	220	37
37	3	1.50 (1.95)	210	35
37	None	0	720	100
37	3	1.0 (1.3)	350	49
37	3	2.0 (2.6)	220	31
37	3	3.0 (3.9)	160	22

[a]Conditions as in Table I.

TABLE III

Inhibition of Phospholipase C Catalyzed Lecithin Hydrolysis by Analogue 4[a]

Temperature (C)	Analogue, mg (μmol)	v, nmol/min	Percent of control
25	0	670	100
25	1.0 (1.3)	294	44
25	2.0 (2.6)	220	37
25	3.0 (3.9)	160	24
37	0	860	100
37	1.0 (1.3)	610	71
37	2.0 (2.6)	420	49
37	3.0 (3.9)	330	38

[a]Conditions given in Table I.

RESULTS

Table I shows the effects of the partially isosteric analogue 1 on the hydrolysis of lecithin by phospholipase C at 25 C and 37 C. The maximal velocities obtained are recorded in the table; these usually are reached after a brief lag period (in this experiment, 10-35 sec). The kinetic curves were quite regular in this experiment; the results indicate that the analogue is a fairly good inhibitor and that it is proportionately more effective at 37 C than at 25 C.

A direct comparison with the previously reported (2) inhibitor 5 in a separate experiment at 25 C indicated that the two are of comparable potency. Using 2.4 μmol of each inhibitor, analogue 1 gave an activity which was 29% control, a value similar to those found above, while analogue 5 gave an activity 47% control.

Table II gives inhibition data for the analogue which is oppositely isosteric with lecithin compared to analogue 1. Since this second analogue was available as an L-(analogue 2), as well as a racemic (analogue 3) form, their inhibitory potency was compared.

The results indicate that the L and DL forms do not differ greatly in inhibitory activity, particularly at 37 C. There is also appreciably more inhibition at the higher temperature. This analogue in either of the optical forms is, thus, appreciably less potent as an inhibitor than is the preceding analogue (1).

The completely isosteric analogue in its L-configuration (analogue 4) then was investigated as an inhibitor. The results, given in Table III, indicate that this is the poorest inhibitor of all those studied. An interesting characteristic of analogue 4 is that, unlike all others tested, it is a distinctly poorer inhibitor at 37 C than at 25 C.

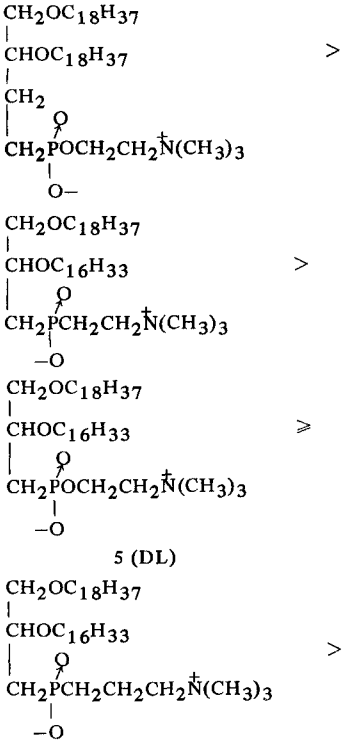
DISCUSSION

In studying an enzyme whose substrate and inhibitor are both in a dispersed state, the separation of physical effects from those effects due to actual molecular geometry is always a formidable task. It is not surprising that the kinetics obtained from such reactions are complex and nonclassical; even the simple competitive inhibition patterns found with analogue 5 (2) and phosphonate analogues of lecithin (1) were obtained after lag phases of varying durations. In an important sense, however, such effects are an inherent part of phospholipase reactions, since their natural substrates are dispersible but not water-soluble.

Not only are particulate interactions among substrate, enzyme, inhibitor, and product likely to be complex; but their effects upon the measuring device (in this case a pH electrode) are not precisely understood. Thus, it is not known what portion, if any, of the complex kinetics found can be ascribed to "artifacts." We do not, however, believe this effect to be significant, at least with reasonably slow reactions.

The available evidence, which is still considerably less complete than desirable, indicates that, at least beyond essential general molecular features, inhibitors of *Cl. perfringens* phospholipase C are not sensitive to the presence or absence of fine structural variables. Noteworthy is the particular insensitivity to steric features, at least by substitution of $-CH_2-$ for -O-, since the completely isosteric phosphinate analogue is a relatively poor inhibitor. On the other hand the corresponding choline-ester phosphonate analogue is the best inhibitor known (1), so that the electronic effects at the base phosphorus linkage may be important for inhibitor specificity. Nevertheless, a compound without the P-O-C- to base linkage (analogue 1) is a good inhibitor, reinforcing the initial conclusion that specific structural moieties are of secondary importance.

It actually is quite difficult to see a clear pattern of inhibitory activity vs. structural features in the order of inhibitor potency vs. known lecithin analogues as shown below:



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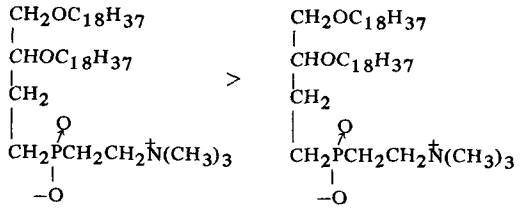

2 (L); 3 (DL) 4 (L)

Although factors beyond molecular geometry must be sought in rationalizing the inhibitor specificity, it is interesting that, so far as can be inferred from casual observation, ease of dispersibility is not a property readily related to the inhibitory order. By far the most easily dispersed of all these analogues is the phosphinate analogue 5; the most difficultly dispersed are the phosphonates, which bracket it in activity.

The melting of the hydrocarbon chains of an analogue appears to enhance inhibitory activity. Although this has not been studied systematically as yet, on the basis of the present report the inhibition by analogues 1, 2, and 3 is increased significantly at 37 C vs. 25 C. Analogue 4, which on the basis of its structure might be expected to have a higher transition temperature than the other analogues, shows an opposite effect. Thus, it can be suggested that, with this analogue, the transition temperature may lie significantly above 37 C, preventing chain melting from increasing the inhibition.

From the point of view of providing good phospholipase C inhibitors, none of the isosteric analogues studied herein presents any practical advantages over analogue 5. If complete nonhydrolyzability or extreme dispersibility is not required, the much more easily synthesized phosphonate analogues can be used as well.

ACKNOWLEDGMENT

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Quantitation of Phosphatidyl N-Methyl and N,N-Dimethyl Aminoethanol in Liver and Lung of N-Methylaminoethanol Fed Rats

S.L. KATYAL and **B. LOMBARDI**, Department of Pathology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

ABSTRACT

A diet supplemented with N-methylaminoethanol was fed to rats, and liver and lung phospholipids were resolved by a two dimensional thin layer chromatographic method affording the separation of phosphatidyl N-methylaminoethanol and phosphatidyl N,N-dimethylaminoethanol, as well as of the major phosphatides. Significant amounts of phosphatidyl N-methylaminoethanol and phosphatidyl N,N-dimethylaminoethanol accumulated within 24 hr in liver and lung. The fatty acid composition of liver phosphatidyl N-methylaminoethanol and phosphatidyl N,N-dimethylaminoethanol was determined. Stearic, arachidonic, and docosahexaenoic acids were the predominant fatty acids of these phosphatides. After injection of (Me-^{14}C)methionine, only phosphatidyl N,N-dimethylaminoethanol and phosphatidylcholine became heavily labeled in both liver and lung. These findings indicate that N-methylaminoethanol is incorporated into phosphatidyl N-methylaminoethanol, and the latter is methylated to phosphatidyl N,N-dimethylaminoethanol and phosphatidylcholine.

INTRODUCTION

Phosphatidyl N-methylaminoethanol (PMME) and phosphatidyl N,N-dimethylaminoethanol (PDME) are known to be intermediates in the metabolic conversion of phosphatidyl aminoethanol (PE) to phosphatidylcholine (PC) (1,2). This conversion constitutes a pathway for biosynthesis of choline and lecithins in liver (1,2) and is active also in lungs of mammals (3,4). However, PMME and PDME usually are present in rat tissues only in trace amounts (5). Thus, their isolation and characterization, as well as the study in vivo of their metabolic role, is rather difficult and has been only partially achieved.

Kobayashi (6) has presented indirect evidence suggesting that a deposition of PMME and PDME may occur in the liver of guinea pigs fed a diet supplemented with N-methylaminoethanol (MMAE). We decided, therefore, to explore this possibility in rats similarly fed and to quantitate the eventual deposition. A two dimensional thin layer chromatographic (TLC) method was developed allowing the separation of PMME and PDME, as well as the other phospholipids of rat liver and lung. Significant amounts of both PMME and PDME were found to accumulate readily in both liver and lung of rats so fed. Sufficient quantities were obtained from the liver to permit analyses of the fatty acid composition. The labeling of liver and lung phospholipids after administration of (Me-^{14}C)methionine to the animals also was investigated. The results of these studies are the object of this report.

MATERIALS AND METHODS

Male and female rats of the Sprague-Dawley strain (Sprague-Dawley, Inc., Madison, Wis.) weighing 100-250 g were used. They were fed either laboratory chow or a semisynthetic, choline-deficient (7) diet supplemented with 1% MMAE. Before addition to the diet, MMAE was neutralized with 9 N HCl. The diet was fed as a single meal to animals fasted overnight. The rats were decapitated 24 hr after, and the liver and lungs were removed and weighed. Homogenates of lungs (10%) were prepared in saline. Aliquots of the homogenates were used for protein determination (8) and lipid analyses.

Liver and lung total lipids were extracted (9) with a mixture of chloroform-methanol (2:1 v/v) and were separated on thin layer plates prepared by coating a slurry of 65 g Silica Gel H (E. Merck, Darmstadt, Germany) in 140 ml distilled water to a thickness of 0.5 mm. Alternatively, precoated plates (Uniplates, Catal. 1111, Analtech, Inc., Newark, Del.) were used. The plates were activated for 70 min at 110 C and allowed to cool in a desiccator. Lipid samples (ca. 50 μg lipid phosphorus) were spotted under nitrogen as a narrow streak, and the plates then were developed in a solvent mixture of chloroform-methanol-ammonium hydroxide (28% w/v) 65:30:5 (v/v) in the first direction and n-butanol-acetic acid-water 90:20:20 (v/v) in the second direction.

Two chromatography tanks lined with filter paper soaked in each solvent system were used.

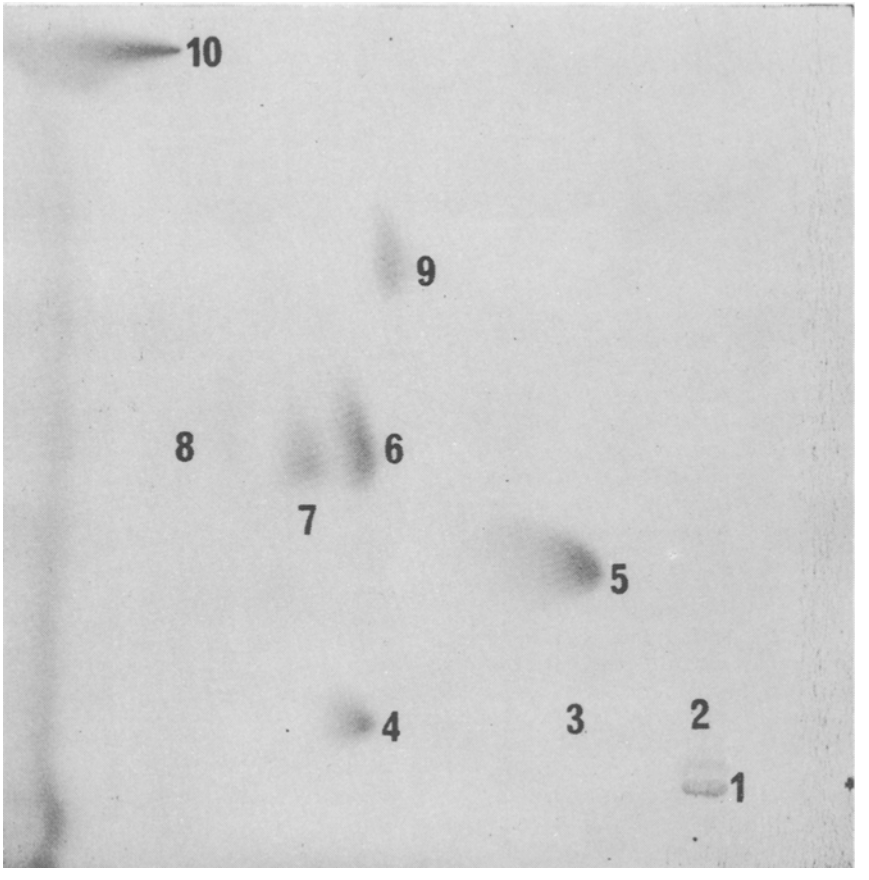

FIG. 1. Two dimensional thin layer chromatograph of lipids extracted from the liver of rats fed N-methylaminoethanol. Spots 1-10 correspond respectively to: origin, lysophosphatidylcholine, sphingomyelin, phosphatidylserine + phosphatidylinositol, phosphatidylcholine, phosphatidyl N-methylaminoethanol, phosphatidylaminoethanol, cardiolipin, phosphatidyl N,N-dimethylaminoethanol, and neutral lipids.

The solvents were allowed to run on the plates to a distance of 15 cm from the line of application in each direction. After development in each solvent mixture, the plates were dried in a tank flushed with nitrogen gas. Lipid spots were located by brief exposure of the plates to iodine vapors or by spraying a solution of 2′,7′-dichlorofluorescein (0.05% in ethanol), when the fatty acid composition of the phospholipid was to be determined. After spraying with the fluorescent dye, the plates were placed for 1-2 min in a tank saturated with ammonia vapors which yielded visible pink spots on a yellow background.

Alternatively, the plates, after spraying with the dye, were viewed under UV light. The spots were scraped from the plates, and the lipids were eluted from the gel with a mixture of chloroform-methanol-acetic acid-water 50:39:1:10 (v/v) (10). Dichlorofluorescein was removed from the eluates, when necessary, as indicated by Arvidson (10). Lipid phosphorus was determined (11) on aliquots of the eluates. A factor of 25 was used to convert lipid P to phospholipids. 2,6-Di-tert-butyl-p-cresol was added to all solvents (10 mg/100 ml) used for extraction and separation of the phospholipids. Fatty acid methyl esters were prepared according to Marinetti (12) using 0.5 N sodium methoxide in methanol. They then were analyzed with an F&M model 400 gas chromatograph fitted with hydrogen flame detector and a column packed with 15% HIEFF 2BP on Gas Chrom P, 80-100 mesh (Applied Science Labs., State College, Pa.). Peak areas were measured by planimetry or with the aid of a computer program relating peak ht and retention time of each peak. Reference PMME, PDME, and PE were obtained from General Biochemicals, Chagrin Falls, Ohio. Phospholipids, other reference compounds, and mixtures of methyl esters of fatty acids were obtained from Applied Science Labs. MMAE and N,N-dimethylaminoethanol (DMAE) were obtained from Eastman

TABLE I

Quantitation of Phosphatides from Laboratory Chow or N-Methylaminoethanol Fed Rats

Diet	Laboratory chow	MMAE-supplemented diet	
Tissue[a]	Liver[b]	Liver[b]	Lung[c]
LPC	0.46 ± 0.03	Trace	Trace
SP	0.98 ± 0.03	1.02 ± 0.05	21.47
PS + PI	2.90 ± 0.11	3.70 ± 0.09	19.44
PE	7.75 ± 0.14	3.87 ± 0.109	24.11
PMME	Trace	7.04 ± 0.54	20.08
PDME	Trace	2.99 ± 0.35	1.69
PC	14.62 ± 0.31	7.29 ± 0.48	71.15

[a]LPC = lysophosphatidylcholine, SP = sphingomyelin, PS = phosphatidylserine, PI = phosphatidylinositol, PE = phosphatidyl aminoethanol, PMME = phosphatidyl N-methylaminoethanol, PDME = phosphatidyl N,N-dimethylaminoethanol, PC = phosphatidylcholine, and MMAE = N-methylaminoethanol.

[b]mg/g wet liver, mean ± standard error of four rats.

[c]μg/mg protein, results of one determination.

Organic Chemicals, Rochester, N.Y. Upon gas chromatograph analysis (5), each base yielded only one peak.

Hydrolysis or methanolysis of PMME and PDME isolated from the liver of MMAE fed rats was performed with 6 N HCl (5) or $NaOCH_3$ (12). The free bases were separated and identified after paper chromatography (1) and gas liquid chromatography (GLC) (5) and the glycerophosphorylbases after paper chromatography (13).

(Me-^{14}C)methionine (specific activity 50 m C/m mole; obtained from Radiochemicals Amersham/Searle Corp., Arlington Heights, Ill.) 10 μC in 0.2 ml saline was injected into a saphenous vein of 100 g body wt rats under light ether anesthesia. Radioactivity was measured in a Packard scintillation spectrometer. Aliquots of the eluted phospholipids were transferred into counting vials and the solvents evaporated under a stream of air. H_2O (0.5 ml) and 15 ml scintillation mixture then were added. The latter had the following composition (ml/1000 ml): toluene, 791.55; Liquifluor (New England Nuclear, Boston, Mass.), 8.45; and Triton X-100 (Packard Instrument, Downers Grove, Ill.), 200. Differences between means were checked with Student's *t* test and regarded to be significant if $P \leq 0.05$.

RESULTS AND DISCUSSION

Figure 1 shows a typical separation of liver lipids from male rats fed the MMAE-supplemented diet. Similar separations were obtained with lung lipids. The major tissue phosphatides are well resolved, and two conspicuous spots corresponding to PMME and PDME are visible. The identity of the latter phosphatides was established on the basis of a positive test with a phospho-molybdenum blue reagent (14), same Rf as reference standards, and by GLC and paper chromatography of the free bases and glycerophosphoryl bases carried out as previously indicated. PMME, but not PDME, also gave a positive ninhydrin reaction.

Table I shows the results of the quantitation of the phosphatides. In laboratory-chow fed rats, the liver contained ca. twice as much PC and PE as the liver of rats fed the MMAE-supplemented diet. However, PMME and PDME were present in undetectable amounts. On the other hand, both PMME and PDME were major phosphatides in the liver of MMAE-fed rats, the content of PMME being the same as that of PC and the content of PDME ca. three-fourths that of PE. The ratio of PMME to PDME was 2.3. Significant amounts of PDME, and especially of PMME, were present also in the lung phosphatides of the MMAE-fed rats. In this tissue, however, the ratio of PMME to PDME was 12. PMME and PDME were not detected in lung phosphatides of laboratory-chow fed rats, and the other phosphatides were not measured. It is evident, therefore, that dietary supplementation of MMAE leads to accumulation, in at least the liver and lung of rats, of PMME and PDME, two phosphatides normally present in these tissues only in trace amounts. It is apparent, furthermore, that the two phosphatides must be synthesized quite actively since a marked accumulation occurs within 24 hr of feeding the MMAE-supplemented diet. However, their rate of synthesis and metabolism appears to be different in liver and lung since the PMME-PDME content ratios are very different in the two tissues. Incorporation of MMAE and DMAE into respective phosphatides has

TABLE II

Fatty Acid Composition[a] of Liver Phosphatides[b] from Laboratory Chow or N-Methylaminoethanol Fed Rats

Diet	Laboratory chow		MMAE-supplemented diet			
Phosphatide	PE	PC	PE	PMME	PDME	PC
16:0	20.41 ± 0.69	26.65 ± 1.09	9.31 ± 1.07	14.83 ± 1.96	10.43 ± 2.51	16.34 ± 1.54
18:0	24.41 ± 0.42	18.19 ± 0.89	28.12 ± 0.43	19.87 ± 0.44	22.94 ± 0.99	22.56 ± 0.44
18:1	5.99 ± 0.10	8.15 ± 0.41	6.24 ± 0.45	7.47 ± 0.10	6.34 ± 0.48	11.95 ± 0.48
18:2	12.92 ± 0.49	20.55 ± 1.27	5.03 ± 0.12	13.67 ± 0.55	13.41 ± 0.71	20.63 ± 0.14
20:3	Trace	Trace	1.13 ± 0.25	1.89 ± 0.29	3.36 ± 0.48	3.75 ± 0.10
20:4	22.24 ± 0.72	18.46 ± 0.10	28.85 ± 0.71	21.94 ± 1.67	27.48 ± 1.48	18.41 ± 1.09
22:6	13.87 ± 0.67	6.28 ± 0.31	21.32 ± 0.42	18.26 ± 1.60	17.13 ± 2.99	6.37 ± 0.69

[a]Percentage of total fatty acids. Each value is the mean ± standard error of four rats.

[b]PE = phosphatidyl aminoethanol, PC = phosphatidylcholine, MMAE = N-methylaminoethanol, PMME = phosphatidyl N-methylaminoethanol, PDME = phosphatidyl N,N-dimethylaminoethanol.

been shown to occur in mammalian tissues (15-18) and insect larvae (19-21). It also has been shown that PMME and PDME accumulate in insect larvae fed on a diet containing MMAE and DMAE (19-22) and in yeast cells grown on a medium supplemented with MMAE (23). Accumulation of PMME and PDME had been previously suggested to occur in the liver of guinea pigs fed a diet supplemented with MMAE (6).

Table II shows the fatty acid composition of some of the major phosphatides isolated from the liver of laboratory-chow or MMAE fed rats. In the former instance, palmitic and stearic acid are the major saturated fatty acids of both PE and PC. Arachidonic acid is the predominant unsaturated fatty acid in PE, while PC contains ca. the same amounts of linoleic and arachidonic acids. In the four phosphatides isolated from the liver of MMAE fed rats, stearic acid is the predominant saturated fatty acid. Arachidonic and docosahexaenoic acids are the major unsaturated fatty acids in PE, PMME and PDME, and linoleic and arachidonic acids in PC. As is the case in the laboratory-chow fed rats, the overall fatty acid composition of PE and PC is, in the MMAE-fed rats, quite dissimilar. Dissimilarity exists also between PMME and PDME on one hand and PE and PC on the other. However, there is a fairly good similarity in the overall fatty acid composition of PDME and PMME, a finding which would be anticipated if PDME were to originate from PMME as a step in the methylation of PE to PC. In this respect it is interesting to note that stearic, arachidonic, and docosahexaenoic acids are the most abundant fatty acids in PE and in both PMME and PDME. Species of PE rich in these same fatty acids are thought to be specifically involved in the conversion of PE to PC via the methylation pathway (24,25).

To learn more about the biosynthesis of PDME and PMME in MMAE-fed animals, two male rats were killed 5 or 60 min after administration of (Me-^{14}C)methionine. Liver and lung phospholipids were isolated and radioactivity measured. In both tissues and at both time intervals, only PDME and PC were labeled heavily. At the 60 min interval, the specific activities (cpm/mg) of liver phosphatides were as follows: PC, 6 x 10^5; PDME, 6 x 10^5; PMME, 7 x 10^2; and PE, 3 x 10^2. At the same time, those of the lung phosphatides were: PC, 2.2 x 10^4; PDME, 1.8 x 10^4; PMME and PE, nil. These findings indicate that no active methylation of PE to PMME has occurred in these animals. It has been suggested by Kobayashi (6) that feeding MMAE blocks such a methylation. On the other hand, the heavy

labeling of PDME and PC indicates that dietary MMAE is incorporated into PMME, either by a CDP-MMAE pathway (18) or by a base exchange (26) and that the phosphatide is then sequentially methylated to PDME and PC. This conclusion is supported further by the observed accumulation of PMME in both liver and lung (Table I), and the similarity in the fatty acid composition of liver PMME and PDME (Table II). It is also evident that whatever the route of PMME synthesis, precursor species of diglycerides (CDP pathways) or phosphatides (base exchange) abundant in stearic and polyunsaturated fatty acids (Table II) must be selectively involved.

Liver phospholipids of rats fed MMAE were separated also by a unidimensional TLC method (27) routinely used in this laboratory. PMME was found to migrate with PE and PDME with phosphatidylserine (PS). The fatty acid composition of the two fractions reflected the contamination with PMME or PDME, respectively. From a quantitative point of view, these findings are of little significance, since, under normal conditions, PMME and PDME are present in rat liver only in trace amounts. However, the unidimensional TLC method should be used with greater caution when isotopic experiments (28) are performed, since considerable radioactivity may be present in PMME and PDME, which would thus be attributed to PE or PS. This fact was clearly shown by the following experiment. One male rat fed laboratory chow was sacrificed 5 min after injection of (Me-^{14}C)methionine, and liver phospholipids were separated by the two dimensional TLC method after addition of carrier PMME and PDME. Recovered radioactivities (cpm/g liver) were as follows: PC, 1.5 x 10^5; PDME, 3.6 x 10^4; PMME, 5.5 x 10^3; and PE, 7 x 10^2.

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Formation of Palmitate Esters of Monohydric Short Chain Alcohols by Swine Arterial Subcellular Fractions

ROBERT J. MORIN and **THOMAS IHRIG**, Department of Pathology, Harbor General Hospital, School of Medicine, University of California, Torrance, California 90509

ABSTRACT

Alcohols of 1-5 carbon chain length were esterified with palmitic acid and palmitoyl CoA substrates by swine aortic cytosol, mitochondrial, and microsomal fractions. Esterification of the palmitic acid substrate with ethanol was enhanced by the addition of adenosine 5'-triphosphate and CoA to the mitochondrial and microsomal fractions. The rate of esterification of ethanol was most rapid in the microsomal fraction and with the palmitoyl CoA substrate. Esterification was linearly related to time of incubation, was optimal at pH 6.8, and could be inactivated by heating at 90 C. Esterification was inactivated by ethanol concentrations above 0.8 M and by lower concentrations of the other alcohols. Rates of esterification were highest with n-propanol and lowest with tert-butanol and the pentanols. The formation of cholesteryl esters by the aortic microsomes was inhibited by ethanol and was inversely related to the rate of ethyl palmitate formation.

INTRODUCTION

Methyl esters of fatty acids (FA) have been noted to form during extraction and storage of tissues in methanol containing solvents (1,2). The natural occurrence of FA methyl and ethyl esters has, nevertheless, been established in yeast (3) and insects (4) and in guinea pig (5), mouse (6), and human liver (7) and in ox (8), dog (9), and human (9,10) pancreas. Evidence has been obtained that formation of these esters in mouse liver (11), rat plasma (12), and porcine pancreas (13) occurs by enzyme catalyzed reactions. The esterification of ethanol by rat plasma in vitro was observed to be a direct reaction, rather than a transesterification reaction utilizing a fatty acyl donor (12). The formation of ethyl esters by rat tissues in vivo has been confirmed by the recovery of labeled long chain FA esters of ethanol from whole body lipids subsequent to the administration of ethanol-^{14}C (14). Similarly, feeding of ethanol to goats results in the appearance of ethyl esters in the milk (15).

During the course of investigation of FA metabolism in swine arteries, it was noted that significant quantities of ethyl esters were formed when ethanol was added to the incubation medium. The mechanisms of the esterification of ethanol and other short chain alcohols were, therefore, investigated in these swine arterial subcellular fraction preparations.

MATERIAL AND METHODS

Aortas and coronary and pulmonary arteries were obtained within 30 min of slaughter from 6 month old male grain-fed Hampshire swine. The intima-media portions of the arteries were dissected free and minced with an Arbor tissue press (Harvard Apparatus, Millis, Mass.) Each arterial mince was suspended in 0.25 M sucrose + 0.001 Methylenediaminetetracetate acid(EDTA) and was homogenized further for 2 min in an ice cooled container using a Vir Tis 45 homogenizer at 2000 rpm. The homogenates were centrifuged for 10 min at 500 g, the supernatant centrifuged at 12,000 g for 15 min, and then recentrifuged at 18,000 g for 15 min (the latter precipitate was discarded). The 12,000 g mitochondrial precipitate was resuspended in an equal vol of 0.25 M sucrose and recentrifuged (this precipitate was used for all mitochondrial assays). The 12,000 g supernatant was centrifuged at 104,000 g for 60 min. After removal of the cytosol, the microsomal precipitate was resuspended in 0.15 M KCl and recentrifuged. The washing (supernatant) from this centrifugation was discarded.

For assay of alcohol esterifying activity, 0.2 ml aliquots of the subcellular fractions were mixed with 0.2 ml aliquots of 0.2 M phosphate buffer, pH 5.8-8.6 (in increments of 0.2 pH units) with or without 6 μmoles adenosine 5'-triphosphate (ATP), 0.4 μmoles CoA, 4.0 μmoles $MgCl_2$, and 2.0 μmoles NaF. A palmitic acid-1-^{14}C substrate (New England Nuclear, Boston, Mass.) 0.2 μc, 4.0 nmoles of each) or palmitoyl-1-^{14}C CoA (0.1 μc, 2.0 nmoles) was added to each homogenate, and the tubes then were incubated at 37 C for 1 hr. Methanol, ethanol, n-propanol, 2-propanol, n-butanol, isobutanol, 2-butanol, tert-butanol, n-pentanol, and isopentanol were added in concentrations ranging from 0.01-1.6M. Control aliquots with no alcohol added were incubated with each group, and other controls were preheated at 90 C for 20 min, stopped at zero time, or incubated for 1 hr with buffer plus substrate

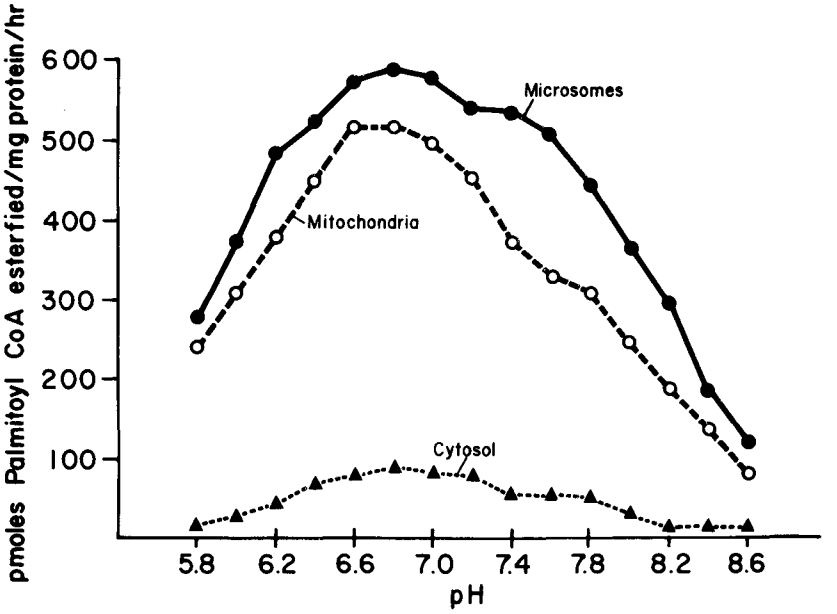

FIG. 1. The influence of pH on the esterification of a palmitoyl CoA substrate with ethanol by the cytosol, mitochondrial, and microsomal fractions from swine aorta. Incubation conditions are given in Table I.

but no tissue. The incubations were stopped by addition of chloroform: methanol, 2:1, and the lipids extracted by the Folch procedure (16). After evaporation under a stream of prepurified nitrogen at 40 C, the extracts were dissolved in chloroform, applied to Silica Gel H 0.5 mm plates, and developed using an ascending solvent mixture of hexane: ethyl ether: acetic acid, 120:20:1. The alcohol ester bands were identified by comparing the R_f of the fractions in the sample to the R_f of known standards. Good separation was achieved between these alcohol esters, the faster migrating cholesteryl esters, and the slower migrating free fatty acids. Radioactivities in these three fractions were determined by liquid scintillation counting using a PPO-POPOP in toluene scintillation solution and a Packard model 3314 automatic refrigerated liquid scintillation counter. Protein content of each fraction was analyzed by the method of Lowry (17) adapted for the Autoanalyzer. The probabilities that apparent differences in the data were due to chance were calculated by the *t* test.

RESULTS

The influence of pH upon the rate of esterification of the palmitoyl CoA substrate to ethanol by swine aortic cytosol, mitochondrial, and microsomal fractions is shown in Figure 1. Activity was highest in the microsomal fraction

TABLE I

Esterification of Palmitic Acid and Palmitoyl CoA Substrates with Ethanol by Subcellular Fractions of Swine Aorta[a]

Substrate	Palmitate -1-^{14}C	Palmitate -1-^{14}C ATP and CoA	Palmitoyl -1-^{14}C
Cytosol	55.8 ± 6.4	62.0 ± 4.9	79.0 ± 18
Mitochondria	59.17 ± 6.4	219 ± 30	512 ± 50
Microsomes	48.2 ± 3.5	294 ± 42	589 ± 66

[a]p Moles esterified/mg protein/hr ± standard deviations of six separate determinations, p moles calculated assuming no equilibrium with endogenous substrates. Each fraction was incubated for 1 hr in 0.1 M phosphate buffer, pH 6.8. Incubations with palmitic acid-1-^{14}C (0.2 μc, 4.0 nmoles) were done with and without addition of 6 μmoles adenosine 5′-triphosphate, 0.4 μmoles CoA, 4 μmoles $MgCl_2$, and 2 μmoles NaF/0.4 ml buffer. Incubations with palmitoyl CoA (0.1 μc, 2.0 nmole) were done without added cofactors. All incubations were made to 0.8 M with ethanol.

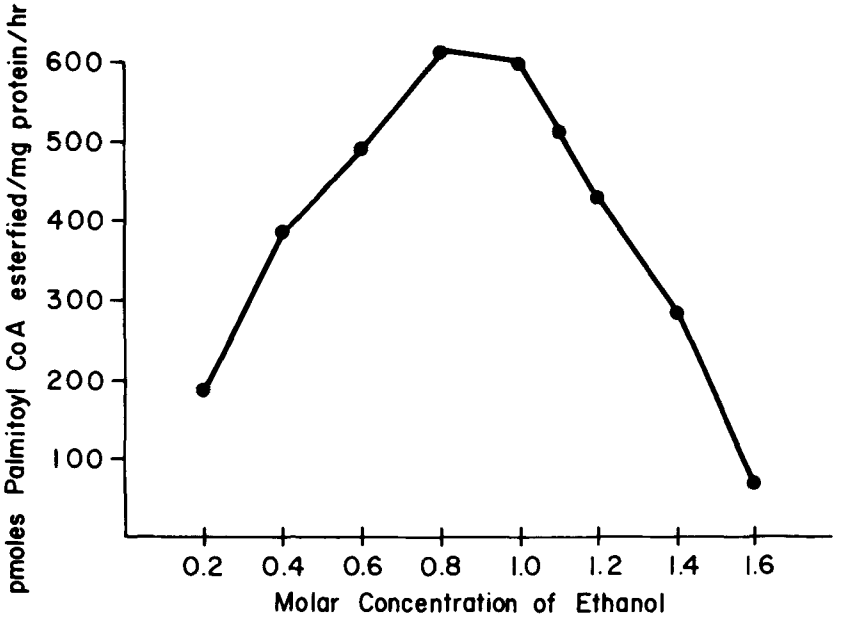

FIG. 2. Effect of ethanol concentration on the esterification of palmitoyl CoA by the aortic microsomal fraction at pH 6.8. Incubation conditions are given in Table I.

and was evident over a broad range of pH values with optimum at pH 6.8. Esterifying activity, when measured in these fractions at pH 6.8 at 5 min intervals up to 2 hr, showed a direct linear relationship to time. Heating these fractions at 60 C for 10 min produced a 10-15% loss of activity, but heating at 90 C for 20 min resulted in complete loss of activity. The influence of ethanol concentration upon the rate of esterification of the palmitoyl CoA substrate by aortic microsomes at pH 6.8 is shown in Figure 2.

TABLE II

Aortic Microsomal Esterification of Palmitoyl-1-^{14}C CoA with Various Monohydric Short Chain Alcohols[a]

	Alcohol concentration	
	0.2 M	0.8 M
No alcohol	0.8 ± 0.3	0.9 ± 0.2
Methanol	152 ± 12	332 ± 51
Ethanol	198 ± 19	589 ± 66
n-Propanol	413 ± 54	58 ± 4.4
2-Propanol	186 ± 36	171 ± 20
n-Butanol	150 ± 13	23 ± 1.1
Isobutanol	131 ± 17	27 ± 1.6
2-Butanol	262 ± 23	33 ± 1.7
Tert-butanol	14 ± 3.0	5 ± 0.7
n-Pentanol	22 ± 4.5	10 ± 3.2
Isopentanol	25 ± 3.6	8 ± 2.2

[a]p Moles esterified/mg protein/hr ± standard deviations of six separate determinations. The no alcohol control values are the means of 10 separate determinations (one with each alcohol group). Incubations were done as described in Table I.

Esterification increased linearly in relation to concentration and was maximal at 0.8 M ethanol. At concentrations greater than 0.8 M, the rate of esterification was reduced significantly.

The rates of esterification by the cytosol, mitochondrial, and microsomal fractions of the palmitic acid substrate, with and without added ATP and CoA, and of the palmitoyl CoA substrate without added cofactors are indicated in Table I. Although some esterification occurred at pH 6.8 without added cofactors, after addition of ATP and CoA, the rate of esterification was increased in the mitochondrial and microsomal fractions but not in the cytosol. Esterification with the palmitoyl CoA substrate was somewhat higher than with palmitic acid plus ATP and CoA. In the controls incubated without ethanol, the rates of alcohol ester formation with each substrate were less than 1.0% of corresponding values indicated in Table I for the 0.8 M ethanol concentrations. Subcellular fractions obtained from the coronary and pulmonary arteries showed esterifying activity with ethanol and the various substrates similar to that of the aortic fractions.

In Table II are shown the results of aortic microsomal esterification of the palmitoyl CoA substrate with 0.2 M and 0.8 M concentrations of various straight and branched chain monohydric alcohols from 1-5 carbons. At the lower concentration level, n-propanol was esterified to palmitoyl CoA most rapidly, followed

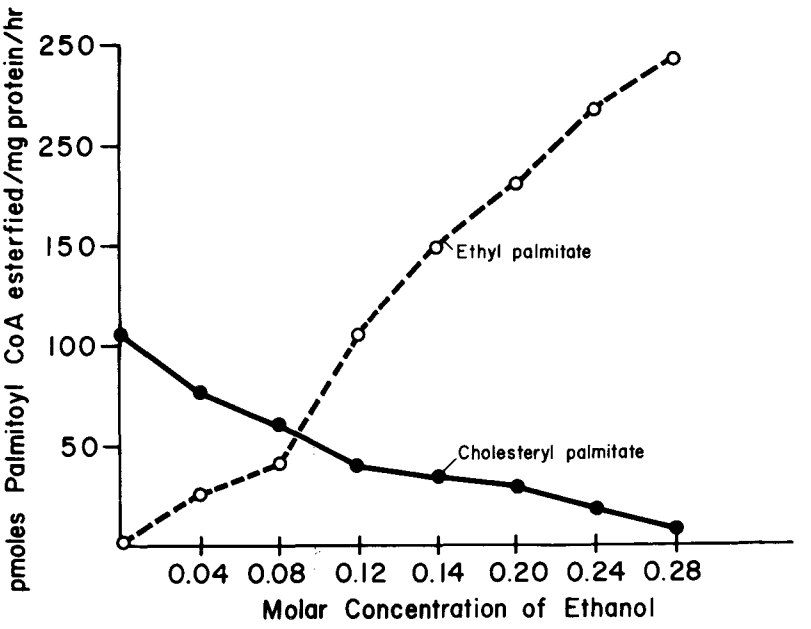

FIG. 3. The influence of ethanol concentration and of the rate of the formation of ethyl palmitate on the incorporation of the palmitoyl CoA substrate into cholesteryl esters by the aortic microsomal fraction.

by 2-butanol and then by ethanol. Tert-butanol, n-pentanol, and isopentanol at 0.2 M concentrations were esterified at relatively lower rates. Increasing the ethanol concentration up to 0.8 M, as previously graphed in Figure 1, resulted in ca. linear increases in the rate of ester formation. Increasing the concentration of n-propanol and the other alcohols to the 0.8 M level, however, resulted in marked reductions in the rates of ester formation.

The influence of ethanol concentrations of 0.02 M-0.3 M on esterification of the palmitoyl CoA substrate to endogenous cholesterol by the aortic microsomal fraction is indicated in Figure 3. At ethanol concentrations of 0.12 M and higher, the esterfication of cholesterol was inhibited markedly. At all concentration levels of ethanol, there was an apparent inverse correlation between the rates of formation of ethyl palmitate vs. cholesteryl palmitate.

DISCUSSION

The formation of ethyl palmitate by the swine arterial subcellular fractions was linearly related to time and to substrate concentration, occurred maximally at pH 6.8, and was prevented by prior heating of the fractions at 90 C. This suggests that this esterfication occurred by an enzymatic mechanism. Previous work with bacteria indicates that methyl esters are formed by transfer of the methyl group of S-adenosylmethionine to FA (18). In rat plasma, ethyl esters are formed by a direct reaction of alcohols with FA, with no involvement of fatty acyl donors (12). In the present experiments, ethyl palmitate formation by swine aortic mitochondrial and microsomal fractions was accelerated by adding ATP and CoA to the media and also by substituting palmitoyl CoA for the palmitic acid substrate. This suggests that esterification occurred by an acyl CoA: alcohol acyltransferase mechanism, similar to that for esterification of cholesterol in arteries (19). A low level of esterification of palmitic acid without added cofactors, however, did occur in all fractions. In the cytosol the addition of ATP and CoA produced no stimulatory effects; and the rate of esterification was not higher with the palmitoyl CoA substrate, suggesting the possibility that an addition direct esterification mechanism may exist in the cytosol and possibly also in the other fractions.

At the lower concentration levels of alcohols, the microsomal acyltransferase esterifying mechanism seemed to exhibit differing specificities for the different types of alcohols; n-propanol was esterified most rapidly, whereas tert-butanol and the five carbon alcohols showed the lowest esterification rates. There was no apparent correlation between the rate of esterification and either the chain length, degree of branching, or position of the hydroxyl group in the alcohols. The rate of hydrolysis of alcohol esters of long chain FA by rat pancreatic lipase has been observed previously to be

influenced by the type of primary alcohol in the ester (20), but the relative rates of hydrolysis of these esters were not similar to the relative rates of esterification of these alcohols observed in the present experiments.

The decrease in rate of esterification at ethanol concentrations above 0.8 M is probably attributable to a denaturation effect of ethanol on the enzymes involved. The other alcohols utilized appeared to denature the enzymes at lower concentrations than 0.8 M, at which the rate of esterification with ethanol was maximal. In contrast to previous studies with rat intestinal mucosa (21) where enzymatic formation of FA esters of methanol, ethanol, butanol, and propanol was noted at 100% alcohol concentrations, no significant enzymatic esterification of alcohols at that concentration level was observed in the present experiments (at 100% alcohol concentrations, formation of esters was less than 0.1% maximal rates and was equal to that obtained with fractions preheated at 90 C).

No evidence is available from the present study to indicate any physiological role of the esterification of short chain alcohols to FA. Formation of ethyl esters has been noted after in vivo administration of ethanol to rats (14) and goats (15) and similarly may occur in mammalian arteries in vivo after ethanol ingestion. At concentrations above 0.12 M in the present experiments, ethanol was noted to inhibit the formation of cholesteryl palmitate by the arterial fractions. Esterification of cholesterol has been observed in arteries and increases markedly with experimental atherosclerosis (19). Although most ethanol concentrations utilized in the present in vitro experiments were higher than would be attained by ethanol ingestion, this does not rule out the possibility that some diversion of arterial FA from cholesterol esterification to ethanol esterification may occur in vivo.

Ethanol has been utilized in many laboratories as a vehicle for solubilization of cholesterol and FA substrates for in vitro studies of tissue cholesterol esterification. Aside from possible competitive effects, the R_f values of ethyl palmitate and cholesteryl esters are close with many types of solvent mixtures utilized for the thin layer chromatographic isolation of cholesteryl esters, and precautions should be taken to avoid artifactual data due to possible inadequate separation of these components.

ACKNOWLEDGMENT

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SYMPOSIUM: EFFECT OF DRUGS ON LIPID METABOLISM

Presented at the AOCS Spring Meeting, New Orleans

ROSLYN B. ALFIN-SLATER, Chairwoman

Oral Contraceptive-α-Tocopherol Interrelationships[1]

L. AFTERGOOD and **R.B. ALFIN-SLATER**, School of Public Health, University of California, Los Angeles, California 90024

ABSTRACT

After observing that some of the side effects of the administration of oral contraceptive drugs to rats resemble those resulting from vitamin E deficiency, a possibility of an increased requirement for vitamin E during oral contraceptive therapy was considered. Female rats were kept on the following diets all of which contained 15% stripped corn oil: (A) basal (no tocopherol), (B) basal + α-tocopherol to provide 1 mg/rat/day, (C) basal + butylated hydroxytoluene, and (D) basal with the α-tocopherol given only during drug administration. At 13 weeks of age, Enovid E was administered orally at a level corresponding to 0.002 mg mestranol and 0.05 mg norethynodrel/day for either 4 or 28 days, at which time the rats were sacrificed. In addition to previously shown changes, lowering of plasma tocopherol levels was observed in rats receiving the drug. On the other hand, the effects of an oral contraceptive were not as drastic in vitamin E deficient rats. Possible implications of these findings are discussed.

INTRODUCTION

For several years now, work on vitamin E in our laboratory has involved two main areas: the effect of oral contraceptives upon lipid metabolism (1) and α-tocopherol-unsaturated fat interrelationships (2). Recently, as might have been expected, both areas of investigation merged.

In our work with female rats, treated with large, unphysiological doses of an oral contraceptive, we repeatedly have observed drastic effects upon several indices of lipid metabolism (3). Cholesterol levels in plasma and adrenals declined, and liver cholesterol levels increased,

[1]One of six papers presented in the symposium "Effect of Drugs on Lipid Metabolism," AOCS Spring Meeting, New Orleans, April 1973.

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TABLE I

Effect of Enovid E and α-Tocopherol Status upon Body Wt after 4 or 28 Days[a]

	Average change in wt, g			
	4 Days		28 Days	
Group	C	E	C	E
T	+0.7	−2.6	+27	+14
B	+1.2	−4.1	+ 5	+ 9
N	−5.0	−7.8	+ 4	+ 6
NT	−2.0	−2.1	+21	+ 9

[a]Groups C (control) contain four and Groups E (Enovid E) seven rats.

with change mainly in the esterified cholesterol moiety, and, in particular, the polyunsaturated fatty acid cholesterol esters. Serum phospholipids also decreased. There was a definite change in lipoprotein distribution, with a lowering of α-lipoprotein.

The response to oral contraceptives could be modified by the type of diet fed, although it was not affected by the decreased food intake that is characteristic of the pill-treated rats. The response also was modified by the type of drug that was administered and obviously by the dose level. After delineating the qualitative direction of changes, we started to explore quantitative changes by administering a physiological dose of the oral contraceptive, namely the minimal dose that produces infertility. Most of the observed effects were similar even at that low a level. Although there still is no clear explanation as to what the primary metabolic derangement is, judging from the many studies in this area, it seems rational to assume the existence of a general hormonal imbalance, whereby some of the changes are the result of several hormonal interferences, while the others may not even be detectable due to mutual cancellation. We believe that some of the differences observed between the effects of oral contraceptives on various species may be explained in this way. In the female rat, cholesterol levels are decreased, whereas in the human female the triglycerides are increased (4) and there is a definite difference between the lipoprotein composition in rats (5) vs. humans (6).

Recently, a series of reports have appeared discussing some of the changes in nutritional requirements observed in subjects on oral contraceptives. For instance, a disturbance in tryptophan metabolism, which often follows oral contraceptive administration, manifests itself by an excessive urinary excretion of its metabolites. This can be traced to a pyridoxine deficiency (7). Similarly, folic acid deficiency anemia has been documented in some oral contraceptive users (8), and folic acid deficiency also may be implicated in disturbances of tyrosine metabolism which, in turn, may result in melanin accumulation leading to frequently observed skin discolorations. Also vitamin B_{12} (9) and vitamin C levels (10) have been reported to be lowered. There is a suggestion of an increased requirement for vitamin A in the oral contraceptive users as well (11).

The requirement for dietary α-tocopherol has been linked to the level of unsaturation of fat in the diet. Lipid peroxidation products have been found in the tissues of vitamin E-deficient animals (12). Some workers have found decreased levels of polyunsaturated fatty acids (PUFA) in the vitamin E deficient tissues, while others were not able to show such an effect (13). Arachidonate was reported to increase in several tissues during vitamin E deficiency (14), possibly due to the interference with the biosynthesis of more highly unsaturated fatty acids, their preferential destruction or retroconversion from higher homologues to $C_{20:4}$, or possibly even due to enhanced biosynthesis from linoleate. In addition, changes in plasma lipid levels have been associated with changes in α-tocopherol content in blood (15). It is known that α-tocopherol is carried preferentially by β-lipoproteins. Changes in lipoprotein distribution might be reflected by changes in α-tocopherol content. In the rat, for instance, since the concentration of tocopherol is proportional to the amount of lipid in each lipoprotein fraction, it has been found mostly in the α-lipoproteins (16).

On this premise we initiated a series of investigations dealing with a possible interinvolvement of oral contraceptives and α-tocopherol.

EXPERIMENTAL PROCEDURES

Four groups each of 22 female weanling rats were placed on a 15% stripped corn oil diet adequate in all required nutrients (2), except α-tocopherol. Group T had 0.01%, d,l-α-tocopheryl acetate incorporated into the diet; Group B had 0.05% butylated hydroxytoluene (BHT); Group N and NT had no additives. After 10 weeks when daily dosing with Enovid E (0.052 mg in 0.1 ml propylene glycol) was initiated, Group NT was changed over to the tocopherol containing diet. The dosing continued for 4 days for half of the animals and for 28 days for the other half (Groups E). Eight animals in each group served as a control and received the propylene glycol carrier only

(Groups C).

At the end of the experimental periods, the rats were killed by removal of blood from the heart under Nembutal anesthesia. Livers and adrenals were trimmed of adhering fat and frozen until extracted with lipid solvents for cholesterol and fatty acids analyses. Cholesterol analyses were carried out by a modification of the Sperry-Schoenheimer method, as reported by Nieft and Deuel (17). Fatty acid analysis was performed on lipid fractions separated by thin layer chromatography, using a Varian Aerograph model 204C. Chromatographic peaks were identified either by comparing retention times with those of standards or from a graph representing the relationship between log retention time and number of carbon atoms.

Red blood cells were tested for their susceptibility to hemolysis using the method of Draper and Csallany (18). Tocopherol analyses were performed by a modification of the method of Bieri, et al. (19) combining thin layer chromatographic separation with $FeCl_3$-bathophenanthroline color reaction. Separations of α- and β-lipoprotein were performed on Cellogel (cellulose acetate gel) strips and measured on a Chromoscan densitometer.

RESULTS

Table I shows changes in wt during the oral contraceptive treatment. During the short experimental period, most of the animals lost wt, whereas the long period of dosing resulted in lower gains in the vitamin E deficient than in E sufficient rats. The trial food consumption study over the period of 15 days on the E sufficient diet showed that the average food intake was insignificantly lower when oral contraceptives were given (Group TE), resulting in an insignificantly lower intake of α-tocopherol (Table II).

Following sacrificing of the rats, the red blood cell hemolysis and α-tocopherol levels in plasma were determined. Both of the E deficient groups showed almost total hemolysis (Table III) and no evidence of α-tocopherol in plasma (Table IV) Hemolysis was negligible, and unaffected by Enovid E in the two other groups. In the control groups, levels were normal (for our strain of rats and our method). The administration of the oral contraceptive resulted in a small but significant decrease in groups receiving α-tocopherol and a slower recovery of plasma α-tocopherol level in groups receiving the vitamin along with the drug. It is interesting to note that recovery from a deficient state is rapid. In 4 days (or less), the levels of α-tocopherol were back to normal.

Table V shows lipoprotein distribution. In this case the values for short and long periods of dosing have been combined since they were quite similar. (The values represent the areas under the peaks traced by a densitometer.) It can be seen that the decrease in α-lipoproteins (uniformly to a greater degree in the E sufficient groups) and the increase in β-lipoproteins (to a lesser degree in the E sufficient group) resulted in a significant uniform decrease of the α/β ratio.

Table VI shows plasma, liver, and adrenal cholesterol levels. As expected, in plasma there is a general tendency toward a decrease in total cholesterol levels as a result of the administration of an oral contraceptive. In the past, we have obtained greater changes on different diets. Changes are more significant when the dosing period is longer and when the diet is E sufficient. Cholesterol levels in the liver increase when oral contraceptive is given. Adrenal cho-

TABLE II

Food Consumption Study (15 Days)

Group	Average wt change g	Average food consumed g/rat/day	Average α-tocopherol mg/rat/day
TC	6	12.7 ± 0.9	1.27
TE	−8	12.0 ± 1.5	1.20

TABLE III

Effect of Enovid E and α-Tocopherol Status upon Red Blood Cell Hemolysis

	Hemolysis, %[a]			
	4 Days		28 Days	
Group	C	E	C	E
T	7.4 ± 1.9	3.8 ± 0.4	5.2 ± 1.8	6.9 ± 1.2
B	93.9 ± 2.1	96.5 ± 1.6	92.2 ± 2.6	90.1 ± 2.3
N	100.0	100.0	97.4 ± 2.6	95.5 ± 2.1
NT	3.4 ± 0.9	2.0 ± 0.5	3.5 ± 0.8	4.5 ± 0.9

[a]Includes standard deviation.

TABLE IV

Effect of Enovid E and α-Tocopherol Status upon Plasma α-Tocopherol Levels[a]

Group	α-Tocopherol, μg/ml			
	4 Days		28 Days	
	C	E	C	E
T	7.5 ± 2.1	6.1 ± 1.9	7.7 ± 0.4[a]	5.8 ± 0.9[a]
B	0	0	0	0
N	0	0	0	0
NT	7.4 ± 1.4[b]	5.4 ± 0.7[b]	7.8 ± 1.2[c]	5.9 ± 2.0[c]

[a]Matched superscripts indicate significance level; a = $p < .001$; b,c = $p < .05$.

TABLE V

Effect of Enovid E and α-Tocopherol Status upon Plasma Lipoprotein Distribution[a]

Group	α-lipoprotein			β-lipoprotein			α/β		
	C(8)[b]	E(14)	%Δ	C	E	%Δ	C	E	%Δ
T	399	260	−34	97	160	+ 64	4.11	1.63	−60
B	428	346	−20	90	191	+101	4.75	1.81	−61
N	393	316	−20	93	203	+110	4.23	1.56	−63
NT	430	293	−31	117	204	+ 74	3.67	1.44	−60

[a]$p < .001$ for the difference between C and E groups in the α/β ratio.
[b]Values in parentheses represent number of rats.

TABLE VI

Effect of Enovid E and α-Tocopherol Status upon Plasma, Liver, and Adrenal Cholesterol Levels[a]

Group	4 Days		4 Days	
	C	E	C	E
	Plasma, total cholesterol, mg/100 ml			
T	63.9 ± 7.8	51.4 ± 11.3	66.6 ± 5.2[g]	41.0 ± 8.2[g]
B	68.7 ± 10.9	56.1 ± 11.1	65.2 ± 8.5[h]	45.5 ± 12.5[h]
N	69.3 ± 9.4[a]	56.3 ± 2.5[a]	60.3 ± 4.2	57.4 ± 4.2
NT	77.4 ± 5.3[f]	48.4 ± 4.2[f]	72.5 ± 8.9[i]	54.5 ± 5.9[i]
	Liver, total cholesterol, mg/g			
T	2.24 ± 0.22	2.30 ± 0.28	1.93 ± 0.10	2.01 ± 0.09
B	2.11 ± 0.13[b]	2.41 ± 0.27[b]	2.09 ± 0.07[c]	2.39 ± 0.29[c]
N	2.16 ± 0.31	2.06 ± 0.25	2.09 ± 0.42	2.12 ± 0.16
NT	1.84 ± 0.03[j]	2.10 ± 0.21[j]	1.84 ± 0.17[m]	2.22 ± 0.15[m]
	Adrenals, total cholesterol, mg/g			
T	38.7 ± 4.1[d]	21.8 ± 3.6[d]	40.1 ± 5.2	30.8 ± 4.3
B	33.1 ± 3.9[e]	22.6 ± 2.9[e]	38.3 ± 4.7	35.8 ± 1.4
N	32.6 ± 5.2	28.7 ± 3.9	31.6 ± 5.8	31.7 ± 1.0
NT	36.1 ± 3.8	28.1 ± 4.0	34.2 ± 2.8[k]	25.5 ± 3.1[k]

[a]Matched superscripts inidcate significance level: a,b,c,d,e = $p < 0.05$; h,i,j,k = $p < .02$; m = $p < .005$.

TABLE VII

Effect of Enovid E and α-Tocopherol Status upon Major Fatty Acids of Plasma Cholesterol Esters

Group[a]	Cholesterol esters, mg/100 ml					
	Total[b]	16:0	18:0	18:1	18:2	20:4[b]
TC	46.7 ± 2.3	5	2	3	8	25
TE	33.3 ± 1.9	4	2	2	6	15
BC	46.9 ± 2.5	5	3	3	8	24
BE	33.6 ± 2.2	4	1	2	6	16
NC	46.1 ± 2.6	6	2	4	8	22
NE	36.1 ± 0.6	4	2	3	6	17
NTC	56.6 ± 1.9	5	2	3	10	33
NTE	35.1 ± 0.9	4	2	3	6	15

[a]E = oral contraceptive treatment.

[b]$p < .001$ between C and E groups.

lesterol levels decrease to a greater degree in the E sufficient groups.

Fatty acid distribution was determined in several lipid fractions of several tissues. Some of the results are shown in Tables VII and VIII. Plasma cholesterol ester fatty acids show the expected lowering of arachidonate in response to the administration of Enovid E, again more pronounced in the E sufficient groups. The accumulation of cholesterol esters in liver appears to be in the form of cholesteryl oleate.

DISCUSSION

The question arises as to whether or not oral contraceptives result in or promote vitamin E deficiency. No decisive, clear-cut answer can be given at this point, albeit some of the changes occurring as a result of oral contraceptive administration follow the direction, if not the extent, of changes taking place when animals are made E deficient. Such changes include decreases in wt gain and in plasma tocopherol levels, adrenal cholesterol levels, and arachidonate in plasma cholesterol ester and an increase in oleate in liver cholesterol esters. There was no apparent effect of tocopherol deficiency upon α/β lipoprotein ratio or plasma and liver cholesterol levels. It is interesting to note that the effects of an oral contraceptive were less pronounced in E deficient rats. The vitamin E deficiency and the stress induced by the administration of an oral contraceptive were definitely not synergistic. It follows then that oral contraceptives do not aggravate biochemical changes when tocopherol status is inadequate.

The possibility exists that the lowering of α-tocopherol level in plasma may be the result of a primary effect of oral contraceptives upon lipoprotein distribution and therefore may reflect the redistribution of α-tocopherol due to an imposed shortage of a protein carrier.

It is tempting to speculate that this observation is related perhaps to the fact that α-tocopherol deficiency affects enzymes, such as

TABLE VIII

Effect of Enovid E and α-Tocopherol Status upon Major Fatty Acids of Liver Cholesterol Esters[a]

Group	Cholesterol esters, mg/g (x 10^{-2})					
	Total	16:0	18:0	18:1	18:2	20:4
TC	24 ± 4.6[a]	6	3[d]	4[e]	4	3
TE	44 ± 4.0[a]	9	6[d]	8[e]	6	5
BC	46 ± 7.9[b]	10	6	9[f]	7[i]	5
BE	64 ± 4.9[b]	10	6	15[f]	12[i]	7
NC	43 ± 6.5	9	6	7[g]	6	6
NE	48 ± 5.9	9	5	11[g]	8	5
NTC	26 ± 3.1[c]	6	3	4[h]	3[j]	4
NTE	43 ± 3.8[c]	8	5	10[h]	7[j]	5

[a]Matched superscripts indicate $p < .001$ between the groups.

oxygenases, in a negative fashion, while, on the other hand, oral contraceptives stimulate the activity of an oxygenase, such as tryptophan oxygenase and possibly others. It would be of further interest to study the excretion of tryptophan metabolites in α-tocopherol deficiency.

Stimulation of steroidogenesis in the adrenal gland as a result of oral contraceptive administration may be affected adversely by enzymatic changes which are due to α-tocopherol deficiency. It is known that heme proteins, oxygenases in particular, play a vital role in the production of adrenal corticosteroids. In vitamin E deficiency, there is a reduced capacity to synthesize heme, the defect occurring at a site of synthesis and subsequent condensation of δ-aminolevulinic acid (20). It has been suggested that deficiency of vitamin E may promote changes in the steroidogenic pathway in adrenals between pregnanolone and progesterone. Changes in urinary excretion of corticoids have been observed following vitamin E therapy (21). Adrenals are one of the main storage areas for α-tocopherol (22) (which in itself might suggest an important site of activity), and steroidogenesis in this tissue is considerably less in vitamin E deficient rats than in those of the control group (21).

Clinical confirmation is necessary before recommendations of dietary changes can be made with assurance. If certain side effects of oral contraceptives are undesirable, is their modification created by vitamin E deficiency an improvement? Thus, is it perhaps beneficial to limit the availability of α-tocopherol to prevent biochemical alterations, imposed by oral contraceptives, from occurring? On the other hand, it is possible that some of the side effects may be a result of the activity of oral contraceptives.

Another observation may be of interest, namely that the group fed the antioxidant (BHT) in place of α-tocopherol responded to the treatment in the same way as the E deficient rats. Obviously, α-tocopherol has functions other than antioxidant activity which can be accomplished by BHT.

Further research should include studies in which the level of oral contraceptives is increased with normal tocopherol intake and where the oral contraceptive administration is kept constant but the vitamin E status is changed to include a condition where vitamin E is given in excess or where a pronounced vitamin E deficiency status is produced.

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New Drugs Affecting Lipid Metabolism[1]

DAVID KRITCHEVSKY, The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104

ABSTRACT

The search for an ideal hypolipidemic agent still is underway. A relatively large number of the newer hypolipemic agents are related to ethyl p-chlorophenoxyisobutyrate–among them are compounds with the trivial names or designations of SU 13,437; SaH 42-348; halofenate, treloxinate, S-8527, and two valeric acid derivatives. Other lipopenic agents, whose modes of action are described, are linoleic acid amides, Colestipol, antidiabetic agents, bis (hydroxyethylthio) 1,10-decane, pyridinol carbamate, and some nicotinic acid derivatives.

INTRODUCTION

Ever since the correlation between hyperlipidemia and coronary disease was observed, the search for a safe, efficacious agent to lower serum lipids has engaged many biomedical scientists. Although initial efforts were directed at lowering circulating lipids, it now has become mandatory to examine all phases of lipid metabolism. Thus, most current hypocholesteremic drugs will be tested for their effects upon serum cholesterol; cholesterol synthesis and degradation; and cholesterol absorption, excretion, and deposition. Hypotriglyceridemic drugs are tested in a similar fashion.

Actually, when discussing lowering serum lipids we really are talking about affecting serum lipoproteins. However, since we only now are beginning to understand lipoprotein synthesis and degradation, we continue to focus our attention upon the lipid components of the lipoproteins. Serum lipids can be lowered by a number of mechanisms, among them: inhibition of cholesterol synthesis, increased cholesterol catabolism, inhibition of reabsorption of cholesterol or bile acids, inhibition of lipolysis (release of free fatty acids [FFA]), inhibition of fatty acid (FA) synthesis, and interference with lipoprotein synthesis or release. Among the currently available hypocholesteremic agents, there are few whose mechanism of action is clear. The two most popular hypocholesteremic agents are ethyl p-chlorophenoxyisobutyrate (CPIB) (Atromid-S; Clofibrate) whose mechanisms of action include a decrease of cholesterol and lipoprotein synthesis (1-3) and increased excretion (4) and D-thyroxine (Choloxin) which, if it mimics the L-isomer, affects cholesterol distribution in the body (5) and alters bile acid spectrum but not quantity (6).

This discussion will limit itself to newer hypolipidemic agents.

The success of ethyl p-chlorophenoxyisobutyrate and its acceptance by the medical profession have stimulated attempts at synthesis of structurally similar compounds. Two congeners, 2-methyl-(p-1,2,-3,4-tetrahydro-1-naphthylphenoxy) propionic acid (SU 13,437) and 1-methyl-4-piperidyl bis (p-chlorophenoxy) acetate (SaH 42-348), were discussed in an earlier review (7). The former is an effective hypolipidemic agent in rats (8,9) but causes hepatomegaly. In man, it has been shown to be

[1]One of six papers presented in the symposium, "Effect of Drugs on Lipid Metabolism," AOCS Spring Meeting, New Orleans, April 1973.

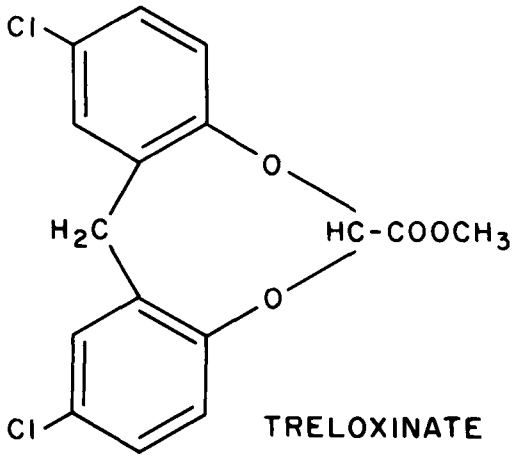
TABLE I

Influence of Halofenate (0.05%) and CIPB[a] (0.3%) upon Serum and Liver Lipids in Rats[b]

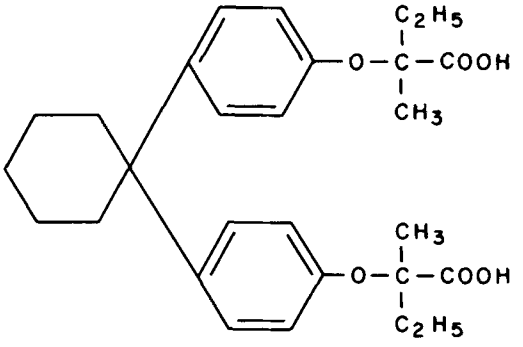
Parameters	Halofenate	CPIB	Control
Number	5/6	6/6	6/6
Wt gain (g)	93 ± 10	81 ± 6	95 ± 7
Liver wt (g)	9.9 ± 0.8	12.7 ± 0.7	8.4 ± 0.7
as % body wt	4.11 ± 0.20	5.54 ± 0.16	3.46 ± 0.19
Serum, mg/dl			
Cholesterol	24.3 ± 1.1	26.0 ± 1.8	44.5 ± 3.4
Triglycerides	57.2 ± 4.0	50.0 ± 4.5	63.0 ± 5.9
Liver, mg/100 g			
Cholesterol	376 ± 7	362 ± 11	345 ± 11
Triglycerides	476 ± 14	408 ± 35	450 ± 19

[a]Ethyl p-chlorophenoxyisobutyrate.

[b]Three week feeding.

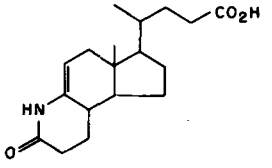


Scheme 1

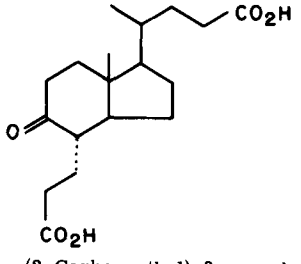


S-8527

Scheme 2



(4R)-4-(2, 3, 4, 6, 6aβ 7, 8, 9, 9aα, 9bβ-Decahydro-6aβ-methyl-3-oxo-1H-cyclopenta[f]quinolin-7β-yl)valeric acid, (321328-S.)



(4R)-4-[4α-(2-Carboxyethyl)-3aα-perhydro-7aβ-methyl-5-oxoindan-1β-yl]valeric acid, (320115-S.)

Scheme 3

$HO{\cdot}CH_2{\cdot}S{\cdot}[CH_2]_{10}{\cdot}S{\cdot}CH_2{\cdot}CH_2OH$

bis-(Hydroxyethylthio) 1,10-Decane

(LL 1558)

Scheme 4

TABLE II

Oxidation of (26-^{14}C) Cholesterol to $^{14}CO_2$ by Liver Mitochondria of Rats fed Halofenate (0.05%) or Clofibrate (0.3%)

Compound	No. experiments	Cytosol	Oxidation
Halofenate	14	+	6.4
	10	–	4.9
CPIB[a]	12	+	8.9
	6	–	4.1
Control	18	+	7.1
	12	–	1.9

[a]See definition Table I.

effective against type II, III, and IV hyperlipoproteinemias (10-12). The latter is hypolipidemic in rats (13,14) with concomitant hepatomegaly and reduces both cholesterol and triglyceride levels in man (15).

Halofenate or 2-acetoamidoethyl (p-chlorophenyl) (m-trifluoro-methylphenoxy) acetate has been found to be hypolipidemic in rats (16). We fed this compound to rats at the 0.05% level and compared it with CPIB (0.3%) (Table I). Liver wt of rats fed halofenate were 18% higher than those of control rats, but it was not as significantly hepatomegalic as CPIB was. Halofenate lowered serum cholesterol levels significantly ($p < .001$) (17). We have reported (18) that liver mitochondrial preparations from rats fed CPIB oxidize significantly more (26-^{14}C) cholesterol to $^{14}CO_2$ in the absence of the cytosol cofactor but not in its presence. Using the cholesterol oxidation system of Whitehouse, et al., (19) we compared mitochondria from livers of rats fed halofenate or CPIB and found that halofenate oxidized 10% less (26-^{14}C) cholesterol to $^{14}CO_2$ in the presence of cytosol but 258% more when cytosol was absent (Table II).

Treloxinate (2,10-dichloro-12H-dibenzo [d,g]-[1,3]-dioxocin-6-carboxylic acid methyl ester) (Scheme 1) has been shown to be a potent hypolipidemic agent in rats (20). It is interesting to note that it affects only triglycerides in Sprague-Dawley rats but both triglycerides and cholesterol in Wistar rats (21). A large number of derivatives of treloxinate have been synthesized and tested for hypolipemic activity in rats (21). In general, the derivatives

TABLE III

Effect of S8527 (0.3%) or CPIB[a] upon Serum and Liver Lipids of Rats[b]

Parameters	S8527	CPIB	Control
Number	14	14	14
Wt gain (g)	57	47	55
Liver wt (g)	8.4	10.4	7.5
as % body wt	4.00	5.18	3.64
Cholesterol			
Serum, mg/dl	22.8	23.1	37.0
Liver, mg/100 g	177.1	129.6	154.2
Triglyceride			
Serum, mg/dl	26.3	32.9	49.9
Liver, mg/100 g	102.0	108.4	119.5

[a]See definition, Table I.

[b]Three week feeding.

have a greater hypotriglyceridemic than hypocholesteremic activity.

Another new aryloxy compound is S-8527 (1,1-bis [4′-(1″-carboxy-1″-methylpropoxy)-phenyl] cyclohexane) (Scheme 2). This compound, when fed to rats at the level of 30 mg/kg, was found to lower serum cholesterol and triglyceride levels by 44 and 53%, respectively. Liver triglyceride levels were 31% lower, but cholesterol levels were 15% higher (22). We fed this compound to rats (0.3% of the diet) and confirmed the hypolipemic effect (23). Table III summarizes data from two 3 week feeding experiments and shows that serum cholesterol and triglycerides were reduced by 38 and 47%, respectively. There was a slight hepatomegalic effect. Cholesterol synthesis from (1-^{14}C) acetate was reduced significantly ($p < .01$) in liver slices from rats fed S-8527 but was unaffected when (2-^{14}C) mevalonate was the precursor. Oxidation of (26-^{14}C) cholesterol to $^{14}CO_2$ by liver mitochondria from rats

TABLE IV

Influence of Two Valeric Acid Derivatives upon Various Parameters of Lipid Metabolism[a]

Parameters	Percent of control	
	32115S	321328S
Wt gain	98	74
Liver wt	106	149
Serum cholesterol	103	119
Liver cholesterol	108	106
Serum triglyceride	109	96
Liver triglyceride	107	103
Cholesterol synthesis		
Acetate	156	11
Mevalonate	95	36
Fatty acid synthesis	137	73
Cholesterol 7α hydroxylation	232	149
(26-^{14}C) Cholesterol oxidation		
+ Cytosol	87	147
− Cytosol	73	282
Cholesterol absorption	102	102
Steroid excretion	93	101

[a]Rats fed 0.3% compound.

fed S-8527 was 31% higher than control level in the presence of cytosol and 21% higher in its absence. The compound did not appear to affect cholesterol absorption.

Two valeric acid derivatives (Scheme 3) also have been tested recently for their effects upon serum and liver lipids, cholesterol and FA synthesis, oxidation and absorption (24). The results are summarized in Table IV. One of the compounds (321328S) inhibits cholesterol synthesis, and both compounds enhance cholesterol hydroxylation.

A series of amides of linoleic acid have been shown to inhibit experimental atherosclerosis in rabbits (25,26). These compounds appear to inhibit cholesterol absorption (27,28). Another inhibitor of cholesterol absorption is Colestipol, which is a high mol wt copolymer of tetraeth-

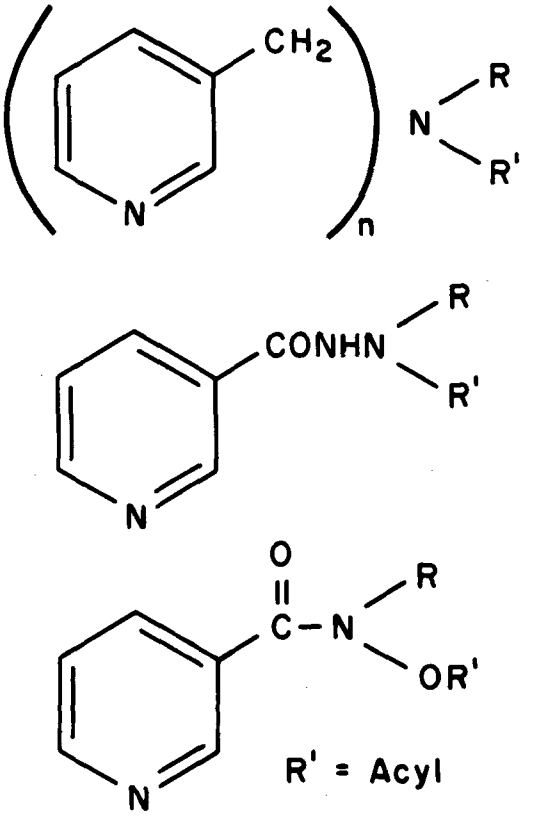
TABLE V

Influence of Antidiabetic Agents upon Atherosclerosis in Rabbits[a,b]

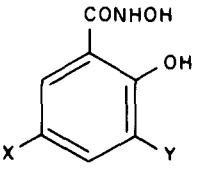
Group	No.	Drug	Cholesterol mg/day	Aortic cholesterol mg/100 g
N	62	—	—	194
C	8	—	200	444
M	8	Metformin	200	208
P	8	Phenformin	200	389
C	8	—	300	805
I	8	Insulin	300	1000
P	12	Chlorpropamide	300	1975
B	16	Carbutamide	300	1950
C	16	—	400	1450
M	16	Metformin	400	610
P	12	Phenformin	400	1000

[a]After Agid and Marquie (31).

[b]Sixty day feeding.



Scheme 5



X	Y	% Fall in Serum Cholesterol
Br	Br	43.2
Br	F	36.9
I	Cl	33.0
Br	Cl	26.0

FIG. 1. Influence of salicylhydroxamic acids on serum cholesterol in rabbits (after Czyzk, et al. [32]).

ylene pentamine and epichlorhydrin. Colestipol is hypocholesteremic in man, dogs, and cockerels (29). We have found (30) that, when fed at the 1% level to rabbits who are ingesting a diet containing 2% cholesterol and 6% corn oil, Colestipol significantly ($p < .05$) inhibits atherosclerosis. The severity of atheromata is reduced by 28% in the aortic arch and by 26% in the thoracic aorta.

There are a number of other compounds on test which resemble no known hypocholesteremic agents and whose mechanism of action is unknown. Agid and Marquie (31) have found that two guanidine containing antidiabetic agents, metformin and phenformin, will reduce atherosclerosis in rabbits by 44 and 21%, respectively. Insulin, chlorpropamide, and carbutamide actually enhance atherosclerosis by 24-145% (Table V). A series of salicylhydroxamic acids have been tested for their effects upon serum cholesterol levels of rabbits (32). The most effective agents are those with halogen atoms in the 3 and 5 positions; the most potent hypocholesteremic agent being 3,5-dibromosalicylhydroxamic acid (Fig. 1). Another interesting new hypocholesteremic agent is bis (hydroxyethylthio) 1,10-decane (LL-1558) (33,34) (Scheme 4). In rats fed a diet containing 4.5% cholesterol and 0.3% propylthiouracil, LL-1558 significantly reduces choles-

TABLE VI

Influence of LL 1558 upon Serum Cholesterol in Rats[a,b]

Diet	No.	Drug	Dose mg/kg/day	Serum cholesterol mg/dl ± SEM	p
Control	125	—	—	90 ± 2.9	—
Cholesterol (4.5%) (A)[c] + PTU (0.3%)	125	—	—	268 ± 16.1	—
A[c]	55	LL[d]	125	158 ± 10.2	<.001
A	30	LL	250	159 ± 12.3	<.001
A	40	CPIB[e]	125	163 ± 11.3	<.001
A	30	CPIB	250	173 ± 15.4	<.001

[a]After Assous, et al. (33).
[b]Fifteen days.
[c]Atherogenic regimen.
[d]bis(hydroxyethylthio) 1,10 decane.
[e]See definition Table I.

TABLE VII

Influence of LL 1558 upon Atherosclerosis in Rabbits[a,b]

Diet	No.	Drug	Dose mg/kg/day	Serum cholesterol mg/dl ± SEM	Average atheroma
Normal	5	—	—	77 ± 14	0
Atherosclerosis	6	—	—	970 ± 69	3+
Atherosclerosis	6	LL[c]	50	520 ± 125	±
Atherosclerosis	6	LL	100	700 ± 177	±
Atherosclerosis	6	CPIB[d]	100	612 ± 93	±

[a]After Assous, et al. (33).
[b]Cholesterol diet 1%; 70 days.
[c]See definition Table VI.
[d]See definition Table I.

terol levels when fed at a dose of 125-250 mg/kg/day. The reduction in cholesterol levels is similar to that observed when CPIB is administered at the same level (Table VI). A dose of 50 mg/kg/day fed to rabbits on an atherogenic regimen will reduce cholesterol levels by 46% and inhibit atherosclerosis (Table VII). In man, a dose of 1.2-3.6 g/day significantly reduces serum cholesterol by 16% in type II patients, triglycerides by 26% in mixed hyperlipidemias, and by 65% in endogenous hypertriglyceridemia (35) (Table VIII).

Nicotinic acid will reduce serum cholesterol levels (36) and will inhibit lipolysis which leads to a reduction in serum FFA. A number of nicotinic acid derivatives has been synthesized and tested in recent years, and one of the most potent inhibitors of lipolysis is ethyl 5-fluoronicotinate (37).

A large number of other derivatives now have been synthesized and tested (38-40). These have included different esters of 5-fluoro and 5-chloronicotinic acid, pyrazoles, and isoxazoles and disubstituted pyridines. A number of compounds were found to be as active as nicotinic acid. Bailey, et al., (41) synthesized "masked" nicotinic acid derivatives—acyl derivatives of 3-pyridyl-methylamines, nicotinic acid hydrazides, and nicotinohydroxamic acids (Scheme 5)—and these have been found to be active on a basis equivalent to equimolar amounts of nicotinic acid. Pyridinol carbamate (2,6-bis[hydroxymethyl] di-N-methyl-carbamate) has been reported to relieve the complications of arteriosclerosis in man and to inhibit atherosclerosis in rabbits (42-44). We have studied the effect of pyridinol carbamate on cholesterol metabolism in rats (45-47), and our findings are summarized in Table IX. In the rat, this compound reduces serum triglycerides, inhibits cholesterol synthesis from acetate, and reduces cholesterol absorption.

When fed (30 mg/day) to rabbits maintained on an atherogenic regimen, pyridinol carbamate had no effect upon the severity of atherosclerosis. Atherosclerosis in the aortic arch was somewhat less severe (87%) than that observed in the controls and that in the thoracic aorta was more severe (106%). Wu (48) has reported that 10 mg/kg/day of pyridinol carbamate will protect rabbits against atherosclerosis. Neither Sanwald and Wagener (49) nor Möttönen, et al., (50) found this compound to affect arterial metabolism. Malinow, et al., (51,52) found that pyridinol carbamate is not effective against experimental atherosclerosis in either squirrel or cynomolgus monkeys.

TABLE VIII

Influence of LL 1558 upon Serum Lipids in Man[a]

Item	Percent reduction		
	Type II	Mixed	Type IV
Total lipid	-13[b]	-10[b]	-28[b]
Cholesterol	-16[b]	- 6[b]	- 8
Triglyceride	- 5	-26[b]	-65[b]
β-Lipoprotein	-11[b]	- 5[b]	-12

[a]After Rouffy and Loeper (35).
[b]Statistically significant.

TABLE IX

Influence of Pyridinol Carbamate (0.3%) upon Cholesterol Metabolism in Rats

Parameters	Percent control
Wt gain	89
Liver wt	99
Serum cholesterol	106
Triglycerides	80
Free fatty acids	101
Liver cholesterol	100
Cholesterol synthesis	
Acetate	32
Mevalonate	93
Oxidation of (26-^{14}C) cholesterol	136
Cholesterol absorption	65

The true test of any hypercholesteremic drug must be its efficacy in reducing the toll of cardiovascular disease in man. There are several drug trials under way, and the results of these trials should give us some idea of the interplay of effects upon serum lipids and mechanism of action in affecting coronary disease in man.

ACKNOWLEDGMENTS

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Effects of Ethanol upon Lipid Metabolism[1]

CHARLES S. LIEBER, Liver Disease and Nutrition Laboratory, Bronx Veterans Administration Hospital, Bronx, New York 10468 and Department of Medicine, Mount Sinai School of Medicine of the City University of New York, New York

ABSTRACT

Ethanol abuse produces fatty liver which cannot be prevented by supplementation in protein, minerals, vitamins, and choline. In rats, protein and choline deficiencies potentiate the effect, whereas replacement of dietary fat by medium chain triglycerides or carbohydrates decreases the capacity of ethanol to produce steatosis. Administration of a single dose of ethanol to rats represents a stressful condition associated with moderate hepatic accumulation of fatty acids derived from adipose tissue. By contrast, chronic ethanol administration produces more pronounced steatosis with a predominance of endogenously synthesized and, when available, dietary fatty acids. These accumulate because of decreased fat oxidation. Ethanol also stimulates hepatic lipogenesis. These various effects can be explained by the increase in the hepatic nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide ratio secondary to the oxidation of ethanol via the alcohol dehydrogenase pathway. In addition there are more lasting changes in intermediary metabolism, such as increased hepatic ketogenesis which could be linked to the persistent alteration in mitochondrial function and structure found after chronic ethanol ingestion. The ultrastructural changes also are characterized by proliferation of the hepatic smooth endoplasmic reticulum. The latter was documented by subfractionation. This led to the description of a new pathway for ethanol metabolism, the microsomal ethanol oxidizing system, which doubles in activity after ethanol feeding. The existence of the microsomal ethanol oxidizing system may contribute to our understanding of increased cholesterol and lipoprotein synthesis. Other effects upon lipid metabolism include decreases in free fatty acids and glycerol concentrations and free fatty acid turnover which result from inhibition of peripheral fat mobilization by acetate, a metabolite of ethanol.

INTRODUCTION

The most common disturbance in lipid metabolism produced by alcohol abuse is that of excessive accumulation of lipids in the liver resulting in a fatty liver. Attempts at elucidating the pathogenesis of the alcoholic fatty liver over the last decade have revealed a variety of effects of ethanol on lipid metabolism not only in the liver but in other organs as well, including the adipose tissue.

ETIOLOGIC ROLE OF ETHANOL IN THE PATHOGENESIS OF THE ALCOHOLIC FATTY LIVER

Until a decade ago, the concept prevailed that malnutrition was primarily responsible for the development of the alcoholic fatty liver. This notion was based largely upon experimental work in rats given ethanol in drinking water (1). With this technique, ethanol consumption usually does not exceed 10-25% of the total caloric intake of the animal. A comparable amount of alcohol, when given with an adequate diet, resulted in negligible ethanol levels in the blood (2). By incorporating ethanol in a totally liquid diet, the amount of ethanol consumed was increased to 36% total calories, a proportion comparable to moderate alcohol intake in man. With these nutritionally adequate diets, isocaloric replacement of sucrose or other carbohydrate by ethanol consistently produces a five-tenfold increase in hepatic triglycerides (2-5). As shown in Figure 1, isocaloric replacement of fat by ethanol also produced steatosis, whereas isocaloric replacement of carbohydrate by fat did not. Hepatic lipid accumulation developed progressively over the first month of alcohol administration and persisted thereafter for at least 1 year in the rat and 3 years in the baboon (7). This contrasts with the results of Porta, et al., (8) who found hepatic fat accumulation to be only transient during the first 4-8 weeks of ethanol consumption; this may be due to the fact that the amount of ethanol consumed by animals studied for 4 months (which did not develop steatosis) was only half that of the short term group. Similarly, when in our studies

[1]One of six papers presented in the symposium "Effect of Drugs on Lipid Metabolism," AOCS Spring Meeting, New Orleans, April 1973.

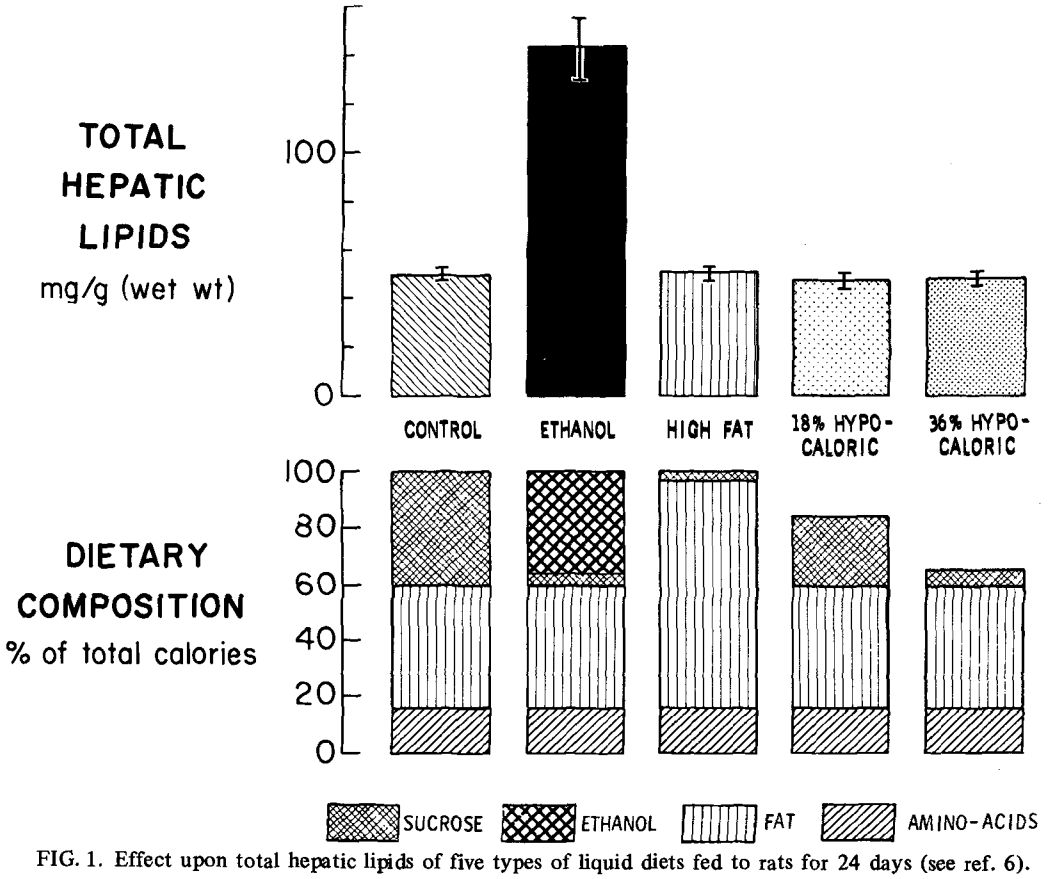

FIG. 1. Effect upon total hepatic lipids of five types of liquid diets fed to rats for 24 days (see ref. 6).

in the rat the ethanol intake was decreased from 36 to 20% of the total calories, no fatty liver was observed (2).

An etiologic role for ethanol in the pathogenesis of human liver disease was suggested by the parallel changes in alcohol consumption and death rate from cirrhosis, as discussed elsewhere (9). Epidemiological studies also indicated that alcohol, rather than malnutrition, is the determining factor (10,11). The importance of the degree of alcohol abuse is illustrated by the fact that, of those with a daily consumption in excess of 160 g ethanol/day, 75% displayed severe liver damage, whereas only 17% who consumed a lesser amount were so affected (10). The duration of alcohol abuse is also important: after 15 years of excessive alcohol consumption, the incidence of severe liver damage was eight times greater than after 5 years. Furthermore, ethanol itself, rather than the congeners of the alcoholic beverages, was implicated (12).

Undernutrition during World War II (13) and starvation as a treatment for obesity (14) or as a consequence of anorexia (15) were not associated with hepatic steatosis.

Volwiler, et al., (16) and Summerskill, et al., (17) failed to detect any deleterious effects from alcohol administration in patients recovering from alcoholic fatty liver. In these studies, however, the amounts of alcohol given were less than the usual intake of alcoholics. With larger amounts of alcohol, Menghini (18) found that the clearance of fat from the alcoholic fatty liver was prevented. Moreover, individuals with a morphologically normal liver (with or without a history of alcoholism) developed a fatty liver when given a variety of nondeficient diets under metabolic ward conditions, with ethanol either as a supplement to the diet or as an isocaloric substitution for carbohydrates (2,3,19,20). This was evident both by morphologic examination and by direct measurement of the lipid content of the liver biopsies which revealed up to a twenty-fivefold rise in triglyceride concentration. Even with a high protein, vitamin supplemented diet, there was a significant increase in hepatic triglycerides, as measured in percutaneous biopsies (Fig. 2). This increase was apparent after a few (20), or even

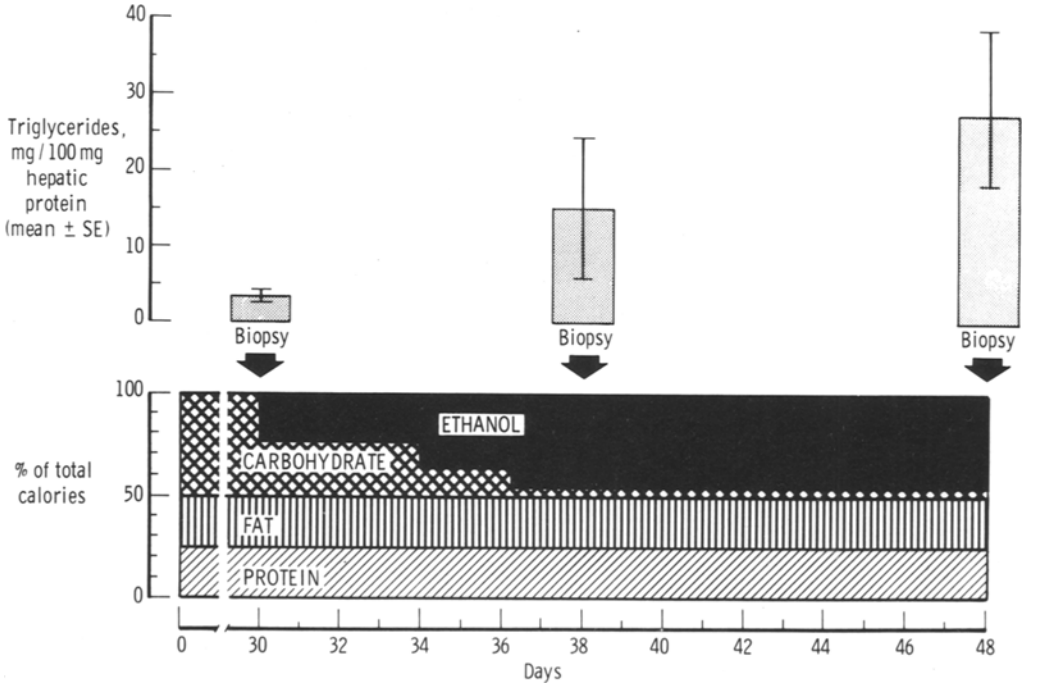

FIG. 2. Effect of ethanol upon hepatic triglycerides in five volunteers given a high protein, low fat diet (see ref. 6).

one (21) days. This steatosis, though reversible, was accompanied by striking ultrastructural changes, as discussed subsequently. Alcohol interferes with intestinal absorptive functions (22-24) and its integrity (25,26). Impairment of lipid absorption, pancreatic function, and intraluminal fat digestion has been reported in alcoholics (27-29) or in subjects after ethanol administration (30,31). Furthermore, in rats, deficiency states can aggravate the effect of alcohol upon the liver (32,33). However, it is unlikely that a deficiency state developed over the short period of some of the studies conducted in volunteers (3,19-21).especially in view of the liberal enrichment of the diets (19,20).

INFLUENCE OF DIETARY FACTORS

Role of Dietary Fat

As discussed subsequently, alcohol ingestion leads to the deposition in the liver of dietary fat. This observation prompted an investigation into the role of the amount and kind of dietary fat in the pathogenesis of alcohol induced liver injury. Rats were given liquid diets containing an adequate amount of protein for rodents (18% total calories), with varying amounts of fat (Fig. 3). In the 2% fat diet, the only lipid given was linoleate to avoid essential fatty acid (FA) deficiency. Reduction in dietary fat to a level of 25% (or less) total calories was accompanied by a significant decrease in the steatosis induced by ethanol (34). These results obtained with alcohol differ from the fatty liver resulting from choline deficiency, the degree of which was found to be independent of the amount of dietary fat (35). The importance of dietary fat was confirmed in volunteers: for a given alcohol intake, much more steatosis developed with diets of normal fat content than with a low fat diet (36). In addition to the amount, the chain length of the dietary fatty acid is also important for the degree of fat deposition in the liver. Replacement of dietary triglycerides containing long chain fatty acids (LCT) by fat containing medium chain fatty acids (MCT) reduces the capacity of alcohol to produce a fatty liver in rats (37). The propensity of MCT to undergo oxidation rather than esterification probably explains this phenomenon (38).

Role of Protein and Lipotropic Factors (Choline and Methionine)

In growing rats, deficiencies in dietary protein and lipotropic factors (choline and methionine) can produce fatty liver (1), but primates are far less susceptible to protein and lipotrope deficiency than rodents (39). Clinically, treatment with choline of patients suffering from alcoholic liver injury has been found to be ineffective in the face of continued alcohol

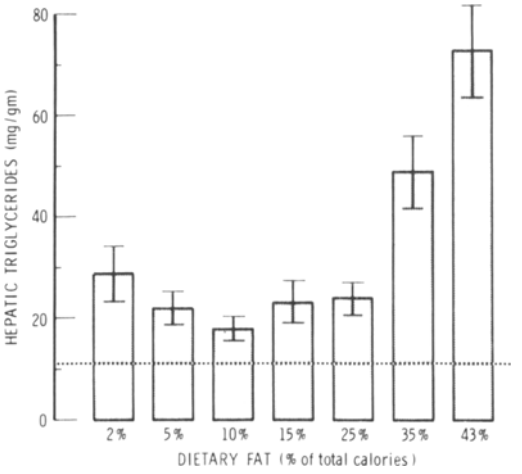

FIG. 3. Hepatic triglycerides in seven groups of rats given ethanol (36% calories) with a diet normal in protein (18% calories) but varying fat content. Average hepatic triglyceride concentration in the control animals is indicated by a dotted line (see ref. 34).

abuse; and, experimentally, massive supplementation with choline failed to prevent the fatty liver produced by alcohol in volunteer subjects (20). This is not surprising, since there is no evidence that a diet which is deficient in choline is deleterious to adult man. Unlike rat liver, human liver contains little choline oxidase activity, which may explain the species difference with regard to choline deficiency. The phospholipid content of the liver represents another key difference between the fatty liver produced by ethanol and that caused by choline deficiency. After the administration of ethanol, hepatic phospholipids increase (2), whereas in the fatty liver produced by choline deficiency they decrease (40). Similarly, hepatic carnitine is decreased by choline deficiency (41) but increased after ethanol feeding (42). Hepatic injury induced by choline deficiency appears to be primarily an experimental disease of rats with little, if any, relevance to human alcoholic liver injury. Even in rats, massive choline supplementation failed to prevent fully the ethanol induced lesion, whether alcohol was administered acutely (43) or chronically (37). Ultrastructurally, the two types of fatty liver also differ (44).

The effect of protein deficiency has not been clearly delineated yet in human adults. In children, protein deficiency leads to hepatic steatosis, one of the manifestations of Kwashiorkor. In adolescent baboons, however, protein restriction to 7% total calories did not result in conspicuous liver injury either by biochemical analysis or by light and electron microscopic examination even after 19 months (45). Significant steatosis was observed only when the protein intake was reduced to 4% total calories (7). Furthermore, an excess of protein (25% of total calories or twice the recommended amount) did not prevent ethanol from producing fat accumulation in human volunteers (Fig. 2). Thus, in man, ethanol is capable of producing striking changes in the liver even in the absence of protein deficiency. When protein deficiency is present, it may potentiate the effect of ethanol. In the rat, a combination of ethanol and a diet deficient in both protein and lipotropic factors leads to more pronounced hepatic steatosis than either deficiency alone (32,33). Other investigators failed to detect differences in fat accumulation between rats given deficient diets alone and those given deficient diets with alcohol (1,46,47). There were however striking differences by other criteria. The most conspicuous was a mortality rate, which ranged from 23-33% in the alcohol fed rats compared to none in the controls. With a severely deficient diet, consisting of only pretzels and alcohol, the morbidity reached 60%, compared to 7% in control rats given pretzels alone (48). This inequality in mortality again illustrates the toxicity of ethanol.

ORIGIN OF THE FA IN THE ALCOHOLIC FATTY LIVER: MECHANISM FOR THEIR ACCUMULATION AND ASSOCIATED CHANGES IN INTERMEDIARY AND DRUG METABOLISM

Lipids which accumulate in the liver can originate from three main sources: dietary lipids, which reach the bloodstream as chylomicrons; adipose tissue lipids, which are transported to the liver as free fatty acids (FFA); and lipids synthesized in the liver itself (Fig. 4). These FA can accumulate in the liver because of a variety of metabolic disturbances (50). The four major mechanisms which have been proposed are: (A) decreased lipid oxidation in the liver, (B) enhanced hepatic lipogenesis, (C) decreased hepatic release of lipoproteins, and (D) increased mobilization of peripheral fat. Depending upon the experimental conditions, any of the three sources and the four mechanisms can be implicated.

Decreased Hepatic FA Oxidation and Enhanced Lipogenesis: Their Link to the Metabolism and Metabolic Effects of Ethanol in the Cytosol and Mitochondria

After consumption of ethanol with lipid containing diets, the FA which accumulate in the liver are derived primarily from dietary FA, whereas when ethanol is given with a low fat diet endogenously synthesized FA are deposited in the liver (33,36,51). Some of these effects can be considered as consequences of

the metabolism of ethanol in the liver.

Effects of hepatic nicotinamide adenine dinucleotide (NADH) generated by the alcohol dehydrogenase pathway on FA oxidation, lipogenesis, lacticidemia, serum uric acid, and acidosis: In vitro, ethanol decreases lipid oxidation (38,52), and the reduction in $^{14}CO_2$ production from palmitate-^{14}C is paralleled by a decrease in $^{14}CO_2$ from acetate-^{14}C (52,53). This suggests that the decrease in FA oxidation may result from reduced citric acid cycle activity (Fig. 5). A dimunition in citric acid cycle activity also was documented by the observation of a reduction in absolute amount of CO_2 produced in isolated livers perfused with ethanol without change in oxygen consumption (38,55). There was a parallel decrease in the oxidation of FFA and chylomicrons (38). Reduced FA oxidation also was recently confirmed in isolation hepatocytes (56). This mechanism may explain the hepatic accumulation of FA originating from the diet, as well as from any other source, including endogenous synthesis. In addition, hepatic accumulation of endogenously synthesized lipids (when ethanol is given with low fat diets) could result from a stimulation of hepatic lipogenesis.

Both decreased lipid oxidation and enhanced lipogenesis can be linked to ethanol oxidation and the associated increased generation of NADH. Ethanol can be synthesized endogenously in trace amounts, but it is primarily an exogenous compound which is readily absorbed from the gastrointestinal tract. Only 2-10% of that absorbed is eliminated through the kidneys and lungs; the rest must be oxidized in the body, and most of this occurs in the liver. The rate of disappearance of ethanol from the blood is indeed remarkably decreased or halted by hepatectomy or procedures damaging the liver (57). Moreover, the predominant role of the liver in ethanol metabolism was shown directly in individuals with portacaval shunts undergoing hepatic vein catheterization (58). Extrahepatic metabolism of ethanol, although it occurs, is small (59,60). This relative organ specificity probably explains why, despite the existence of intracellular mechanisms responsible for redox homeostasis, ethanol oxidation produces striking metabolic imbalances in the liver. This is aggravated by the lack of a feedback mechanism to adjust the rate of ethanol oxidation to the metabolic state of the hepatocyte and the inability of ethanol, unlike other nutrients, to be stored.

The main hepatic pathway for ethanol disposition involves alcohol dehydrogenase (ADH), an enzyme of the cell sap (cytosol) which catalyzes the conversion of ethanol to

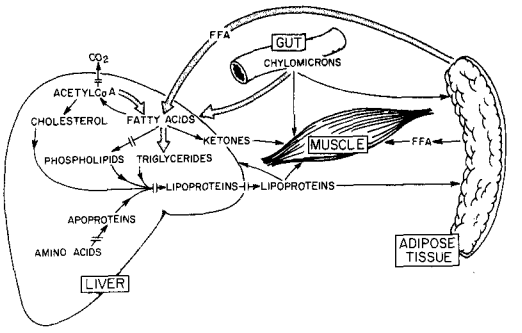

FIG. 4. Possible mechanisms of fatty liver production through either increase (→) or decrease (—|→) of lipid transport and metabolism (see ref. 49).

acetaldehyde. Hydrogen is transferred from ethanol to the cofactor nicotinamide adenine dinucleotide (NAD), which is converted to NADH (Fig. 5). The acetaldehyde produced is then converted to acetate again with loss of hydrogen and reduction of NAD to NADH. As a net result, ethanol is converted in the liver to acetate which is released into the bloodstream, leaving an excess of reducing equivalents in the liver (as NADH). This increased NADH generation is reflected in an enhanced NADH/NAD ratio, which in turn produces a change in the ratio of those metabolites whose reduction is dependent upon the NADH-NAD couple. For instance, the increased NADH/NAD ratio raises the concentration of α-glycerophosphate (61) which favors hepatic triglyceride accumulation by trapping FA. In addition, excess NADH promotes lipogenesis (52,62), possibly by the mitochondrial elongation pathway or transhydrogenation to reduced nicotinamideadenine dinucleotide phosphate (NADPH). Theoretically, enhanced lipogenesis can be considered a means for disposing of the excess hydrogen generated by ethanol oxidation in the liver. Some of the excess hydrogen equivalents can be transferred into the mitochondria by various shuttle mechanisms.

These hydrogen equivalents supplant the citric acid cycle as a source of hydrogen. Ethanol can block the activity of the citric acid cycle in at least two ways, both of which are consequences of the change in the NADH/NAD ratio. Increased NADH/NAD slows those reactions of the cycle which require NAD. Indeed, a major site of interaction of ethanol on the citric acid cycle was found to be on α-ketoglutarate oxidation (56). Moreover, the redox change associated with ethanol oxidation decreases hepatic concentration of oxaloacetate (63), the availability of which controls the activity of citrate synthetase. Under these conditions, the mitochondria will utilize the hydrogen equiv-

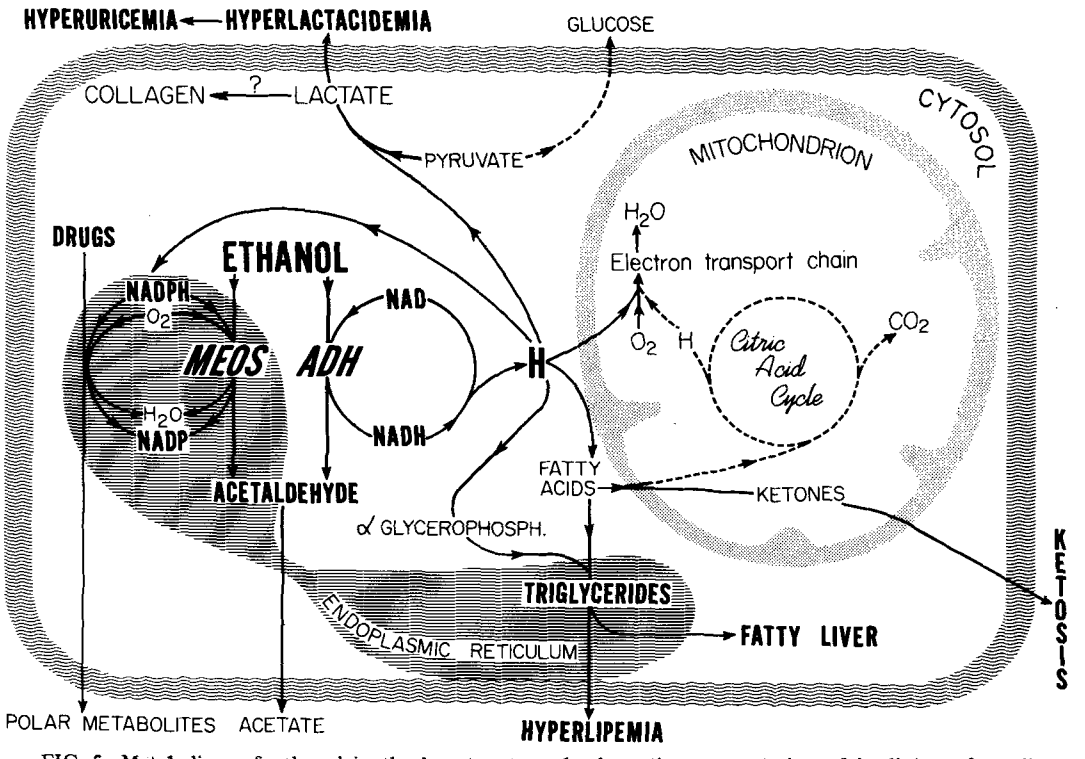

FIG. 5. Metabolism of ethanol in the hepatocyte and schematic representation of its link to fatty liver, hyperlipemia, hyperuricemia, hyperlactacidemia, and ketosis (ADH = alcohol dehydrogenase and MEOS = microsomal ethanol oxidizing system). Pathways that are decreased by ethanol are represented by dashed lines (see ref. 54).

alents from the ethanol, rather than oxidize the two carbon fragments derived from the FA. FA, which normally represent the main mitochondrial fuel (64), are, thus, supplanted by ethanol. Therefore, the liver burns ethanol in preference to fat. This decreased activity of the citric acid cycle results in the deposition in the liver of dietary fat when available or FA derived from adipose tissue and endogenous synthesis in the absence of dietary fat.

The enhanced NADH/NAD also reflects itself in an increased lactate/pyruvate ratio which results in hyperlactacidemia, thus contributing to the acidosis which follows ethanol consumption. Lactate also decreases the capacity of the kidney to excrete uric acid which leads to secondary hyperuricemia (65).

Persistent changes in the structure and function of mitochondria and associated alterations in lipid and ketone metabolism: In addition to the changes in mitochondrial functions which are a direct consequence of the metabolism of ethanol, chronic ethanol abuse results in more persistent changes in the mitochondria. Indeed, alcoholics are known to have profound mitochondrial changes in their liver (66,67), including the swelling and disfiguration of mitochondria, disorientation of the cristae, and intramitochondrial crystalline inclusions. Increased serum activity of the intramitochondrial enzyme glutamate dehydrogenase also was reported in alcoholics (68). From these clinical observations, however, it was impossible to assess whether the mitochondrial changes were a direct result of chronic ethanol intake or were secondary to other factors, such as dietary deficiencies. This question was resolved by the observation of Iseri, et al., (44) who showed that in the rat, isocaloric substitution of ethanol for carbohydrates in otherwise adequate diets leads to enlargement and alterations of the configuration of the mitochondria.

Mitochondrial changes similar to those seen in chronic alcoholics also were produced by isocaloric substitution of ethanol for carbohydrate in baboons (7) and in man, both in alcoholics (19,69) and in nonalcoholics (20). Mitochondrial alterations occurred under a variety of conditions, which included high protein, low fat, and choline supplemented diets (19,20). Degenerated mitochondria were conspicuous, and the debris of these degraded

organelles also was found within autophagic vacuoles and residual vacuolated bodies (70).

These ultrastructural changes in the mitochondria are associated with increased fragility and permeability (71-73) and decreased phospholipid content (74) and altered FA composition (75). Essential FA deficiency partially protected the mitochondria from developing the increased fragility induced by chronic ethanol feeding (76). The mechanism of the alteration of mitochondrial membranes is unknown but could possibly be linked to depression of mitochondrial protein synthesis by ethanol consumption (71). These altered mitochondria have a reduction in cytochrome a and b content (71) and in succinic dehydrogenase activity (71,77). Though in one study (78), succinic dehydrogenase activity measured in total liver homogenates was reported to be increased in ethanol fed rats.

The striking structural changes of the mitochondria are associated with corresponding functional abnormalities. Indeed, the respiratory capacity of the mitochondria was found to be depressed (79-81), using pyruvate and succinate as substrates. Other substrates also were found to have reduced oxidation by the mitochondria of ethanol fed rats, except for α-glycerophosphate, the oxidation of which was reported by some to be increased (82) or unchanged (79), whereas others found it to be decreased (81). Oxidative phosphorylation was found to be either unaltered (81), decreased (83), or variable depending upon the substrate (79) or perhaps upon the duration of treatment.

The major function of the mitochondria is FA oxidation. As discussed before, ethanol depresses hepatic FA oxidation, and this inhibition can be attributed to the associated redox change and the inhibition of the activity of the citric acid cycle. This redox change has now been demonstrated in mitochondria of rats fed ethanol chronically (84), but this alteration returns rapidly to normal after cessation of alcohol intake (84). Since the structural changes of the mitochondria persist, however, the question arose as to whether these, in turn, could be responsible for some alterations in lipid metabolism beyond those produced by the altered redox change. The first indication that ethanol consumption may result in more persistent metabolic changes arose from the observation that alcohol ingestion is associated with a progressive increase in ketonemia and ketonuria, which was most pronounced in the fasting state in the absence of ethanol (85). Moreover, in experimental animals this is associated with enhanced ketogenesis in liver slices, in the absence of ethanol (85), which contrasts with the inhibition of ketogenesis reported in the presence of ethanol (56). The ketonemia may aggravate the acidosis and hyperuricemia resulting from hyperlactacidemia (as discussed subsequently) and, on occasion, may lead to severe alcoholic ketoacidosis (86). The capacity for ethanol to produce ketones was found to be greater than that of fat itself, provided, however, that fat was present in the diet. Thus, fat seems to play a permissive role (85). Preliminary observations indicated that mitochondria obtained from ethanol fed rats, when incubated in vitro, even in the absence of ethanol, display decreased capacity to oxidize FA but enhanced β-oxidation, possibly responsible for the increased ketogenesis (87). In any event, decreased FA oxidation, whether as a function of the reduced citric acid cycle activity (secondary to the altered redox potential) or whether as a consequence of permanent changes in mitochondrial structure, offers the most likely explanation for the deposition of fat in the liver after chronic alcohol ingestion, especially fat derived from the diet.

Hepatic Lipoprotein Production and Interaction of Ethanol with Hepatic Microsomal Functions

Alcoholic hyperlipemia: The initial phase of hepatic lipid deposition after ethanol is accompanied by an increased release of lipoproteins into the blood; this tends to counteract lipid accumulation in the liver. However, this adaptive mechanism is generally insufficient to prevent fully the development of hepatic steatosis. In any event, decreased hepatic lipoprotein secretion and release, which has been proposed as an explanation for the fatty liver produced by a variety of toxic agents, apparently does not play a role in initiating the development of the fatty liver produced by the usual ethanol abuse. The effect is dose dependent. In vitro, high ethanol concentration may decrease hepatic lipoprotein release (88). More recently, when livers were perfused with ethanol in concentrations more in keeping with in vivo conditions, no inhibition of lipoprotein secretion was found (89). Similarly, contrasting with the hyperlipemia which is commonly associated with the administration of moderate to large amounts of ethanol (3,90,91), an extremely high dose has been reported to decrease serum triglycerides (92), very low density lipoproteins (93), high density lipoproteins (94), and the incorporation of glucosamine into the carbohydrate moiety of serum lipoproteins (95) in the rat. However, both in man (3,90) and in rats (91), ethanol administration usually produces hyper- rather

than hypolipemia, and the most striking changes take place in the very low density lipoprotein fraction. The alcohol induced hyperlipemia can occur in the fasting state (90); it is markedly exaggerated, however, when alcohol is given with a fat containing diet (96-98). This alcohol effect does not result solely from caloric overload, since no comparable hyperlipemia was produced by isocaloric amounts of either carbohydrate or lipids (99). Incorporation into lipoprotein of intragastrically administered ^{3}H-palmitate and intravenously injected ^{14}C-lysine was increased significantly by alcohol administration (91). These, as well as other studies, suggested that the ethanol induced hyperlipemia results from enhanced lipoprotein production. This contrasts with choline (100) and protein (101,102) deficiencies which produce the opposite effect. Ethanol could act by enhancing the availability of FA, which, in turn, can induce hepatic synthesis of lipoproteins (103). Furthermore, FA are esterified (104), and lipoproteins are formed (105) in the endoplasmic reticulum. Moreover, ethanol consumption enhances the activity of hepatic microsomal L-α-glycerophosphate acyl transferase (106), as well as that of other acyl transferases, depending upon the dietary conditions (107). The mechanism of the alterations of these microsomal functions produced by ethanol has not been clarified. It could be linked directly to the fact that ethanol can be oxidized at this key metabolic site and results in a proliferation of the membranes of the smooth endoplasmic reticulum, the morphologic counterpart of the microsomal fraction obtained by ultracentrifugation (Vide infra). Ethanol feeding also was found to enhance the activity of glycosyltransferase in the Golgi apparatus (108).

Ethanol-induced hyperlipemia is usually moderate. However, some alcoholic patients develop marked hyperlipemia, which suggests that other factors in addition to ethanol itself contribute to this alteration. A possible role of postheparin lipoprotein lipase activity (PHLA) in alcoholic hyperlipemia has been suggested on the basis of the finding that six of eight patients with marked hyperlipemia reported by Losowsky, et al., (99) have a decreased PHLA. Furthermore, a mild decrease in the fractional turnover rate of intravenously injected exogenous triglycerides has been reported in alcoholics who develop a marked degree of hyperlipemia (109). However, in most of the subjects reported by Losowsky, et al., (99) the PHLA remained decreased after the hyperlipemia had subsided and after alcohol had been withdrawn for weeks or months. This alteration could not be reproduced either by ethanol in vitro (99) or by administration of ethanol in vivo (98,110,111). Thus, one factor in the development of hyperlipemia could be the existence of a defective removal of serum lipids in some patients. Furthermore, some of the reported alcoholic patients with marked hyperlipemia had other conditions which can contribute to the hyperlipemia, such as diabetes (99,111) or pancreatitis (112). The latter condition has been reported to be associated with the production of an inhibitor of PHLA (113).

Another possible mechanism could be an increased capacity to secrete serum lipoproteins. This may account for the observation that some alcoholic patients appear to have an unusual sensitivity to the hyperlipemic effect of ethanol (111). Thus, patients with normal PHLA have been shown to develop hyperlipemia with doses of ethanol (120-160 g/day) that do not produce hyperlipemia in normal subjects or individuals with endogenous hypertriglyceridemia (type IV). The mechanism for the increased capacity of these patients to develop alcoholic hyperlipemia remains unknown. Since ethanol consumption results in an increased capacity to secrete lipoproteins in response to a lipid load (114), one may wonder whether the difference in response to ethanol between some alcoholics and some individuals with type IV hyperlipemia may be secondary, at least in part, to a difference in prior alcohol consumption.

Concerning the site of the ethanol effects, it is noteworthy that both in men and in rats, ethanol-induced hyperlipemia results in increased concentrations of the various serum lipoprotein fractions, but the main change occurs in the lipoproteins of $d<1.006$. In the postprandial state, this fraction includes very low density lipoproteins and chylomicrons. In patients with alcoholic hyperlipemia, chylomicron-like particles have been observed in the fasting state (109). In the rat rendered hyperlipemic by ethanol feeding, the lipid/protein ratio of the $d<1.006$ lipoproteins approaches that of chylomicrons (114). However, the site of origin of these particles cannot be deduced with certainty from physical or chemical characteristics. Indeed, in other states of accelerated lipoprotein production, such as carbohydrate-induced hyperlipemia, the lipid/protein ratio and particle size of the $d<1.006$ lipoproteins increases even in the absence of dietary fat. The increase in serum lipoproteins of higher density both in man (98) and in rats (91) indicates that the hyperlipemia is not merely of intestinal origin and that the liver participates in this process.

The possibility still remains that, after alcohol feeding, the intestine releases more lipid into the lymph, either by decreasing oxidation of FA or by increasing the synthesis of lipids from sources other than dietary fat (115,116). A decreased production of $^{14}CO_2$ from labeled FA by intestinal slices after an acute load of alcohol (117) and an increased incorporation of these FA into intestinal triglycerides by slices obtained from rats fed ethanol (118) have been reported. To what extent these alterations contribute to alcoholic hyperlipemia is unknown. Recently, Mistilis and Ockner (119) have shown that intraduodenal infusion of 10% ethanol to the fasted rat in a dose of 5 g/kg produces a mild increase in the very low density lipoprotein output in the lymph. They postulated that this increase in nondietary lymph lipid could contribute to the hyperlipemia, although the peak serum rise actually preceded the maximum increase in intestinal lymph lipids. Furthermore, lymph lipoproteins can derive in part from plasma lipoproteins (120). Moreover, although a single intragastric administration of a diet containing ethanol (3 g/kg) increased both intestinal lymph flow and lipid output in rats not previously fed alcohol, postprandial hyperlipemia was not produced under these conditions (114). Actually, the acute load of an ethanol containing diet did not increase lymph lipid output in rats fed alcohol for several weeks, compared to their pair fed controls; however, marked hyperlipemia developed in these alcohol fed rats. Moreover, when a similar lymph lipid load was infused intravenously to alcohol pretreated and control rats with diversion of intestinal lymph, the alcohol fed rats developed hyperlipemia. If lymph depletion was not prevented by intravenous replacement, hepatic and plasma lipids decreased, and alcoholic hyperlipemia did not occur. This indicates that, although an adequate supply of dietary lipids represents a permissive factor needed to induce alcoholic hyperlipemia in the rat, changes in lymph lipid output do not seem to play a major role in the lipemic effect of ethanol and that the site of origin of the increased production of serum lipoprotein is a nonintestinal one, most likely hepatic. Similarly, the contribution of lymph lipids to the steatosis appears to be a minor one (121).

The mechanism for the increase in lipids other than triglycerides in the course of alcoholic hyperlipemia remains unknown. This is due partly to the fact that the role of cholesterol and phospholipids in serum lipoproteins has not been clarified. The changes in the plasma concentration of these lipids could be a reflection of variations in the mass of serum lipoprotein secondary to changes in triglyceride transport. Ethanol also increases cholesterogenesis in the liver (122) and in the small intestine (123). Furthermore, ethanol feeding decreases bile acid excretion (122) though after cessation of ethanol administration, the opposite was observed (124).

After the initial development of fatty liver associated with hyperlipemia, the blood lipids return towards normal (3). Progressive deterioration of liver function, including lipoprotein production and secretion, could be responsible, and may secondarily aggravate fat accumulation in the liver. In any event, whenever there is lipid deposition in the liver, removal is obviously inadequate to offset the increased availability of lipids in the liver.

Microsomal ethanol oxidizing system (MEOS) and adaptive increase in ethanol metabolism: In addition to its main oxidation pathway via ADH, ethanol also can be metabolized by a MEOS (125). MEOS, which is localized in the endoplasmic reticulum, utilizes NADPH and oxygen (Fig. 5) and increases in activity after chronic ethanol consumption. This is associated with a proliferation of the smooth endoplasmic reticulum (44) which now has been substantiated by microsomal subfractionation (126). The increase in MEOS activity may explain the moderate acceleration of ethanol metabolism (127) which, in addition to central nervous system tolerance, contributes to the adaptation of alcoholics to ethanol. Since this MEOS pathway utilizes reduced cofactors, it theoretically could be considered as another way for the liver to dispose of the excess hydrogen generated by the oxidation of ethanol via its main ADH pathway (Fig. 5). It also may help us to understand the changes in lipoprotein metabolism, discussed before, as well as the interaction of ethanol with drug metabolism.

Interaction of ethanol with drug metabolism and other microsomal functions, including lipid peroxidation: Ethanol shares many characteristics with other microsomal substrates, especially a relative lack of specificity as a microsomal inducer: prolonged ethanol intake increases the activities of a variety of microsomal enzymes other than MEOS, including those involved in the detoxification of barbiturates and tranquilizers (126,128,129) with a corresponding acceleration of drug metabolism (127). This, in addition to a decreased responsiveness of the central nervous system, contributes to the known tolerance of alcoholics to various drugs, including sedatives, (130) and the accelerated drug metabolism in alcoholics (131), at least as long as the liver functions remain relatively intact. Severe liver damage

however, which may decrease drug detoxification (132) can offset any induction. Moreover, the adaptive response is observed only when the alcoholic is sober. In inebriated individuals, ethanol potentiates the effects of other drugs, including tranquilizers, both by an additive action on the central nervous system and by inhibition of drug metabolism (133). The latter probably is accomplished through competition for an at least partially common detoxifying pathway in the liver. Enhanced microsomal activity also may favor biotransformation of CCl_4, thereby promoting its hepatotoxicity (134).

A similar microsomal pathway requiring O_2 and NADPH is also capable of generating lipid peroxides. Enhanced lipid peroxidation has been proposed as a mechanism for ethanol induced fatty liver (135), but its role is still controversial (136-140). The accumulation of lipid peroxide may be secondary to the lipid accumulation (141), rather than represent its cause. However, theoretically, increased activity of microsomal NADPH oxidase following ethanol consumption (142) could result in enhanced H_2O_2 production, thereby also favoring lipid peroxidation.

FA Mobilization from Adipose Tissue: Effect of Ethanol and Acetate on FFA Metabolism

In rats given one large, sublethal dose of ethanol, it was observed that FA resembling those of adipose tissue accumulate in the liver (51,143). Experimental procedures or agents which reduce the normal rate of peripheral fat mobilization, i.e. adrenalectomy, spinal cord transsection, or ganglioplegic drugs, prevent or decrease this type of hepatic fat accumulation (143-145). More direct approaches, however, such as studies in rats with prelabeled epididymal fat pads yielded conflicting information, with evidence for increased (146) or unchanged (147) FA mobilization. Similarly, in rats one large dose of ethanol has been reported to result in an increased (143,148) or unchanged (149) circulating levels of FFA. In man, even with amounts of ethanol as large as 300 g/day, the concentration of circulating FFA did not increase; it rose only after ingestion of large doses of ethanol (400 g/day) (3). In short term studies, ethanol administration produced a fall in the level of circulating FFA in man (90,150) with reduced peripheral venous-arterial differences in FFA (150), decreased FFA turnover (151) and concomitant reduction in circulating glycerol (152). This effect of ethanol upon FFA mobilization from adipose tissue was found to be mediated by acetate (153). Acetate is the end product of ethanol metabolism in the liver (Fig. 5) and is released into the bloodstream. Since stressful doses of ethanol probably both stimulate FA mobilization (via catecholamine release) and depress it (via the acetate produced), the net effect may depend upon the particular experimental conditions. This may account for some of the apparent contradictions of the literature.

Actually, whether enhanced peripheral fat mobilization is responsible for hepatic fat accumulation after one large sublethal dose of ethanol in the rat is of little clinical relevance after chronic ethanol consumption. Under the latter conditions, the FA deposited in the liver do not derive primarily from adipose tissue (36,51).

Prevention and Treatment of the Alcoholic Fatty Liver

Various chemicals and procedures to reduce or prevent the alcoholic fatty liver have been described before (154). As already mentioned here, decreasing dietary fat (34,36,51) or replacing it with MCT (37) reduces the capacity of ethanol to produce a fatty liver. Differences in dietary fat may explain some of the discrepancies in reports concerning the effect of antioxidants which reduced or prevented hepatic steatosis in some studies (155) but not in others (37). The negative results were obtained with diets containing 43% total calories as fat (an amount comparable to that of the average U.S. diet), whereas partial protection was observed with a relatively low fat diet. Since dietary fat potentiated the steatogenic effect of ethanol, it is quite conceivable that antioxidants may be moderately active with low fat diets but incapable of counteracting the much stronger effects of ethanol combined with dietary fat. Chlorophenoxyisobutyrate, a drug used to reduce hyperlipemia, partially protected against the alcoholic fatty liver (156,157), possibly through a reduction in glycerolipid formation (158). The protective action also could be related to the abolition of the redox change (159).

Among the drugs capable of decreasing the capacity of ethanol to produce a fatty liver, one must list the barbiturates (160), an effect recently confirmed (161), and antihistamine derivatives (162). Asparagine, previously reported as protective (163), now has been found ineffective after acute (164) and prolonged (37) ethanol intake. To the list of measures previously reported to prevent the fatty liver produced by one large dose of ethanol (154), one can add the β-sympathicolytic agents (165), pyridyncarbinol (166), and cold exposure (167). In rats, hyperbaric oxygen was found to

be protective (168).

ATP previously has been reported to protect against acute ethanol induced fatty liver (169). However, when given in moderate amounts, it restored liver ATP levels to normal without preventing the ethanol induced fatty liver (170); the partial protection afforded by much larger doses (169) possibly can be attributed to nonspecific effects. Chlorpromazine, which inhibits ADH activity, failed to prevent the fatty liver produced by an acute large ethanol dose (171). There is a controversy over whether or not pyrazole, another ADH inhibitor, prevents the fatty liver produced by a single large dose of ethanol: some found no reduction (172), whereas others found prevention (173,174); the difference is perhaps due to the dose of the drug (175) or the sex of the animal (176) used. Though some acute effects of ethanol on lipid metabolism were prevented by pyrazole (177), the effects of pyrazole on the consequences of chronic ethanol ingestion were inconclusive (178), a not unexpected result in view of the hepatotoxicity of pyrazole (179). A derivative of pyrazole, 3,5-dimethyl pyrazole, was shown to reduce the fatty liver resulting from a single large dose of ethanol by blocking FFA mobilization from adipose tissue (180).

Anabolic steriods were reported to be ineffective by some (181) but not by others (182,183) in accelerating the disappearance of fat from the alcoholic fatty liver.

To characterize the complex sequence of events which leads from ethanol ingestion to the development of fatty liver, it appears that ethanol replaces FA as a fuel for the hepatic mitochondria. This results in FA accumulation, directly because of decreased lipid oxidation and indirectly because one way for the liver to dispose of excess hydrogen generated by ethanol oxidation is to synthesize more lipids. Lipids accumulate in the liver despite the fact that the release of lipoproteins from the liver into the bloodstream is stimulated by ethanol, at least during the initial stage of the intoxication.

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Effects of Anabolic and Progestational Agents upon Triglycerides and Triglyceride Kinetics in Normals and Hyperlipemic Patients[1]

RONALD FALLAT, Lipoprotein Research Laboratory, and CHARLES J. GLUECK,[2]
General Clinical Research Center, Cincinnati General Hospital, Cincinnati, Ohio 45229

ABSTRACT

Progestational and anabolic-androgenic compounds may have extensive effects upon plasma triglycerides, triglyceride clearing enzymes, and upon mechanisms of triglyceride clearing from the plasma. This article centers upon recent advances in the understanding of the mode of action of these compounds, both in normals and in patients with hypertriglyceridemia.

ORAL CONTRACEPTIVES AND PLASMA LIPIDS IN NORMALS

Much of the interest in purely progestational oral contraceptives (or low estrogen-progestin compounds) was stimulated by reports of increased plasma triglycerides in normal women of virtually every ethnic and national origin taking a variety of estrogen-progestin oral contraceptives (1-11). The triglyceride levels on these varied oral contraceptives were primarily within broad normal limits (usually less than 170 mg%) and generally were accompanied by increased prebeta-lipoproteins (3,4). The estrogen component alone of the mixed oral contraceptive appeared to cause the elevation of plasma triglyceride (1,2,8,10-12). Stokes, et al., (10) reported that the "most estrogenic" mixed oral contraceptives gave the highest triglyceride values and the "most progestational," the highest cholesterol values. In most studies (1-9, 11) there were inconstant changes in plasma cholesterol, which, for the most part, was relatively unchanged by oral contraceptives. Plasma triglyceride levels did not always return to normal immediately after cessation of the oral contraceptive or estrogen (4,8). Qualitatively, the changes in plasma triglyceride on oral contraceptives resembled some changes observed in pregnancy, probably because the estrogen-progestin dose in most oral contraceptives is pharmacologic, not physiologic (11).

The significance of moderate increases in plasma triglycerides in women taking oral contraceptives remains unclear. Where moderate increases in triglycerides are present (4,8,10), the duration of their elevation over long-term therapy periods remains to be determined. Although no definite causal relationship has been shown between elevated triglycerides on oral contraceptives and vascular disease, the incidence of myocardial infarctions (13) and cerebrovascular accidents (14,15) in women on oral contraceptives may be increased as compared to premenopausal women on no oral contraceptives. There is also concern that long-term moderate triglyceride elevations in otherwise normal women might augment one of the multifactorial risks associated with premature morbidity and mortality from heart disease (16).

MECHANISMS OF ACTION OF ORAL CONTRACEPTIVES ON LIPID METABOLISM

The triglyceride elevating effects of estrogen-progestin oral contraceptives have fueled interest in evaluation of possible mechanisms of action. Interaction with postheparin lipolytic activity (PHLA) and triglyceride clearing, on the one hand, and with glucose, insulin, and growth hormone, on the other, are currently major areas of concentration. Interactions with carbohydrate and insulin metabolism have been reviewed extensively by many groups (4, 17-22) who implicated an estrogen mediated increase in hepatic triglyceride synthesis, fueled by increased glucose, insulin, and free fatty acids (FFA).

Several groups (4,8, 23-25) have shown that estrogen-progestin oral contraceptives depress plasma PHLA rather uniformly. The estrogenic component of mixed oral contraceptives has been identified as the culprit in depression of PHLA (4,8). Despite estrogen induced depression of PHLA to levels comparable to those seen in familial type I hyperlipoproteinemia (4,26), fasting chylomicronemia has not been observed in normal women with depressed PHLA (4,8,24,25). Intravenous fat tolerance (an indirect method of measuring exogenous triglyceride clearance) also appears to be normal in women on oral contraceptives, even in the face of severely depressed PHLA (8,24,25).

[1]One of six papers presented in the symposium "Effect of Drugs on Lipid Metabolism," AOCS Spring Meeting, New Orleans, April 1973.

[2]Author to whom reprint requests should be addressed.

The dichotomy of reduced PHLA and normal exogenous fat clearance is difficult to resolve within the limits of the current methodology. Better understanding of effects of oral contraceptives upon triglyceride clearance and upon lipoprotein lipase will require a more specific lipase assay than is currently available. Demonstration of reduced clearance of prebeta-lipoprotein triglyceride in normals on oral contraceptives would add further wt to speculations that depressed triglyceride clearing efficiency is a primary mechanism of estrogen action.

PROGESTATIONAL ORAL CONTRACEPTIVES AND PLASMA LIPIDS IN NORMALS

The effects of mixed estrogen-progestin oral contraceptives upon triglycerides and triglyceride clearing have prompted study of several purely progestational oral contraceptives. Beck reported that the purely progestational oral contraceptive, chlormadinone, had insubstantial effects upon triglyceride and cholesterol levels (27,28) in normal women. The synthetic progestin, norethindrone, (0.4 mg/day), given as an oral contraceptive, also did not appear to elevate plasma cholesterol or triglyceride levels in normal women (25).

We have evaluated a newly available purely progestational oral contraceptive, quingestanol acetate (29), (300 micrograms/day given continuously). We prospectively studied the effects of this compound upon plasma cholesterol, triglycerides, and PHLA. This synthetic steroid is quite similar to norethindrone in structure and progestational activity. In 10 normal women on quingestanol acetate for 1 year, serial determinations of fasting plasma triglyceride were either unchanged or slightly decreased; and plasma cholesterol was unchanged (29). There were no serial changes in PHLA, triglyceride hydrolase, or monoglyceride hydrolase. In a separate set of 10 women followed for 6 months, cholesterol was unchanged, plasma triglycerides fell ca. 20%, and PHLA did not change. This compound and the structurally related progestational oral contraceptive, norethindrone acetate, offer promise as effective oral contraceptives that have insubstantial effects upon plasma lipids and post-heparin lipases. The absence of triglyceride elevation on purely progestational oral contraceptives (27-29) further implicates the estrogenic component in the triglyceride elevations seen on mixed oral contraceptives (1-11).

The potentially opposite effects of progestational and estrogenic compounds upon plasma lipids and lipases have stimulated evaluation of the effects of these compounds upon triglyceride clearance.

TRIGLYCERIDE CLEARANCE AND KINETICS: EFFECTS OF ORAL CONTRACEPTIVES AND ANABOLIC-ANDROGENIC COMPOUNDS

The development of elevated plasma triglyceride levels in patients with hypertriglyceridemia or in normals on oral contraceptives probably relates to a complex interplay of the rate of synthesis, maximal removal velocity, and the Km of the removal enzymes (30). The Vmax of both synthesis and removal may be under genetic control but can be modified by diet and drugs (6, 30). Frank, et al., (31) reported that the fractional rate of loss of very low density lipoprotein triglyceride from the plasma compartment appeared to be lower in patients with type IV hyperlipoproteinemia than in normals. Frank, et al., did not find evidence for abnormally increased synthesis of very low density lipoprotein triglyceride.

Drugs which lower triglyceride levels also have varying effects upon triglyceride kinetics. Sodhi (32) reported that reduction in plasma triglycerides in some patients with endogenous hypertriglyceridemia (on clofibrate) was associated with an increase in the fractional turnover rate for labeled triglycerides in the plasma. Reaven, et al., (33) observed diminished very low density lipoprotein triglyceride turnover in three hypertriglyceridemic subjects given chlorpropamide with attendant lowering of their triglyceride levels. Reaven (33) proposed that increased triglyceride synthesis, rather than impaired triglyceride removal, was responsible for elevated plasma triglyceride levels. In contrast, Havel, et al., (34) reported that impaired triglyceride removal (in extrahepatic tissues) was a prominent abnormality in fasting hypertriglyceridemic subjects.

The diversity of opinion regarding the balance between triglyceride synthesis and removal rates in normals and hypertriglyceridemic patients has been paralleled in studies of the effects of oral contraceptives upon triglyceride kinetics in normals (6). Kekki, et al., (6) reported increased triglyceride clearing efficiency in normal women taking mixed estrogen-progestin oral contraceptives. Kekki (6) proposed that the progestational component of the oral contraceptive was responsible for increasing triglyceride clearance but that this was over-balanced by an estrogen induced increment in synthesis of very low density lipoprotein triglyceride.

Stimulated by the work of Kekki, et al., (6) we evaluated the effects of the anabolic-androgenic synthetic steroid, oxandrolone, upon triglyceride kinetics and triglyceride clearing (35). Very low density lipoprotein triglyceride turnover rate, fractional turnover rate, and half-life

were studied using Glycerol-^{3}H to label very low density lipoproteins in 16 patients with familial type IV hyperlipoproteinemia. Mean half-life of labeled very low density lipoprotein triglyceride was 10.3 hr on placebo and was shortened to 5.2 hr on drug. The fractional turnover rate on placebo was .104 hr^{-1} and rose to .166 on drug. Mean turnover rate did not change appreciably. As we previously reported (36), both PHLA and postheparin triglyceride lipase were increased appreciably on drug, rising from .297 μEq FFA/ml/min and 11.7 mμEq FFA/ml/hr on placebo to .396 and 23.7 on drug. The changes in very low density lipoprotein triglyceride correlated with changes in half-life ($r = .76$) and with fractional turnover rate ($r = -.53$). It was postulated that oxandrolone might lower very low density lipoprotein triglyceride by increasing fractional turnover rate, shortening half-life, and improving efficiency of very low density lipoprotein triglyceride removal. This apparent effect upon increasing triglyceride clearance agrees with the speculations of Kekki (6) on effects of progestational compounds upon triglyceride clearance. Although the kinetic changes on oxandrolone (36) were concurrent with substantial increases in plasma postheparin triglyceride lipases, no correlations between triglyceride clearing rate and triglyceride clearing activities could be shown. Since triglyceride lipase levels in postheparin plasma may not accurately reflect the in vivo tissue capacity for triglyceride clearance (37), more concrete conclusions as to the relationship (or lack of relationship) of lipases and very low density lipoprotein removal efficiency probably await more sensitive and specific lipase assays (37).

Both estrogens, which may elevate plasma triglycerides, and progestins or anabolic-androgenic agents, which may lower plasma triglycerides, offer experimental perturbations which may help in evaluation of mechanisms of hypertriglyceridemia. Progestational or anabolic-androgenic compounds appear to increase the efficiency of triglyceride clearance, while effects of estrogen on triglyceride clearing mechanisms remain in question. Progestational compounds have little effect upon triglyceride synthesis rate, while estrogens may increase hepatic triglyceride synthesis. Application of information on metabolic effects of estrogens and progestins may allow for development of metabolically safe oral contraceptives and compounds useful in the treatment of hyperlipidemia.

ACKNOWLEDGMENTS

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Effect of (-)-Hydroxycitrate upon the Accumulation of Lipid in the Rat: I. Lipogenesis[1]

ANN C. SULLIVAN, JOSEPH TRISCARI, JAMES G. HAMILTON, O. NEAL MILLER, and **VICTOR R. WHEATLEY,**[2] Department of Biochemical Nutrition, Hoffmann-LaRoche Inc., Nutley, New Jersey 07110

ABSTRACT

The purpose of these investigations was to ascertain the effect of (−)−hydroxycitrate on the accumulation of lipid in the meal fed rat by examining the rates of lipogenesis after acute and chronic treatment. Oral administration of (−)−hydroxycitrate depressed significantly the in vivo lipogenic rates in a dose-dependent manner in the liver, adipose tissue, and small intestine. The hepatic inhibition was significant for the 8 hr period, when control animals demonstrated elevated rates of lipid synthesis. The kinetics of this reduction of in vivo hepatic lipogenesis were identical after acute or chronic administration of (−)−hydroxycitrate. However, in vitro rates of lipogenesis were elevated after chronic administration of (−)−hydroxycitrate for 30 days. Rats receiving (−)−hydroxycitrate consumed less food than the untreated controls; however, this decreased caloric intake was not responsible for the drug induced depression of hepatic lipogenesis, as shown by studies using pair fed rats.

INTRODUCTION

(−)−Hydroxycitrate, the principal acid of the fruit rinds of *Garcinia cambogia*, (1-3) was shown to be a competitive inhibitor of adenosine 5′-triphosphate (ATP) citrate lyase (EC 4.1.3.8) (4,5), the enzyme catalyzing the extramitochondrial cleavage of citrate to oxaloacetate and acetyl CoA. This action of (−)−hydroxycitrate should reduce the acetyl CoA pool, thus limiting the availability of 2 carbon units required for fatty acid (FA) and cholesterol biosynthesis. Our previous investigations substantiated this hypothesis, since the acute admnistration of (−)−hydroxycitrate inhibited in a dose-dependent manner the in vitro rates of lipogenesis in hepatic cell-free and slice systems and the in vivo rates of hepatic FA and cholesterol synthesis (6). Of the four stereoisomers of hydroxycitrate, only (−)−hydroxycitrate reduced significantly the in vitro and in vivo rates of lipid synthesis (6). The depression of 3-β-hydroxysterol biosynthesis by (−)−hydroxycitrate was confirmed in a perfused rat liver system (7,8). The inhibition of FA synthesis was demonstrated after intraperitoneal administration of (−)−hydroxycitrate (9).

It seemed important to ascertain how the chronic administration of (−)−hydroxycitrate would affect the rat's metabolic pattern of lipid biosynthesis and storage. This investigation was designed to examine the effect of the chronic oral administration of (−)−hydroxycitrate upon in vivo rates of lipogenesis and to analyze in greater detail the characteristics of the inhibition of lipogenesis after acute administration. These studies were performed under the conditions of induced rates of lipid synthesis, which have been described previously (10). In vivo rates of lipogenesis were determined using [^{14}C] alanine and [^{3}H] water; the latter being employed to determine the total rate of FA (9, 11-16) and cholesterol synthesis (7) independent of the source of carbon precursors of the acetyl groups.

EXPERIMENTAL PROCEDURES

Female rats of the Charles River CD strain (Charles River Breeding Laboratories, Wilmington, Mass.) weighing 120-160 g (ca. 16 wk old) were housed individually in wire-bottomed cages in a temperature-regulated (22 C), light-controlled room (12 hr light, 6 A.M.-6 P.M., and dark 6 P.M.-6 A.M.). They had free access to water and were fed a commercial diet (Purine Rodent Chow, Ralston Purina Co., St. Louis, Mo.) ad libitum for at least 1 week prior to the experiment. Animals were fasted 48 hr, then meal fed a synthetic diet (G-70) daily from 8-11 A.M. for the remainder of the experiment. Food consumption and body wt were measured during the meal feeding period. Body wts were randomized, so that each experimental group had an identical wt spread. Food spillage was measured daily.

The G-70 diet consisted of 70% glucose, 23% vitamin-free casein, 5% Phillips and Hart salt mixture IV (17), 1% corn oil, 1% complete

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[2]Present address: Department of Dermatology, New York University School of Medicine, New York, New York.

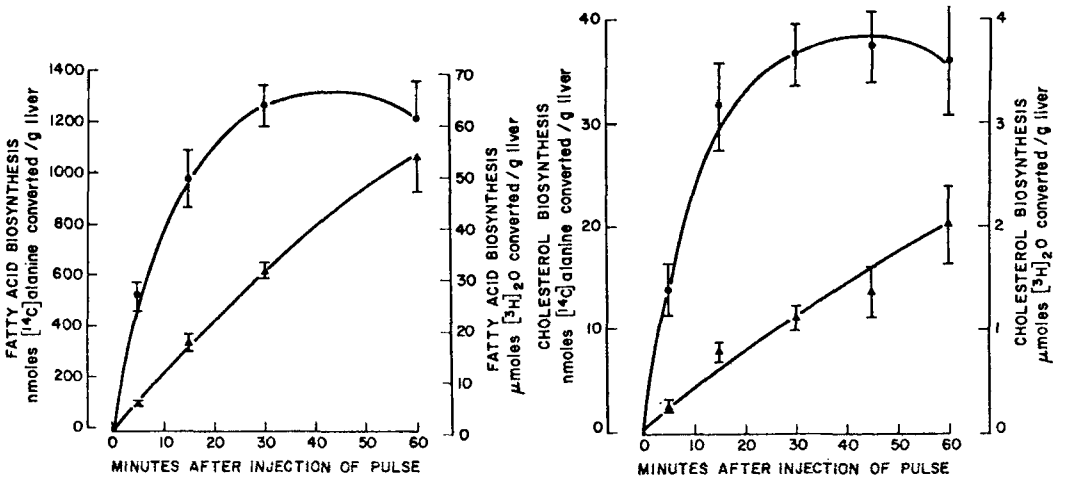

FIG. 1. Relation between the in vivo rates of hepatic fatty acid (FA) and cholesterol synthesis and the time of administration of [^{14}C]alanine and [^{3}H] water. Rats were prefasted 48 hr, then meal fed the G-70 diet for 9 days. The rates of FA and cholesterol synthesis were determined using a pulse of [^{14}C]alanine and [^{3}H]water injected immediately after the 3 hr meal. FA were separated from cholesterol by anion exchange chromatography as described in the text. Each group consisted of 9-10 rats. The vertical bar gives the standard error of the mean for FA synthesis (above) and cholesterol synthesis (below). • [^{14}C] alanine ▲ [^{3}H] $_2$O.

vitamin mixture, and 40 g/kg cellulose. When equimolar amounts of (−)−hydroxycitrate $(Na)_3$ and citrate $(Na)_3$ were administered as dietary admixtures, an equivalent wt of glucose was deleted from the diet. To ensure complete uniformity, all diets were mixed with a twin-shell dry blender equipped with an intensifier bar (Patterson-Kelley Co., East Stroudsburg, Pa.).

Measurement of In Vivo Rate of Lipid Synthesis

Immediately after the 3 hr feeding period, rats were anaesthetized lightly with Penthrane (methoxyflurane, Abbott Laboratories, North Chicago, Ill.) administered intravenously a 0.25 ml saline (pH 7.4 to 7.6) solution with the following composition: 12.3 mg alanine, 5 μCi [^{14}C]alanine (specific activity = 156 mCi/mmole), 30.6 mg α-ketoglutarate (as an amine acceptor for transaminase) and 1 mCi [^{3}H] water (specific activity = 100 mCi/g). Experiments indicated that [^{14}C]alanine was equivalent to either [^{14}C]pyruvate or [^{14}C]lactate as a carbon precursor for lipogenesis. [^{3}H]Water was employed to determine the total rate of lipogenesis, since tritium is incorporated into FA independent of the source of carbon precursors (11,12). Animals were killed by decapitation and blood collected in centrifuge tubes 30 min after the radioactive pulse, unless otherwise indicated. The specific radioactivity of the body water of each rat was determined by counting a diluted serum aliquot in 10 ml following cocktail: toluene (2.4 l), 2-methoxyethanol (1.6 l), naphthalene (320 g), and BBOT (2,5-bis-2-(5-tert-butylbenzoxazolyl)-thiophene, 16 g). Liver, perirenal adipose tissue, and small intestine were excised rapidly, weighed, and homogenized in 15 ml H_2O in a Virtis 45 Macro Homogenizer for 15 sec at ca. 30,000 rpm. The contents of the small intestine were removed by repeated washing before homogenization. Duplicate 3 ml aliquots whole homogenates were saponified, extracted, and the absolute radioactivity (dpm) determined, as described previously (6,10). Liver lipids were extracted totally or separated into FA and cholesterol by anion exchange chromatography, as described previously (6). It was determined by anion exchange chromatography that the total lipid extract of liver contained FA (96-97%) and cholesterol (3-4%). Data are expressed as nmoles [^{14}C]alanine or μmoles [^{3}H] water converted into lipid/g tissue/30 min. The nmoles [^{14}C]alanine were calculated according to the injected load of alanine, as reported previously (6,10). The μmoles [^{3}H] water were determined as described previously (9,11).

Determination of Radioactive Neutral Lipids and FA in Serum

Serum lipids were extracted by the method of Bligh and Dyer (18). Sera from three rats were pooled (5 ml) and the following reagents added in order after adequate mixing: 5 ml chloroform, 10 ml methanol, 5 ml chloroform, then 5 ml H_2O. The extract was filtered over Whatman 1 paper, the upper phase discarded, the lower phase evaporated to dryness under

N_2, and 5 ml methanol added. The extract was added to a 1 x 10 cm column containing Dowex 1-X2. Neutral lipids were eluted with 45 ml methanol:ethyl ether (1:1), and FA were eluted with 80 ml ether:80% methanol: acetic acid (10:8:2). Both fractions were evaporated under N_2 to dryness and analyzed for absolute readioactivity, as described previously (6).

Measurement of In Vitro Rate of Lipogenesis

The details for determining the incorporation of [1,5-^{14}C] citrate (specific acitivity = 6.4 mCi/mmole) into saponifiable lipid by a hepatic cell-free (100,000 x g for 30 min) system have been reported previously (19). The procedures used for the saponification and extraction of lipid were the same as those described previously (6). Protein was determined by a modification of the method of Lowry (20). The amount of radioactivity incorporated from [1,5-^{14}C]-citrate into saponifiable lipid was a measure of the in vitro rate of lipogenesis dependent upon the activities of ATP citrate lyase, acetyl CoA carboxylase, and FA synthetase. Data are expressed as nmoles [^{14}C] citrate converted into lipid/g liver/30 min. Data may be converted to [^{14}C] citrate incorporated into lipid/mg protein by dividing by 20.

Sources of Chemicals

(−)−Hydroxycitric acid lactone was isolated from the dried fruit rinds of the Indian plant *Garcinia cambogia*. Trisodium salt was used; solutions of this salt were prepared from the crystalline form or by hydrolysis of the lactone (30 min heating at 90 C with three equivalents of NaOH).

Constituents for the synthetic diets were obtained from Nutritional Biochemicals, Cleveland, Ohio. Other chemicals were purchased from Sigma Chemical, Milwaukee, Wis.

Statistical Analysis

The *t* test was used to analyze all experimental results (21). Data were processed statistically for outliers (22).

RESULTS

Measurement of In Vivo Rate of Lipid Synthesis

Figure 1 demonstrates the in vivo rate of hepatic FA and cholesterol biosynthesis as a function of min after the injection of the [^{14}C] alanine and [^{3}H] water pulse. The conversion of [^{3}H] water into FA and cholesterol was linear for 60 min after pulse administration. During this 60 min period, the specific activity of the body water remained constant.

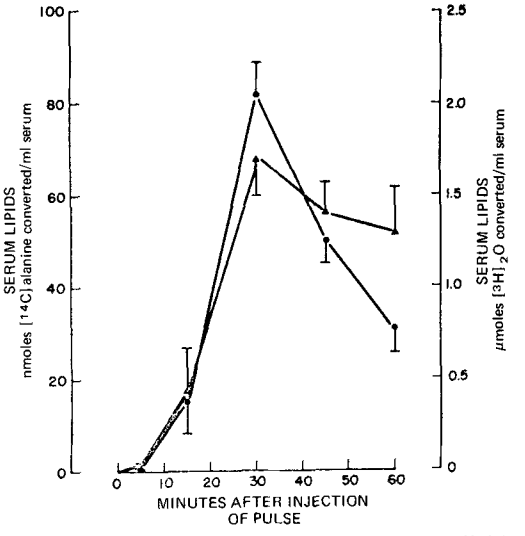

FIG. 2. Relation between radioactive serum lipids and the time of administration of [^{14}C] alanine and [^{3}H] water. Rats were prefasted 48 hr then meal fed the G-70 diet for 9 days. A pulse of [^{14}C] alanine and [^{3}H] water was injected immediately after the 3 hr meal. Serum lipids were extracted as described in the text. Each group consisted of 9-10 rats, and the vertical bar gives the standard error of the mean. • [^{14}C] alanine ▲ [^{3}H] $_2$O.

However, [^{14}C] alanine conversion was maximal at 30 min and ca. linear for only 15 min.

When the sera of the rats receiving [^{14}C] alanine and [^{3}H] water were examined for the amount of radioactive lipid present, both [^{14}C] alanine and [^{3}H] water conversion were linear for 30 min (Fig. 2). Since the standard assay time employed was 30 min, it was important to determine whether the radioactive serum lipids present at 30 min were neutral lipids (indicating liver biosynthesis) or FA (indicating adipose tissue biosynthesis). Sera FA and neutral lipids were separated, and all the radioactivity was recovered in the neutral lipid fraction, indicating that the [^{14}C], [^{3}H] lipids present in the serum at 30 min were of liver origin.

Characteristics of the Inhibition of In Vivo Rates of Lipid Synthesis by (−)−Hydroxycitrate

The effect of the oral administration of (−)−hydroxycitrate (2.63 mmoles/kg) upon the in vivo rate of hepatic lipogenesis was examined over a 24 hr period to determine the extent and duration of the inhibition and to establish whether the lipogenic rates in the (−)−hydroxycitrate-treated animals increased during the time when control rates decreased. Figure 3 illustrates the in vivo rates of lipogenesis determined by the conversion of [^{14}C] alanine and [^{3}H] water into lipid. To

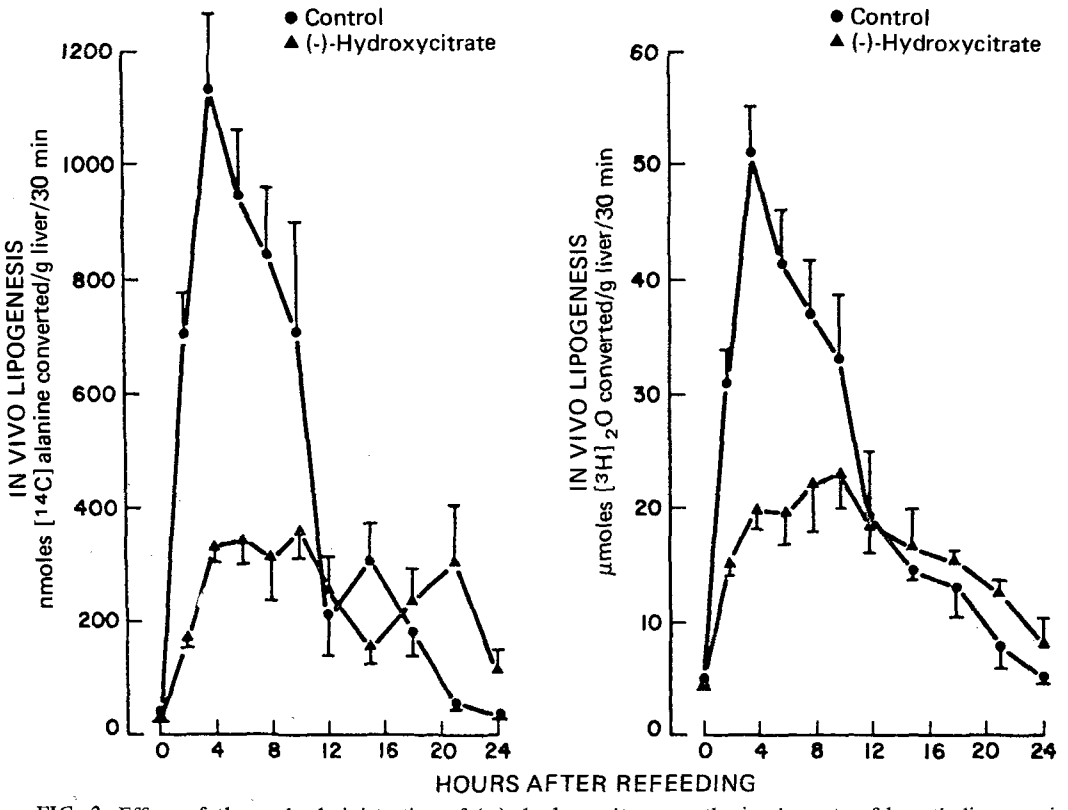

FIG. 3. Effect of the oral administration of (−)−hydroxycitrate on the in vivo rate of hepatic lipogenesis determined over a 24 hr period. Rats were prefasted 48 hr, then meal fed the G-70 diet for 6 days. On day 7, 50 rats each were given orally either saline or (−)−hydroxycitrate (2.63 mmoles/kg) directly before receiving 8.7 g food. The in vivo rate of lipogenesis was determined using the [^{14}C]alanine and [^{3}H]water pulse at the indicated times. The animals were killed 30 min after pulse administration. The vertical bar gives the standard error of the mean (five rats/point). Nmoles [^{14}C]alanine and μmoles [^{3}H]water converted into lipid in the (−)−hydroxycitrate treated animals were significantly different from controls at hr 2, 4, 6, and 8 ($p < 0.05$). ● Control ▲ (−)−Hydroxycitrate.

standardize the amount of food consumed on the experimental day, each rat was given 8.7 g G-70 diet, which equals the food intake of rats receiving 2.63 mmoles/kg of (−)−hydroxycitrate. Lipogenic rates increased to a maximum at 3-5 hr after feeding and declined subsequently to a minimum at 24 hr. (−)−Hydroxycitrate caused a significant inhibition of the lipogenic rate for 8 hr after refeeding. During this period, lipid synthesis from [^{14}C]alanine and [^{3}H]water was decreased by 68% and 72%, respectively. By 12 hr, the rates of lipogenesis were indistinguishable in controls and treated rats. Although there was a tendency towards elevated lipogenic rates 14-24 hr in the (−)−hydroxycitrate treated rats compared to controls, these results were not significant.

The oral administration of (−)−hydroxycitrate inhibited significantly the in vivo rates of lipogenesis in several tissues known to convert carbohydrate into FA, namely liver, adipose tissue, and small intestine (Table I). Although the rates at which [^{14}C]alanine was converted into lipid by the three tissues differed markedly (adipose tissue > liver > small intestine), all were depressed significantly by 10.52 and 5.26 mmoles/kg of (−)−hydroxycitrate. After oral administration of 2.63 mmoles/kg of (−)−hydroxycitrate, the lipogenic rates in liver and adipose tissue were significantly inhibited.

Effect of the Chronic Oral Administration of (−)−Hydroxycitrate on Hepatic Lipogenesis

Figure 4 demonstrates the in vitro rate of lipogenesis in rats administered varying concentrations of (−)−hydroxycitrate orally for 30 days. The upper curve illustrates the observed in vitro rate, and the lower curve gives the rate of lipogenesis when 1 mM (−)−hydroxycitrate was added to each assay. Animals receiving (−)−hydroxycitrate at daily concentrations of 2.63, 1.32, and 0.66 mmoles/kg for 30 days demonstrated a significantly higher rate of lipogenesis compared to controls (1.5-2.1-fold).

TABLE I

Inhibition of In Vivo Lipogenesis in Liver, Adipose, and Small Intestine by Oral Administration of (−)–Hydroxycitrate

Treatment[a]	Dose mmoles/kg	Rate of lipogenesis					
		Liver		Adipose		Small intestine	
		nmoles [^{14}C]alanine converted[b]	Inhibition percent	nmoles [^{14}C]alanine converted[b]	Inhibition percent	nmoles [^{14}C]alanine converted[b]	Inhibition percent
Saline		855 ± 99		2473 ± 656		84 ± 9	
(−)–Hydroxycitrate	10.52	93 ± 33[c]	89	393 ± 136[d]	84	37 ± 13[c]	56
(−)–Hydroxycitrate	5.26	188 ± 28[c]	78			28 ± 4[c]	67
(−)–Hydroxycitrate	2.63	498 ± 86[d]	42	754 ± 59[d]	69	72 ± 9	14

[a]Four groups of five rats each were prefasted, then meal fed the G-70 diet for 9 days. On day 9 they were given (−)–hydroxycitrate dissolved in saline at the indicated doses 60 min before feeding. Immediately after the 3 hr feeding period, the in vivo rates of lipogenesis were determined in rat liver, perirenal adipose tissue, and washed small intestine, as described in the text.

[b]Data are expressed as nmoles [^{14}C]alanine converted into lipid/g tissue/30 min. Each value is the group mean ± standard error.

[c]$p < 0.01$.

[d]$p < 0.05$.

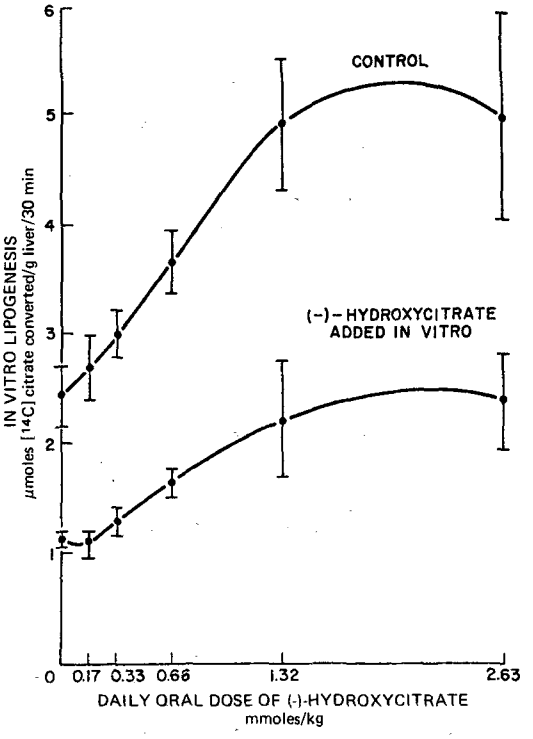

FIG. 4. In vitro rate of hepatic lipogenesis in rats administered (−)–hydroxycitrate orally for 30 days. Rats were prefasted 48 hr, then meal fed the G-70 diet for 5 days. From day 6-36, they received saline or varying concentrations of (−)–hydroxycitrate 1 hr before feeding the G-70 diet. Immediately after the 3 hr feeding period on day 36, livers from four rats in each group were assayed for in vitro rates of lipogenesis in 10 mM citrate as described in the text. The upper curve demonstrates the observed rate of lipogenesis; the lower curve gives the rate of lipogenesis when 1 mM (−)–hydroxycitrate was added to each assay. The vertical bar gives the standard error of the mean.

Lower concentrations of (−)–hydroxycitrate (0.17 and 0.33 mmoles/kg) produced small, but insignificant, increases in rates of lipogenesis. When (−)–hydroxycitrate was added, a similar level of significant inhibition was observed regardless of the control in vitro lipogenic rate.

Figure 5 illustrates the reduction in the in vivo rate of hepatic lipogenesis determined by the conversion of [^{14}C]alanine and [^{3}H]water into lipid in rats given varying concentrations of (−)–hydroxycitrate orally for 30 days. Animals receiving (−)–hydroxycitrate at daily doses of 2.63 and 1.32 mmoles/kg demonstrated a significantly depressed in vivo rate of lipogenesis.

The effect of chronic oral administration of (−)–hydroxycitrate for 11 days upon in vivo hepatic lipogenesis is shown in Table II. Here, as in Figure 5, significant inhibition was observed at concentrations of 2.63 and 1.32

TABLE II

Reduction in the In Vivo Rate of Lipogenesis in Rats Administered (−)−Hydroxycitrate Orally for 11 Days[a]

Daily oral dose of (−)−hydroxycitrate mmoles/kg	Rate of lipogenesis			
	nmoles [^{14}C]alanine converted[b]	Inhibition percent	μmoles [^{3}H]$_2$O converted[b]	Inhibition percent
0	1043 ± 131	0	64.3 ± 5.3	0
0.66	760 ± 107	27	50.4 ± 5.1	22
0.33 bid	824 ± 84	21	56.2 ± 5.8	13
2.63	248 ± 27[c]	76	32.5 ± 2.0[c]	49
1.32 bid	592 ± 151[c]	43	40.7 ± 6.2[c]	37

[a]Five groups of 10 rats each were prefasted 48 hr, then meal fed the G-70 diet for 5 days. They then were given saline or (−)−hydroxycitrate by stomach tube 1 hr before feeding the G-70 diet and 4 hr after the completion of the meal (where bid [twice a day] administration is indicated) for 11 days. Immediately after the 3 hr feeding period on day 11, the in vivo rate of lipogenesis was determined in rat liver, as described in the text.

[b]Data are expressed as nmoles [^{14}C]alanine and μmoles [^{3}H]$_2$O converted into lipid/g liver/30 min. Each value is the group mean ± standard error.

[c]$p < 0.01$.

mmoles/kg, whereas 0.66 and 0.33 produced insignificant decreases.

As will be discussed, in the next paper, (−)−hydroxycitrate significantly reduced appetite, body wt gain, and body lipid levels. In an attempt to separate the effects upon lipogenesis from the effects upon appetite, three groups of rats were treated as follows: (A) the first group (control) was allowed free access to the G-70 diet, (B) the second group [(−)−hydroxycitrate)] was permitted unlimited access to the G-70 diet, which contained (−)−hydroxycitrate as a dietary admixture (daily dose equivalent to 2.63 mmoles/kg), and (C) a third group (paired control) was allowed only the quantity of G-70 diet which its (−)−hydroxycitrate-treated pair consumed on the preceding day (Table III). The paired controls, even under dietary restriction, demonstrated rates of lipogenesis equivalent to unrestricted controls. However, in vivo rates were inhibited significantly in the (−)−hydroxycitrate-treated rats.

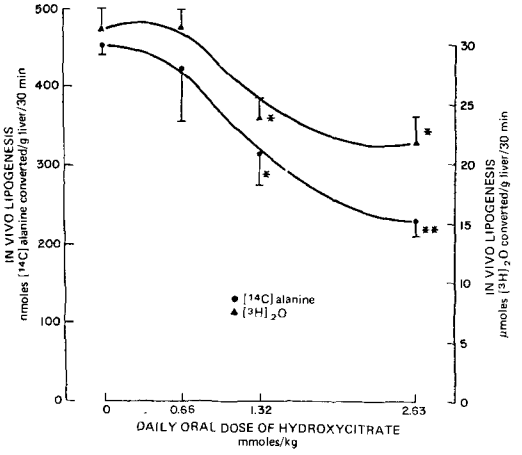

FIG. 5. Reduction in the in vivo rate of hepatic lipogenesis in rats administered (−)−hydroxycitrate orally for 30 days. Rats were prefasted 48 hr, then meal fed the G-70 diet for 5 days. From day 6-36, they received saline or varying concentrations of (−)−hydroxycitrate 1 hr before feeding the G-70 diet. Immediately after the 3 hr feeding period on day 36, the in vivo rates of lipogenesis were determined in 8-10 rat livers/group as described in the text. The vertical bar gives the standard error of the mean. *$p < 0.05$ **$p < 0.01$ • [^{14}C]alanine ▲ [^{3}H]$_2$O.

DISCUSSION

The preferential usefulness of [^{3}H]water for the measurement of in vivo rates of FA and cholesterol synthesis was apparent from the results illustrated in Figure 1. The rate of incorporation of [^{3}H]water was linear for at least 60 min and the total net rate was substantially greater than that determined from [^{14}C]alanine at each interval. This was expected, since the protons of [^{3}H]water were converted into FA (9, 11-16) and cholesterol (7) independent of the source of carbon precursors of acetyl CoA. In the studies reported here, [^{14}C]alanine was employed simultaneously to provide a measurement of the lipogenic rate from a specific carbon precursor.

We previously demonstrated that the oral administration of (−)−hydroxycitrate inhibited significantly in vivo rates of FA and cholesterol synthesis in rat liver, as measured by the conversion of [^{14}C]alanine. These rate measurements were made immediately following the 3 hr meal (6). The results presented in Figure 3 amplify and extend these observations,

TABLE III

In Vivo Lipogenesis in Pair fed Rats Administered (−)−Hydroxycitrate in the Diet and Meal fed for 38 Days

Treatment[a]	Rate of lipogenesis: nmoles [^{14}C]alanine converted[b]	Percent of control	μmoles [^{3}H]$_2$O converted[b]	Percent of control
Control	581 ± 64		33.6 ± 3.4	
Paired control	781 ± 86	134	39.2 ± 2.9	117
(−)−Hydroxycitrate	163 ± 16[c]	28	21.1 ± 2.4[c]	63

[a]Three groups of nine rats each were prefasted 48 hr, then meal fed the G-70 diet for 5 days. Two groups were then meal fed the G-70 diet for 38 days, while the third group received a dietary admixture of (−)−hydroxycitrate (52.6 mmoles/kg diet) in G-70. This amount of (−)−hydroxycitrate was equivalent to ca. 3 mmoles/kg body wt/day. One group receiving G-70 was pair fed to the (−)−hydroxycitrate treated rats. Immediately after the 3 hr feeding period on day 38, the in vivo rate of lipogenesis was determined in rat liver, as described in the text.

[b]Data are expressed as nmoles [^{14}C]alanine and μmoles [^{3}H]$_2$O converted into lipid/g liver/30 min. Each value is the group mean ± standard error.

[c]$p < 0.01$.

since they provide the hepatic lipogenic profile using [^{14}C]alanine and [^{3}H]water over a 24 hr period. The rise and fall in the rates of conversion of [^{3}H]water over a 24 hr period in meal fed, control rats corresponded to that of [^{14}C]alanine, previously reported (10). A single oral dose of (−)−hydroxycitrate administered to rats given the same amount of food as controls depressed hepatic lipogenesis for the 8 hr period when control animals demonstrated elevated rates of synthesis. During the next 16 hr, it was possible that the hepatic lipogenic rates in the (−)−hydroxycitrate-treated rats would increase or plateau, while the control rates were decreasing, i.e. a frame shift of lipogenic rates would occur with (−)−hydroxycitrate. The possibility that the carbons and electrons diverted from hepatic lipid synthesis during the 8 hr after feeding would be incorporated into lipid at a later time was not substantiated, since the increased rates observed during the next 16 hr in the (−)−hydroxycitrate-treated animals compared to controls were not significant. Results not reported here indicated that the amount of liver lipid, determined gravimetrically, in the (−)−hydroxycitrate-treated animals durng the 8 hr period when lipogenesis was significantly inhibited was 11% less than controls. Although these differences were not significant, at 10 hr the liver lipid content of the (−)−hydroxycitrate treated rats was significantly less than controls (21%). Liver lipid levels were similar in both groups from 12-24 hr after refeeding.

The metabolic fate of the carbons and electrons that were diverted from conversion into lipid posed an interesting question. Rates of lipogenesis determined at a single interval, 3 hr after feeding, were reduced equivalently by (−)−hydroxycitrate administration in liver and adipose tissue, although inhibition was less dramatic in small intestine (Table I). The possibility that carbon and electron flux into fatty acids increased in adipose tissue and small intestine at later intervals in (−)−hydroxycitrate treated animals appeared unlikely. Currently, we are investigating other metabolic fates of these diverted carbons and electrons.

The dose-dependent inhibition of the in vivo hepatic rates of lipogenesis after 11 and 30 days of (−)−hydroxycitrate administration (Fig. 5, Table II) was similar to that observed after acute administration (6). It was expected that rates determined from [^{3}H]water were less depressed than rates from [^{14}C]alanine, since [^{3}H]water provided a measure of the total rate of lipid synthesis. However, the significant increases (1.5-2.1-fold) in the in vitro rate of hepatic lipogenesis in animals receiving 2.63, 1.32, and 0.66 mmoles/kg (−)−hydroxycitrate for 30 days were unexpected. Since the in vitro assay provided a measure of the activities of ATP citrate lyase, acetyl CoA carboxylase, and FA synthetase, the observed rate increases suggested that the lipogenic enzyme activities and levels were increased as a result of a prolonged period of daily depression of FA synthesis resulting from the competitive inhibition of ATP citrate lyase. The fact that a constant level of (−)−hydroxycitrate added exogenously depressed the in vivo rates equivalently suggested that ATP citrate lyase may be the elevated enzyme. This theoretical, compensatory mechanism was provocative but unproved. It was interesting that dietary restriction to the level consumed by (−)−hydroxy-

citrate treated rats still produced elevated control rates of hepatic lipogenesis (Table III).

The following paper examines the influence of the chronic oral administration of (−)–hydroxycitrate on appetite, wt gain, and body lipid levels and discusses whether these effects are related to the antilipogenic action of (−)–hydroxycitrate described here.

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Effect of (-)-Hydroxycitrate upon the Accumulation of Lipid in the Rat: II. Appetite[1]

ANN C. SULLIVAN, JOSEPH TRISCARI, JAMES G. HAMILTON and **O. NEAL MILLER,**
Department of Biochemical Nutrition, Hoffmann-La Roche Inc., Nutley, New Jersey 07110

ABSTRACT

These studies were designed to determine the effect of (−)−hydroxycitrate upon the accumulation of lipid in the rat by examining appetite, wt gain, and total body lipid profiles. The chronic oral administration of a nontoxic dose of (−)−hydroxycitrate to growing rats for 11-30 days caused a significant reduction in body wt gain, food consumption, and total body lipid. The administration of equimolar amounts of citrate did not alter wt gain, appetite, or body lipid. No increase in liver size or liver lipid content occurred with either treatment. Pair feeding studies demonstrated that the reduction in food intake accounted for the decrease in wt gain and body lipid observed with (−)−hydroxycitrate treatment.

INTRODUCTION

The accumulation of lipid is an important process in the mammal, since he is primarily an intermittent eater, thus requiring mechanisms for the storage of chemical energy ingested in food and for the utilization of this energy during postabsorptive periods. This diversion of the metabolic flux toward energy storage when caloric intake exceeds immediate demand is clearly the major homeostatic function of fatty acid (FA) synthesis.

Using as a model system the meal fed rat induced to synthesize lipids at an elevated rate (1), we demonstrated that (−)−hydroxycitrate interfered with the metabolic flux of carbohydrate and its metabolites into lipid by inhibiting significantly in vivo rates of FA and cholesterol synthesis (2). In the preceding paper, we reported that the extent of this diversion of carbohydrate carbons and electrons from lipid biosynthesis was considerable, since it was shown to occur in three major lipogenic tissues (adipose tissue, liver, and small intestine) and to persist in the liver for the 8 hr period when control animals exhibited significant lipogenic rates (3).

The present report examines the question of how the long term administration of (−)−hydroxycitrate would affect wt gain and the size of body lipid stores. Since the accumulation of lipid in the mammal involves not only the conversion of carbohydrate and its metabolites into lipid but also the size and content of the dietary intake, it seemed important to study the effect of chronic (−)−hydroxycitrate treatment on appetite.

EXPERIMENTAL PROCEDURES

Female rats of the Charles River CD strain (Charles River Breeding Laboratories, Wilmington, Mass.) weighing 120-160 g (ca. 16 week old) were housed individually, fasted 48 hr, then meal fed a synthetic diet (G-70) daily from 8-11 A.M. for the remainder of the experiment. The G-70 diet consisted of 70% glucose, 23% vitamin free casein, 5% Phillips and Hart salt mixture IV (4), 1% corn oil, 1% complete vitamin mixture and 40 g/kg cellulose. Further details on housing, diets, and feeding regimens are given in the preceding paper (3). When equimolar amounts of (−)−hydroxycitrate $(Na)_3$ and citrate $(Na)_3$ were administered as dietary admixtures, an equivalent wt of glucose was deleted from the diet. Food consumption and body wt were determined two-three times/week, and food spillage was measured daily. At the beginning of the experiment body wt were randomized so that each group had an identical wt spread.

Gravimetric Determination of Liver Lipids

Immediately after the 3 hr feeding period the rats were anaesthetized, injected intravenously with the [^{14}C]alanine and [^{3}H]water pulse, and killed by decapitation 30 min later as described in the preceding paper (3). Livers were excised rapidly, weighed, and homogenized in 15 ml H_2O in a Virtis 45 Macro homogenizer for 15 sec at ca. 30,000 rpm.

Duplicate 3 ml aliquots of liver:H_2O homogenate were saponified in 2.1 ml 5N NaOH for ca. 15 hr at 90 C. Both aliquots were acidified with 2.6 ml 5N HCl and extracted three times with 5 ml petroleum ether (bp 30-60 C). The petroleum ether supernatants were transferred to preweighed glass vials, evaporated immediately to dryness under N_2, and reweighed. Total lipid data are expressed in g and % of liver wt.

[1]One of six papers presented in the symposium "Effect of Drugs on Lipid Metabolism," AOCS Spring Meeting, New Orleans, April 1973.

TABLE I

Total Wt Gain and Food Consumption in Rats Administered (−)−Hydroxycitrate Orally for 30 Days[a]

Daily oral dose of (−)−hydroxycitrate mmoles/kg	Wt gain		Food consumption	
	g[b]	Percent of control	g[b]	Percent of control
0	45 ± 2	0	419 ± 23	0
0.17	46 ± 6	102	405 ± 16	97
0.66	38 ± 2	84	404 ± 13	96
1.32	37 ± 5	82	371 ± 8[c]	89
2.63	29 ± 3[c]	64	349 ± 11[c]	83
0.33 bid	28 ± 2[d]	62	359 ± 10[c]	87

[a]Sixty rats were prefasted 48 hr, then meal fed the G-70 diet for 5 days. The mean body wt ± standard deviation after this pretreatment period was 195 ± 19 g. The rats then were divided into six groups of 10 each and given saline or (−)−hydroxycitrate by stomach tube 1 hr before feeding (and 4 hr after the completion of the meal where bid administration is indicated) for 30 days.

[b]Each value is the group mean ± standard error.

[c]$p < 0.05$.

[d]$p < 0.01$.

Gravimetric Determination of Body Lipids

Carcasses (minus blood and liver) were weighed and saponified in 600 ml (carcass wt < 250 g)-1000 ml (carcass wt > 250 g) 10% alcoholic KOH (100 g KOH dissolved in 150 ml H_2O and ethanol added to 1 liter) for 18-48 hr at 70 C. Duplicate 15 ml aliquots were acidified with 6 ml 5N HCl and extracted three times with 10 ml petroleum ether. The ether supernatants were transferred to preweighed tubes, evaporated to dryness under N_2, and reweighed. Body lipid data are expressed in g and % of body wt.

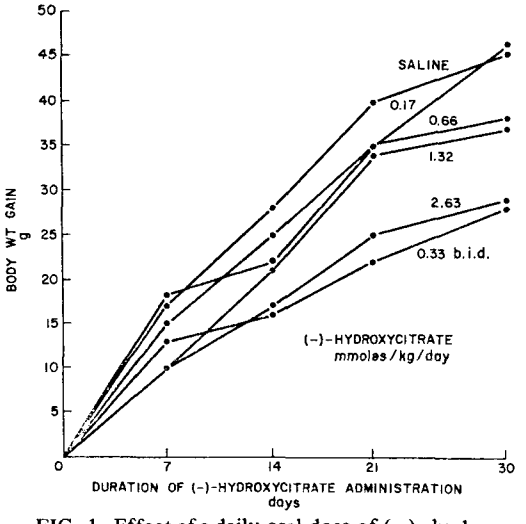

FIG. 1. Effect of a daily oral dose of (−)−hydroxycitrate on body wt gain in growing rats. Sixty rats were prefasted 48 hr, then meal fed the G-70 diet for 5 days. The mean body wt ± standard deviation after this pretreatment period was 195 ± 19 g. The rats then were divided into six groups of 10 each. From day 6-36, they were administered saline or varying concentrations of (−)−hydroxycitrate 1 hr before feeding (and 4 hr after the completion of the meal where bid administration is indicated). The body wt gains of rats receiving 2.63 mmoles/kg and 0.33 mmoles/kg bid of (−)−hydroxycitrate were significantly less than controls ($p < 0.05$).

Sources of Chemicals

(−)−Hydroxycitrate lactone was isolated from the dried fruit rinds of the Indian plant *Garcinia cambogia*, and the trisodium salt was prepared. Constituents for the synthetic diets were obtained from Nutritional Biochemicals, Cleveland, Ohio. Other chemicals were purchased from Sigma Chemical, Milwaukee, Wis.

Statistical Analysis

The *t* test was used to analyze all experimental results (5). Data were processed statistically for outliers (6).

RESULTS

Effect of the Chronic Oral Administration of (−)−Hydroxycitrate on wt Gain, Appetite, and Total Body Lipid

Figure 1 demonstrates the effect of administering saline or various concentrations of (−)−hydroxycitrate either once a day (0.17, 0.66, 1.32, 2.63 mmoles/kg) or twice a day (0.33 mmoles/kg for 30 days on body wt gain in growing rats (160 g). A dose related reduction in wt gain was observed with (−)−hydroxycitrate treatment. These decreases were significant at concentrations of 2.63 mmoles/kg once a day and 0.33 mmoles/kg bid (twice a day). However, no significant reductions were observed with the single daily ad-

ministration of 0.17, 0.66, and 1.32 mmoles/kg.

Table I provides the cumulative wt gain and food consumption data for these rats. Significant depression of appetite paralleled significant wt reduction.

(−)−Hydroxycitrate was administered chronically for 11 days, and its effect upon wt gain, food consumption, and body lipids was determined (Table II). Significant depression of food intake and wt gain occurred with the oral administration of 0.33 mmoles/kg bid, 1.32 mmoles/kg bid, and 2.63 mmoles/kg once a day of (−)−hydroxycitrate compared to saline-treated controls. These reductions were reflected metabolically in significant decreases of the total body lipid stores (minus liver and blood lipids). No differences were detected in liver lipids in control and (−)−hydroxycitrate-treated rats, and serum lipids were normal. It was apparent from these data and those presented in Table I that bid administration increased significantly the effectiveness of (−)−hydroxycitrate.

Comparison of the Chronic Oral Administration of Citrate $(Na)_3$ and (−)−Hydroxycitrate $(Na)_3$

The effect of the chronic oral administration for 11 days of citrate $(Na)_3$ was compared with (−)−hydroxycitrate $(Na)_3$ (Table III). The citrate $(Na)_3$-treated rats gained significantly more wt than saline controls. However, their cumulative food intake and body lipid levels were not significantly different. When an equimolar concentration (1.32 mmoles/kg bid) of (−)−hydroxycitrate $(Na)_3$ was given, a significant reduction in wt gain and body lipid was observed. Food consumption also was decreased. No differences in liver wt or liver lipid content were detected in either treatment group.

Effect of the Chronic Oral Administration of (−)−Hydroxycitrate in a Pair feeding Study

(−)−Hydroxycitrate produced a significant depression of body wt gain, food consumption, and total body lipid. Pair feeding studies proved that these effects were due to the decreased food intake induced by (−)−hydroxycitrate treatment. Figure 2 demonstrates that when the food intake of rats in the saline treated group (paired control) was restricted to the exact quantity consumed by their (−)−hydroxycitrate-treated pair, an equivalent reduction in wt gain was observed. There were no significant differences between the wt gain of the (−)−hydroxycitrate-treated and paired control groups, but both were significantly less than controls ($p < 0.05$) at each time. Food consumption data paralleled the body wt gain

TABLE II

Effect of Oral Administration of (−)−Hydroxycitrate for 11 Days Upon Total Wt Gain, Food Consumption, and Body Lipid[a]

Daily oral dose of (−)−hydroxycitrate mmoles/kg	Wt gain		Food consumption		Body lipid[b]	
	g[c]	Percent of control	g[c]	Percent of control	g[c]	Percent of carcass wt[c]	Percent of control
0	12 ± 3		102 ± 3		9.97 ± 0.96	5.7 ± 0.5	
0.66	12 ± 3	100	94 ± 6	92	9.46 ± 0.71	5.5 ± 0.4	96
0.33 bid	6 ± 2	50	89 ± 3[d]	87	6.77 ± 0.82[d]	4.1 ± 0.5[d]	70
2.63	1 ± 2[e]	8	89 ± 3[d]	87	6.01 ± 0.31[e]	3.5 ± 0.1[e]	61
1.32 bid	2 ± 3[d]	17	85 ± 4[d]	83	6.34 ± 0.64[e]	4.0 ± 0.4[d]	67

[a]Forty-five rats were prefasted 48 hr, then meal fed the G-70 diet for 5 days. The mean body wt ± standard deviation after this pretreatment period was 178 ± 13 g. The rats then were divided into five groups of nine each and given saline or (−)−hydroxycitrate by stomach tube 1 hr before feeding (and 4 hr after the completion of the meal where bid administration is indicated) for 11 days.

[b]Body lipid minus liver and blood lipid was determined as described in the text.

[c]Each value is the group mean ± standard error.

[d]$p < 0.05$.

[e]$p < 0.01$.

TABLE III

Comparison of the Effect of Oral Administration of Citrate $(Na)_3$ and (−)−Hydroxycitrate $(Na)_3$ for 11 Days[a]

Treatment	Wt gain		Food consumption	Body lipid[b]			Liver wt	Liver lipid
	g[c]	Percent of control	g[c]	g[c]	Percent of carcass wt	Percent of control	g[c]	Percent of liver wt[c]
Saline	20 ± 3		120 ± 5	7.4 ± 1.0	4.5 ± 0.7		5.8 ± 0.3	3.2 ± 0.1
Citrate $(Na)_3$	27 ± 2[d]	135	131 ± 5	6.8 ± 0.8	4.1 ± 0.5	92	5.9 ± 0.2	3.2 ± 0.1
(−)−Hydroxycitrate $(Na)_3$	12 ± 2[d]	60	112 ± 4	4.4 ± 0.5[d]	2.9 ± 0.5[d]	62	5.8 ± 0.2	2.9 ± 0.1

[a]Forty-two rats were prefasted 48 hr, then meal fed the G-70 diet for 5 days. The mean body wt ± standard deviation after this pretreatment period was 151 ± 13 g. The rats then were divided into three groups of 14 each and administered saline, citrate $(Na)_3$ (1.32 mmoles/kg), or (−)−hydroxycitrate $(Na)_3$ (1.32 mmoles/kg) 1 hr before feeding and 4 hr after completion of the meal for 11 days.

[b]*Body lipid minus liver and blood lipid was determined as described in the text.*

[c]Each value is the group mean ± standard error.

[d]$p < 0.05$.

results. The average daily food intake of the unrestricted control, paired control, and (−)−hydroxycitrate-treated rats was 13, 9, and 10 g, respectively.

Table IV illustrates the body lipid levels of the same rats pair fed to the (−)−hydroxycitrate treated rats for 22 days. Body lipid levels were significantly less than controls in the (−)−hydroxycitrate treated and the pair fed controls, indicating that the effect of (−)−hydroxycitrate upon body lipid was due to caloric restriction. Liver lipid levels were identical in the control and treated groups.

DISCUSSION

The chronic oral administration of certain levels of (−)−hydroxycitrate to growing rats produced a significant reduction in wt gain and food consumption when compared to controls (Fig. 1, Table I, Table II). These decreases were reflected metabolically in a significantly reduced level of total body lipid (Table II). The dose of (−)−hydroxycitrate required to produce these changes was lowered by bid administration. Results not reported here demonstrated that serum lipids were unaltered by (−)−hydroxycitrate treatment. Subsequent studies on the effect of ad libitum feeding a diet containing (−)−hydroxycitrate to mature rats (6-14 months) demonstrated equivalent reductions in wt gain, appetite, and body lipid.

The chronic oral administration of (−)−hydroxycitrate $(Na)_3$ to growing rats decreased significantly wt gain and total body lipids (Table III). Food consumption also was depressed. However, equimolar concentrations of citrate $(Na)_3$ given under the same experimental conditions failed to demonstrate any significant differences when compared to controls. No hepatomegaly was observed with either treatment. These results argued against a nonspecific tricarboxylate effect upon appetite. More cirtical proof of the selective action of (−)−hydroxycitrate upon appetite would be obtained by analyzing the effect of the stereoisomers of hydroxycitrate. It would be interesting to determine whether the stereospecific inhibition of in vitro and in vivo rates of hepatic lipogenesis (2) extended to the (−)−hydroxycitrate induced depression of appetite.

Pair feeding studies proved that these effects upon wt and body lipids were due to the decreased caloric intake which resulted from (−)−hydroxycitrate treatment (Fig. 2, Table IV). Similar reductions in wt gain and body lipids were observed in rats whose food intake was restricted to that of the (−)−hydroxycitrate treated rats.

TABLE IV

Body Lipid Levels of Rats Pair fed to (−)−Hydroxycitrate Treated Rats for 22 Days[a]

Treatment	Body lipid[b] g[c]	Body lipid[b] Percent of carcass wt[c]	Body lipid[b] Percent of control	Liver lipid Percent of liver wt[c]
Control	10.1 ± 1.1	5.1 ± 0.5		3.2 ± 0.0
Pair fed control	5.4 ± 0.7[d]	3.4 ± 0.4[e]	67	3.1 ± 0.0
(−)−Hydroxycitrate	6.3 ± 0.7[e]	3.8 ± 0.3[e]	75	3.1 ± 0.0

[a]Thirty rats were prefasted 48 hr, then meal fed the G-70 diet for 5 days. The mean body wt ± standard deviation after this pretreatment period was 153 ± 6 g. The rats then were divided into three groups of 10 each. The control groups then were administered saline, and the experimental group received (−)−hydroxycitrate (1.32 mmoles/kg bid) directly before feeding and 4 hr after the completion of the meal for 22 days. During this time the pair fed controls received the exact quantity of food that the (−)−hydroxycitrate treated rats had consumed on the previous day.

[b]Body lipid minus blood and liver lipid were determined as described in the text.

[c]Each value is the group mean ± standard error.

[d]$p < 0.01$.

[e]$p < 0.05$.

(−)−Hydroxycitrate inhibited lipogenesis (2,3) by functioning as a competitive inhibitor of adenosine 5′-triphosphate (ATP) citrate lyase (7,8) and suppressed appetite by an unknown mechanism. Whether the depression of lipogenesis caused by (−)−hydroxycitrate was related to the appetite suppression was an important question. In the experiments described here, (−)−hydroxycitrate was administered orally 1 hr before feeding, and 3 hr later the food consumption and lipogenic rates were determined. Thus, from these studies, it was impossible to state whether the appetite suppression was caused by the antilipogenic activity of (−)−hydroxycitrate. It was interesting that smaller doses of (−)−hydroxycitrate would suppress food intake if the compound was given bid, i.e. directly before feeding and 4 hr after the completion of the meal. Although the lipogenic rates occurring 4 hr after the meal were declining, this was insufficient evidence to disprove a cause and effect relationship between antilipogenesis and antiappetite. Experiments in which one of these systems (lipogenesis and appetite) is altered or removed from the other are in progress.

Since the appetite suppression induced by (−)−hydroxycitrate appeared to be selective (equimolar concentrations of citrate were inactive), the possibility of central nervous system involvement was provocative. The problems inherent in designing experiments to prove mediation of the central nervous system are apparent when one considers some of the complex factors which have been implicated in appetite regulation and feeding behavior. Glucose utilization rates (9), serum amino acid pattern and concentration (10,11), unidentified humoral factor(s) in blood from satiated rats (12,13), enterogastrone (14) and other gastrointestinal hormones, plasma and brain tryptophan and brain serotonin interrelationships (15,16,17,18), and brain catecholamines (19) have all been suggested.

Since (−)−hydroxycitrate was demonstrated to inhibit FA and cholesterol synthesis pre-

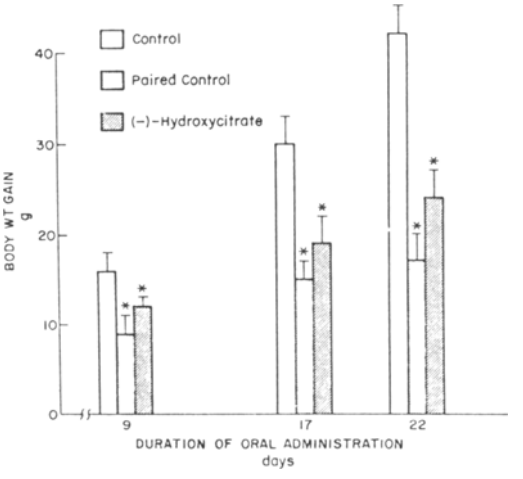

FIG. 2. Effect of oral administration of (−)−hydroxycitrate for 22 days in a pair feeding study. Thirty rats were prefasted 48 hr, then meal fed the G-70 diet for 7 days. The mean body wt ± standard deviation after this pretreatment period was 153 ± 6 g. The rats then were divided into three groups of 10 each and were administered saline (control and paired control) or (−)−hydroxycitrate (1.32 mmoles/kg bid) directly before feeding and 4 hr after the completion of the meal. The paired control group was pair fed to the (−)−hydroxycitrate treated rats. The vertical bar gives the standard error of the mean. *$p < 0.05$. □ Control ■ Paired control ▩ (−)−Hydroxycitrate.

sumably through a reduction in the acetyl CoA pool in the lipogenic tissues (2), the same effect would be expected in any cell possessing ATP citrate lyase. If (−)−hydroxycitrate could penetrate the blood brain barrier, then the depression of acetylcholine levels or rate of turnover in the brain resulting from a decreased precursor pool could affect cholinergic receptor systems that may be involved in feeding behavior.

ACKNOWLEDGMENTS

R.W. Guthrie and R.W. Kierstead, Roche Chemical Division, prepared (−)−hydroxycitrate (trisodium salt) and isolated (−)−hydroxycitrate lactone from the dried fruit rinds of *Garcinia cambogia*. G.P. Mackie provided technical assistance.

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Location of Double Bonds in Long Chain Esters by Methoxymercuration-Demercuration Followed by Mass Spectroscopy

D.E. MINNIKIN, P. ABLEY, and **F.J. MCQUILLIN,** Department of Organic Chemistry, The University, Newcastle upon Tyne, NE1 7RU, Great Britain, and **K. KUSAMRAN, K. MASKENS,** and **N. POLGAR,** Dyson Perrins Laboratory, Oxford, OX1 3QY, Great Britain

ABSTRACT

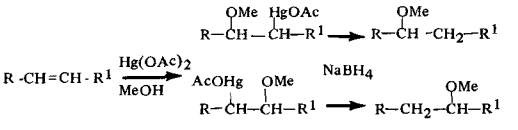
Methyl esters of simple mono-, di-, and triunsaturated long chain fatty acids quantitatively react with mercuric acetate in methanol to produce methoxyacetoxymercuri derivatives. Demercuration of these derivatives with sodium borohydride yields methoxylated fatty acid esters, readily isolable by thin layer chromatography. Mass spectra of the methoxylated esters are characterized by intense peaks due to cleavage adjacent to methoxy-functions which allow the position of the original double bond in the chain to be ascertained. The methoxylated derivatives are conveniently analyzed by gas liquid chromatography and also by combined gas chromatography-mass spectrometry. In comparison with other methods for double bond location, the procedure described is simple, reliable, and rapid.

INTRODUCTION

The determination of double bond position in unsaturated fatty acid esters by mass spectrometric techniques requires examination of suitable derivatives since the intact unsaturated esters do not produce useful spectra. Most of the successful procedures employed so far involve the formation of oxygenated derivatives.

Epoxidation of a double bond produces a derivative which is suitable for mass spectra of monoenes but not polyenes (1,2). Partial epoxidation, however, followed by hydrogenation and mass spectrometry of the resulting monoepoxides allows polyunsaturated esters to be characterized (3). Conversion of an epoxide to isomeric ketones (4) or dimethylamino-alcohols (5) allows the position of the original double bond to be determined by mass spectrometry. Hydrolysis of epoxides or direct hydroxylation of double bonds, most conveniently with osmium tetroxide, produce vicinal diols which may be analyzed intact (1) or after conversion to more volatile derivatives. Isopropylidene ketals and ethylidene acetals have been investigated and show characteristic cleavages adjacent to the 1,3-dioxolane ring (6,7); such fragmentations also have been observed in 1,3-dioxan derivatives prepared from the naturally occurring phthiocerols (8). Trimethylsilyl ethers of vicinal diols are suitable for the positional identification of double bonds in unsaturated long chain compounds (9-11). Methyl ethers also have been demonstrated as being suitable for combined gas chromatography-mass spectrometry (GC-MS) of a range of diols derived from polyunsaturated fatty acid methyl esters (12). These procedures have been reviewed (11,13,14).

Sodium borohydride reduction of oxymercuri adducts of olefins is a convenient demercuration procedure (15). Reduction of methoxymercuriacetate adducts of simple olefins leads to methoxy derivatives according to the following scheme:



Methoxy derivatives of long chain esters or hydrocarbons are expected to produce relatively simple mass spectra suitable for the assignment of the structure of the parent olefin. This paper describes a simple method for the location of the double bonds in nonconjugated mono-, di-, and triunsaturated long chain esters and hydrocarbons; the procedures described here represent an improvement on those outlined in a preliminary report (16).

PROCEDURES

Sources of long chain substrates: *Trans*-dec-5-ene and methyl esters of oleic, elaidic, linoleic, linolenic, and erucic acids were commercial products. Lyophilized cells of *Lactobacillus casei* NCIB 6375, cultivated as described previously (17), were extracted with chloroform-methanol (2:1) and methyl esters prepared from the extract by treatment with sodium methoxide in methanol.

Thin layer chromatography (TLC): TLC was performed on analytical (0.4 mm) or preparative

TABLE I

Equivalent Chain Lengths of Methoxylated Derivatives of Unsaturated Fatty Acid Esters[a]

Long chain ester substrate	Stationary phase SE 30	QF 1
Oleate) Elaidate)	19.32	20.26
Lactobacillus casei		
(C_{18} (*cis*-vaccenate)	19.40	20.00
(C_{16})	17.74	18.36
Linolenate	20.72	22.40
Linolenate (upper band)	21.80	24.08
Linolenate (lower band)	22.04	24.28

[a]The derivatives from linolenate are the upper and lower bands isolated by thin layer chromatography.

(1 mm) layers of Merck silica gel $PF_{254+366}$. Mixtures of hexane and diethyl ether (80:20) were used as development solvent in all cases, except for *trans*-dec-5-ene and its derivative when the proportions 95:5 were employed.

Gas liquid chromatography (GLC): A Pye 104 flame ionization instrument was employed for GLC; nitrogen was used as carrier gas. Glass columns (2 m x 8 mm outside diameter) were packed separately with methyl silicone (SE 30, 2.5%) and fluorosilicone oil (QF 1, 2.5%) coated on acid-washed Celite (85-100 mesh).

Mass spectrometry: Mass spectra (70 ev) were determined on A.E.I.MS 9 spectrometers. Combined gas GC-MS experiments were carried out (Dyson Perrins Laboratory, Oxford, England) using a Pye 104 gas chromatograph (SE 30 column, helium as carrier gas) linked by a Biemann separator to an MS 9 mass spectrometer.

Proton magnetic resonance spectra (PMR): NMR spectra were measured on a Perkin Elmer R14 100 MHz spectrometer in deuteriochloroform solution.

Methoxymercuration and demercuration procedures: Two procedures were employed for the preparation of methoxymercuriacetate derivatives. The traditional procedure of Jantzen and Andreas (18) was satisfactory in the case of monoenes; but for derivatization of polyenes (and indeed monoenes) the modified method of White (19) is more convenient. The former procedure involves treatment of the olefin in methanol solution with a 5-10% excess of mercuric acetate at room temperature for 24 hr; the latter method utilizes the same proportions of reactants, but the reaction is performed by heating under reflux for 1 hr. Solid sodium borohydride was added to the crude reaction mixtures from both procedures until no more mercury was precipitated. A few drops of acetic acid were added to decompose any unreacted borohydride and the mixture evaporated to the dryness and partitioned between water and diethyl ether. The ethereal layer was dried (Na_2SO_4) and evaporated to dryness. Pure methoxy-esters were isolated by preparative TLC of the crude reaction products.

RESULTS

TLC: The products of the methoxymercuration-borohydride reduction procedures gave the following results on TLC. *Trans*-dec-5-ene (R_f 0.95, hexane-ether, 95:5) was transformed into a methoxy derivative (R_f 0.48, hexane ether, 95:5). The methyl esters under consideration all had the same initial chromatographic behavior (R_f 0.66, hexane-ether, 80:20). Methyl oleate, elaidate, and erucate were transformed completely into monomethoxy derivatives (R_f 0.51). Methyl linoleate gave a dimethoxy-ester (R_f 0.39), but analysis of the product from methyl linolenate showed the presence of two chromatographically separable spots (R_f 0.12 and 0.20). The methyl esters from *L. casei* after reaction showed the presence of unreacted esters (R_f 0.66) and monomethoxy esters (R_f 0.51). The methoxylated esters were purified by preparative TLC; two separate fractions were obtained from the methoxylated derivative of methyl linolenate.

GLC: The gas chromatographic behavior of the methoxylated derivatives of the unsaturated fatty acid esters (excluding methyl erucate) is summarized in Table I.

PMR: PMR spectra of all the methoxylated derivatives contained singlets at τ 6.68 attributable to the protons of methoxyl groups; the remaining areas of the spectra showed signals characteristic of long chain esters and hydrocarbons. The intensities of the signals due to methoxyl protons (τ 6.68) in comparison with that due to the methyl ester function (τ 6.38) confirmed that each olefinic linkage gave rise to a single methoxyl function. The PMR spectra of the two derivatives isolated from methyl linolenate were closely similar.

Mass spectrometry: The isomeric methoxy-esters derived from mono-unsaturated fatty acid methyl esters may be represented by the general formula (I):

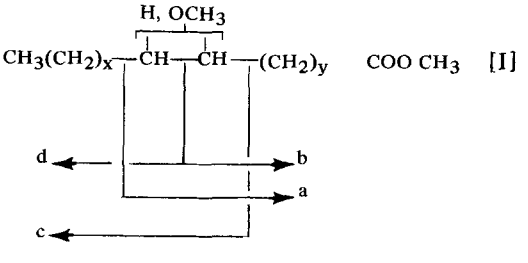


TABLE II

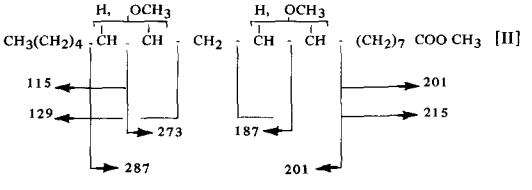
Principal Mass Spectral Fragments of Methoxylated Derivatives of Monounsaturated Esters[a]

Long chain ester substrate	Mass spectral fragment (m/e), relative intensity inbrackets						
	a	a-32	b	b-32	c	d	Base peak
Oleate elaidate x=y=7	215(75)	183(7)	201(98)	(169(11)	171(44)	157(62)	69
Erucate x=7, y=11	271(19)	239(5)	257(21)	225(5)	171(16)	157(19)	57
Lactobacillus casei x=5, y=7	215(90)	183(13)	201(97)	169(11)	143(75)	129(100)	129
Lactobacillus casei x=5, y=9	243(76)	211(14)	229(92)	197(15)	143(100)	129(98)	143

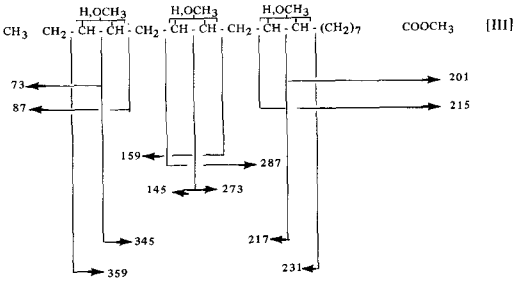
[a]The origin of the fragments a-d is shown in the formula (1).

Fragments of the type $R\text{-}\overset{+}{C}H\text{-}OCH_3 \leftrightarrow R\text{-}CH{=}\overset{+}{O}\text{-}CH_3$ are expected to be particularly prominent in the mass spectra of such methoxy-esters. The mass spectrum of the methoxylated esters derived from methyl oleate (I, x=y=7) is shown in Figure 1, and the assignment of the major fragments arising from cleavages adjacent to methoxyl groups is included in Table II. It is immediately apparent that the structure of the methoxylated derivatives, and hence their olefinic precursors, can be deduced from the principal fragments shown in Figure 1 and Table II. Closely corresponding mass spectra were obtained from the methoxylated esters derived from the other mono-unsaturated esters including the natural esters from *L. casei*; the principal fragments are summarized in Table II.

The mass spectral breakdown of the dimethoxylated derivative (II) of methyl linoleate is shown in Figure 2 and is interpreted as shown below; peaks at m/e 155, 169, 241, and 255 may arise by loss of the elements of methanol from the primary fragments:



The two separated trimethoxylated fractions derived from methyl linolenate were analyzed separately, and the mass spectra are shown in Figure 3. The expected fragmentations of these trimethoxylated components (III) is shown below; peaks at m/e 113, 127, 153, 167, 185, 199, 241, 255, 281, 295, 313, and 327 are due to successive loss of the elements of methanol from the primary fragments:



Methoxymercuration-demercuration of *trans*-dec-5-ene gives rise to a single product, 5-methoxydecane:

$$CH_3(CH_2)_3\,\underset{\displaystyle OCH_3}{\underset{|}{CH}}\,(CH_2)_4\,CH_3$$

The mass spectrum of 5-methoxydecane is dominated by intense peaks at m/e 101 (base peak) and 115 (71%) representing cleavages adjacent to the $>CH\text{-}OCH_3$ group. These two fragments ($CH_3(CH_2)_n\,CH{=}O^+\text{-}CH_3$, n=3 and 4 respectively) lose the elements of methanol to produce peaks at m/e 69 (26%) and 83 (32%), both these processes being confirmed by metastable ions at m/e 47.1 and 59.9, respectively. A molecular ion, m/e 172 (2%), is observed and loses methanol to produce a peak at m/e 140 (2%).

DISCUSSION

The methoxymercuration-demercuration procedure described above provides a convenient procedure for the preparation of derivatives suitable for the location of olefinic linkages in simple unsaturated and polyunsaturated long chain compounds by mass spectrometry. Methoxymercuriacetate (18) and methoxy-

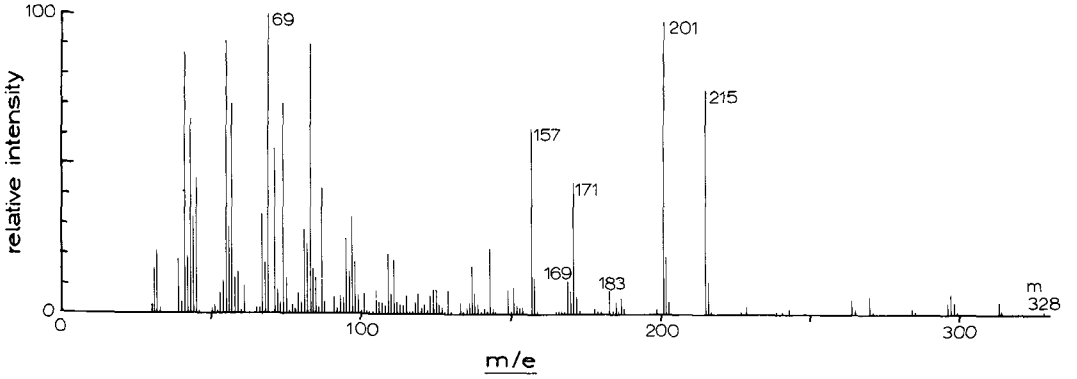


FIG. 1. Mass spectrum of methoxylated esters derived from methyl oleate by methoxymercuration-demercuration.

mercuribromide (19) derivatives have been used for the isolation and sub-fractionation of olefinic esters by chromatographic procedures since the adducts are readily and stereospecifically decomposed to the original esters by treatment with mineral acid. An extension of this procedure to include reaction of these adducts, or a small portion of them, with sodium borohydride produces a derivative suitable for mass spectrometry.

It will be noted that the gas chromatographic behavior of the methoxylated derivatives from methyl oleate (9, 10 double bond) and *cis*-vaccenate (11,12 double bond) is distinct (Table I); cochromatography of a mixture of these materials, however, gave a single peak. The dimethoxy esters derived from methyl linoleate were not resolved by the gas chromatographic columns employed. The trimethoxy derivatives from methyl linolenate were separable by TLC and GLC into two main fractions whose mass spectral fragmentation patterns (Fig. 3) were different in some respects. The component having the higher mobility on TLC (Fig. 3a) had a higher proportion of peaks corresponding to methoxyl groups in 9 and 16 positions (m/e 201, 231, 199, 167 and 73, 359, 327, 295, respectively) than those corresponding to 10- and 15-methoxyl groups (m/e 215, 217, 183, 153 and 87, 345, 313, 281, respectively); the position was reversed for the isomer having lower mobility (Fig. 3b). No conclusive opinion regarding the relative distribution of methoxyl groups at the 12 and 13 positions could be obtained from the mass spectra. The mass spectrum of an unresolved mixture of the trimethoxylated esters derived from methyl linolenate was essentially a synthesis of those of the two separated isomers.

The procedure described here for the location of olefinic linkages in unsaturated fatty acid esters has several distinct advantages. The overall procedure is fast. If the reaction mixture is heated under reflux and if the organic layer from the reaction is injected directly into the GC-MS instrument, a result may be obtained in a matter of 2-3 hr or less. The chemistry of the reaction is carried out in a single flask. Cyclic derivatives, e.g. isopropylidene or ethylidene derivatives, of diols prepared from hydroxylation products of olefins (7,8) can give information regarding the stereochemistry of the origi-

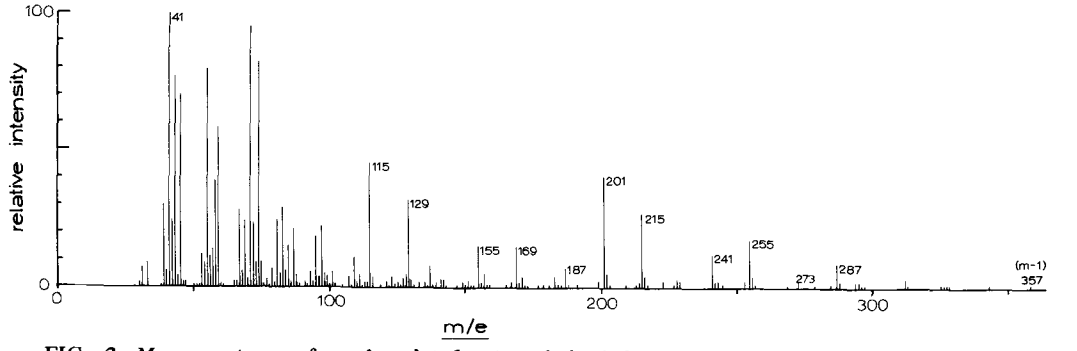


FIG. 2. Mass spectrum of methoxylated esters derived from methyl linoleate by methoxymercuration-demercuration.

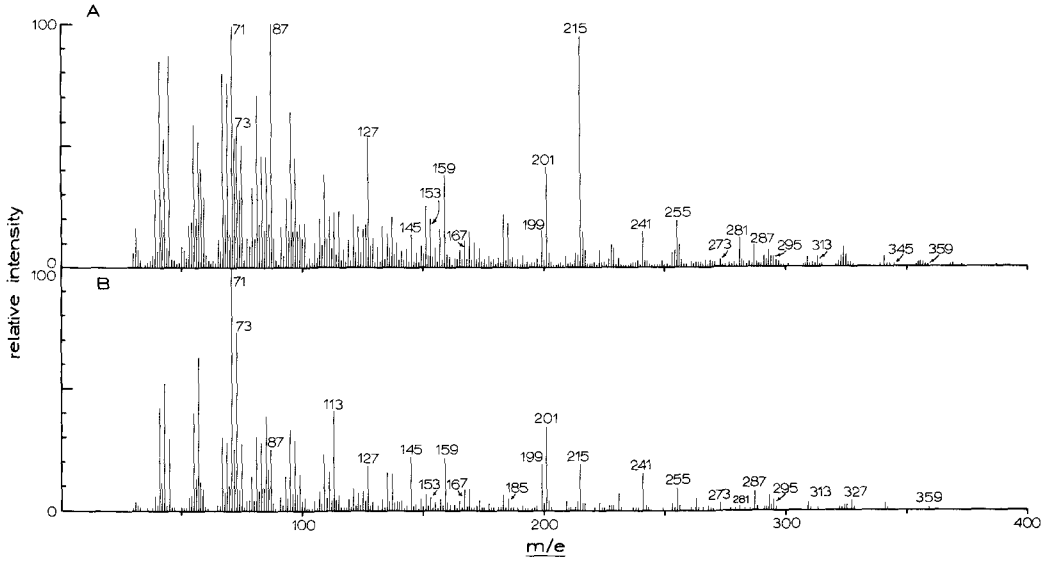


FIG. 3. Mass spectra of methoxylated esters derived from methyl linolenate by methoxymercuration-demercuration (A) is faster moving and (B) is slower moving component from thin layer chromatography purification.

nal double bond since the derivatives of *cis*- and *trans*- olefins are separable by GLC (20); the mass spectra are, however, rather complicated and unsuitable for esters containing more than a single double bond. Methyl (12) and trimethylsilyl (10,11) ethers of diols derived from olefinic links give relatively simple mass spectra, but their preparation is less convenient. Each double bond also gives rise to two polar ether groups, whereas the oxymercuration procedure produces only one. The latter derivatives are consequently more volatile than the dimethoxy derivatives, and a wider range of unsaturated long chain compounds may be studied by combined GC-MS. This advantage is offset by the hetereogeneity of the derivatives from polyunsaturated esters, though this could have potential as a diagnostic tool.

The present procedure is particularly convenient for the analysis of certain bacterial fatty acid mixtures containing unsaturated and cyclopropane acids. Treatment of the total methyl esters with the procedure described here allows isolation of the methoxylated derivatives of the olefinic esters. The unreacted esters then may be treated with 50% boron trifluoride-methanol (21) and methoxylated derivatives from the cyclopropane esters isolated. Both sets of methoxylated derivatives then may be analyzed by GC-MS under the same conditions.

A complementary series of experiments on the chemistry of oxymercuration-demercuration reactions of long chain olefins has been carried out by Gunstone and Inglis (22,23). Various nucleophilic reagents (methanol, ethanol, water, acetic acid, acetonitrile, and ethane-1,2-diol) were found to attack olefinic esters in the presence of mercuric salts; a reaction time of 2-4 days was employed. The previously reported results (16) on the methoxymercuration-demercuration products from methyl oleate and linoleate were confirmed, but no detailed studies on the reaction of methyl linolenate were reported.

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Lipids of Cultured Hepatoma Cells: III. Triglyceride and Phosphoglyceride Biosynthesis in Minimal Deviation Hepatoma 7288C[1]

REX D. WIEGAND and **RANDALL WOOD**, Division of Gastroenterology, Departments of Medicine and Biochemistry, University of Missouri School of Medicine, Columbia, Missouri 65201

ABSTRACT

Minimal deviation hepatoma 7288C cells were cultured on media containing 25% serum to the confluent stage. The growth media was replaced with serum-free media containing 1-^{14}C-palmitate, and incubations were continued for 0.75, 1.5, 3, 6, 12, and 24 hr. The distribution of radioactivity among the major neutral lipids and phosphoglycerides was determined for cells and culture media. Radioactivity in individual fatty acids of cellular triglyceride, phosphatidylcholine, and phosphatidylethanolamine also was determined. After 24 hr, more than 95% of the administered radioactivity was recovered in neutral and phosphoglycerides, indicating that only a small amount of the fatty acid was oxidized. At any time period examined, over 80% of the incorporated radioactivity was found in triglyceride, phosphatidylcholine, and phosphatidylethanolamine. Incorporation of the label into cellular triglyceride and phosphatidylcholine plateaued at 12 hr, whereas incorporation of radioactivity into phosphatidylethanolamine still was increasing at 24 hr. In contrast, during the entire incubation period the relative distribution of ^{14}C among esterified lipid classes in the culture media remained constant. Elongation of palmitic acid to stearic acid and its subsequent desaturation to oleic acid suggests that these cells possess an active elongation and monoenoic desaturation system. Labeled glycerol ether diesters were not detected in the cells or culture media. Positional distribution of the ^{14}C label in the triglyceride and phosphatidylcholine suggests that minimal deviation hepatoma cells do not exhibit diglyceride selectivity in the biosynthesis of these two lipid classes.

INTRODUCTION

It has been well established that lipids are involved intimately with metabolism and cellular structure and function, especially at the membrane level. Continuing investigations are providing a clearer understanding of how these varied metabolic events are related. Striking differences in lipid metabolism between the normal and neoplastic tissues have been shown to occur (1). Information regarding lipids and their metabolism could be pertinent in any attempt to develop and evaluate methods for prevention and treatment of neoplasia. Mammalian cells grown in culture provide a potentially useful system for studying lipid metabolism under normal and pathological conditions. One important advantage offered by the tissue culture technique is that the lipid composition of the cell can easily be manipulated either qualitatively or quantitatively. Such alterations in lipid character may be of direct or indirect involvement in our overall understanding of neoplasia.

Cultured cells derived from Morris minimal deviation hepatoma (HTC) cells have been widely employed in gaining information on enzyme induction by steroid hormones (2-4). However, to date only limited information is available on the lipid metabolism by cultured HTC (5-9). Therefore, we have initiated studies directed toward a better understanding of the regulation of glyceride synthesis in HTC cells. An earlier report from this laboratory has shown that nutritional variation had little effect upon the neutral lipid and phospholipid class and fatty acid distribution of HTC cells (9). The objective of the present investigation was to assess the ability of HTC cells to incorporate and metabolize exogenous palmitic acid.

EXPERIMENTAL PROCEDURES

Culture Conditions

HTC 7288C cells were cultured in 75 cm^2 flasks as monolayers on Swim's 77 medium supplemented with 20% bovine and 5% fetal calf serums using conventional sterile techniques as described previously (9). After cell densities reached ca. 20 x 10^6 cells/flask, the growth medium was decanted and the attached cells were washed twice with serum-free Swim's 77 media. Fresh serum-free Swim's 77 media (20 ml) and potassium-1-^{14}C-palmitate (2.07

[1]Presented at the AOCS Spring Meeting, New Orleans, April 29, 1973.

TABLE I

Percentage of Administered 1-^{14}C-Palmitate Recovered from Minimal Deviation Hepatoma Cells and Culture Media after Various Incubation Times[a]

Incubation time (hr)	Percentage of radioactivity[b]	
	Cell lipids	Media lipids
0.75	14.8	81.9
1.5	22.6	62.6
3.0	31.5	59.0
6.0	49.5	40.9
12.0	70.1	27.2
24.0	75.9	19.3

[a]Culture conditions, isotope administration, extraction, and quantification of cell and media lipids at the various incubation times are given in the text.

[b]The percentages of radioactivity are based on recoverable ^{14}C.

TABLE II

Percentage of Administered 1-^{14}C-Palmitate Recovered in Media Lipid Classes[a]

Incubation time(hr)	Percent of administered radioactivity recovered[b]	Lipid class[c]		
		PL	FFA	TG
0.75	81.9	1.7	73.4	1.8
1.5	62.6	2.2	53.5	2.8
3.0	59.0	4.1	49.1	4.0
6.0	40.9	7.7	22.9	6.2
12.0	27.2	6.1	11.4	6.2
24.0	19.3	6.2	3.4	5.9

[a]Culture conditions, isotope administraction, extraction, and quantification of media lipids at the various incubation times are given in the text.

[b]The percentages of radioactivity are based on recoverable ^{14}C.

[c]The difference between the sum of the percents of media lipid classes PL (phospholipid), FFA (free fatty acid), and TG (triglyceride) in each row and the percent of administered radioactivity recovered represents the sum of minor lipid classes not given in the table.

μc/flask) dispersed in aqueous 2.7% bovine serum albumin were added to the culture flasks. Incubations were carried out for six time periods: 0.75, 1.5, 3, 6, 12, and 24 hr. At specified times the culture media were collected and the cells harvested by enzymatic release.

Lipid Analysis

Lipids were extracted from the cells using ca. 20 volumes of acidified chloroform-methanol (2:1, v/v). Clarification of the solvent phases was accomplished by centrifugation, and the chloroform layer was removed and washed twice with 0.2 volumes of the Folch theoretical upper phase (10). Lipids were extracted from the culture media by adding 0.5 volumes of acidified chloroform-methanol. The chloroform layer was removed and the aqueous-methanol phase was extracted again. The chloroform extracts were combined and washed twice with 0.2 volumes of Folch theoretical upper phase. The chloroform extracts from the cells and curture media were evaporated to dryness under a stream of nitrogen. The dried lipids were diluted to a specific volume with benzene and aliquots taken for radioactive counting and thin layer chromatographic (TLC) separations. Neutral lipids were separated by TLC on adsorbent layers of Silica Gel G developed in a solvent system of hexane-diethyl ether-acetic acid (80:20:1, by vol). Phospholipid class separations were accomplished by TLC on adsorbent layers of Silica Gel HR developed in a solvent system of chloroform-methanol-acetic acid-0.9% saline (50:25:8:4, by vol). Methyl esters of the fatty acids were prepared by transesterification of the glycerides in methanol with 2% sulfuric acid (11). Gas liquid chromatography (GLC) of the methyl esters was performed on a 6 ft. x 0.25 in. stainless steel column packed with 10% diethylene glycol succinate coated on Gas Chrom Q (100-200 mesh) support in an Aerograph model A90-P chromatograph equipped with a thermal conductivity detector. The column was operated at 190 C with a flow rate of helium carrier gas at 60 ml/min. Radioactive fatty acid methyl esters emerging from the gas chromatograph were trapped by means of a collecting device described previously by Wood and Reiser (12). The fatty acid esters were rinsed from the collection tubes into scintillation vials with diethyl ether. The ether was evaporated to dryness, and 15 ml scintillation mixture (13) was added to the vial and counted in a Packard Tricarb 3375 liquid scintillation spectrometer operating at 65% efficiency for ^{14}C. Collected methyl ester fractions corresponding to methyl palmitoleate and methyl oleate also may have contained other positional isomers. Double bond position and configuration of these acids were not established.

Positional distribution of the radioactive fatty acids in phosphatidylcholine and phosphatidylethanolamine was determined by phospholipase A hydrolysis as described previously (14). Lipolysis of the triglyceride fraction by pancreatic lipase and analysis of the reaction products were determined by the method of Luddy, et al. (15).

Materials

Plastic tissue culture flasks and dishes were purchased from Falcon Plastics, Oxnard, Ca.

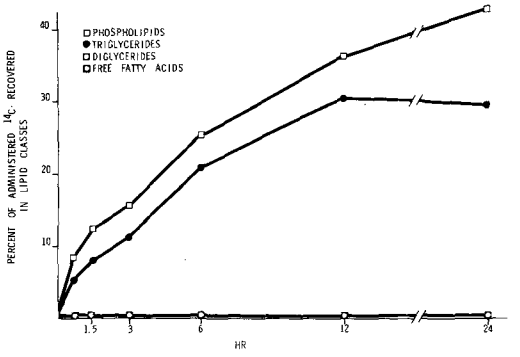

FIG. 1. Percentage of administered radioactivity in minimal deviation hepatoma cellular lipids at various incubation times. Culture conditions, isotope administration, extraction, and quantification of cellular lipids are given in the text. The difference between the sum of the percentages of these four cellular lipid fractions at each time period and 100% represents the sum of the other minor cellular lipid components and the amount of radioactivity recovered from the media.

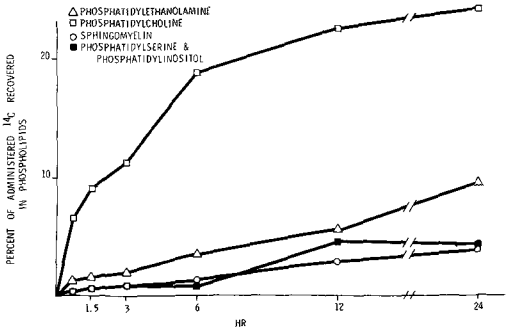

FIG. 2. Percentage of administered radioactivity recovered in minimal deviaiton hepatoma cellular phospholipid at various incubation times. Culture conditions, isotope administration, extraction, and quantification of the phospholipids are given in the text.

Fetal calf serum, bovine serum, and Swim's 77 medium were purchased from Grand Island Biological Co., Grand Island, N.Y. 1-^{14}C-Palmitic acid (specific activity 55.2 mc/mM) was purchased from Nuclear-Chicago, Des Plaines, Ill. Fatty acids and neutral lipid standards were purchased from Nu-Check-Prep, Inc., Elysian, Minn. Phospholipid standards were purchased from Supelco, Inc., Bellefonte, Pa. All solvents were glass distilled and purchased from Burdick and Jackson Laboratories, Muskegon, Mich. Other chemicals were reagent grade or better and were used without further purification.

RESULTS

Recovery of Administered Radioactivity

Table I shows the percentage of the administered ^{14}C radioactivity incorporated into lipids of the HTC cells and that found in the media after the six incubation times. Ca. 50% of the administered radioactivity was incorporated into cellular lipid within 6 hr. The rate of uptake slows with time but does not reach a plateau even after 24 hr. Decreases with time in the media ^{14}C radioactivity were found to accompany the uptake of the fatty acid by the cells. Total radioactivity recovered during the six incubation periods varied from a low of 85% at 1.5 hr to a high of 97% at 12 hr.

Media Lipids

Table II shows that not all of the activity remaining in the media was the administered substrate. As was expected, the levels of media ^{14}C free fatty acid were reduced as the incubation time progressed. At 45 min ca. 3.5% administered radioactivity appeared in media phospholipid and triglyceride. The recovery of radioactively labeled media lipids increased with time and reached peak incorporation at the sixth hr. During the 24 hr culture period, the percentage of administered radioactivity recovered in the media phospholipid and triglyceride remained relatively constant. The distribution of radioactivity on developed neutral lipid chromatoplates did not reveal any radioactivity in the region between triglyceride and cholesterol ester suggesting that glycerol ether diesters were not excreted into the media by these cells under the culture conditions used.

Cellular Lipids

The percentage of administered ^{14}C recovered in the major lipid classes of the cells is shown in Figure 1. The proportion of radioactivity recovered in phospholipid to that of triglycerides remained constant through the twelfth hr. After 12 hr, a reduction in the incorporation rate of the isotope occurred in triglyceride, whereas that of the phospholipid continued to increase. Throughout the 24 hr culture period, the levels of labeled diglyceride and free fatty acids remained low.

Percentages of administered ^{14}C recovered in the cellular phospholipids at the various times are present in Figure 2. Phosphatidylcholine was found to incorporate the largest amount of the radioactivity. A leveling off in the incorporation rate of the isotope into phosphatidylcholine occurred after 6 hr. Throughout the entire period of incubation, phosphatidylethanolamine was incorporating the ^{14}C label, but at a slower rate than that observed for phosphatidylcholine. Minor amounts of radioactivity appeared in the combined fractions of phosphatidylserine and phos-

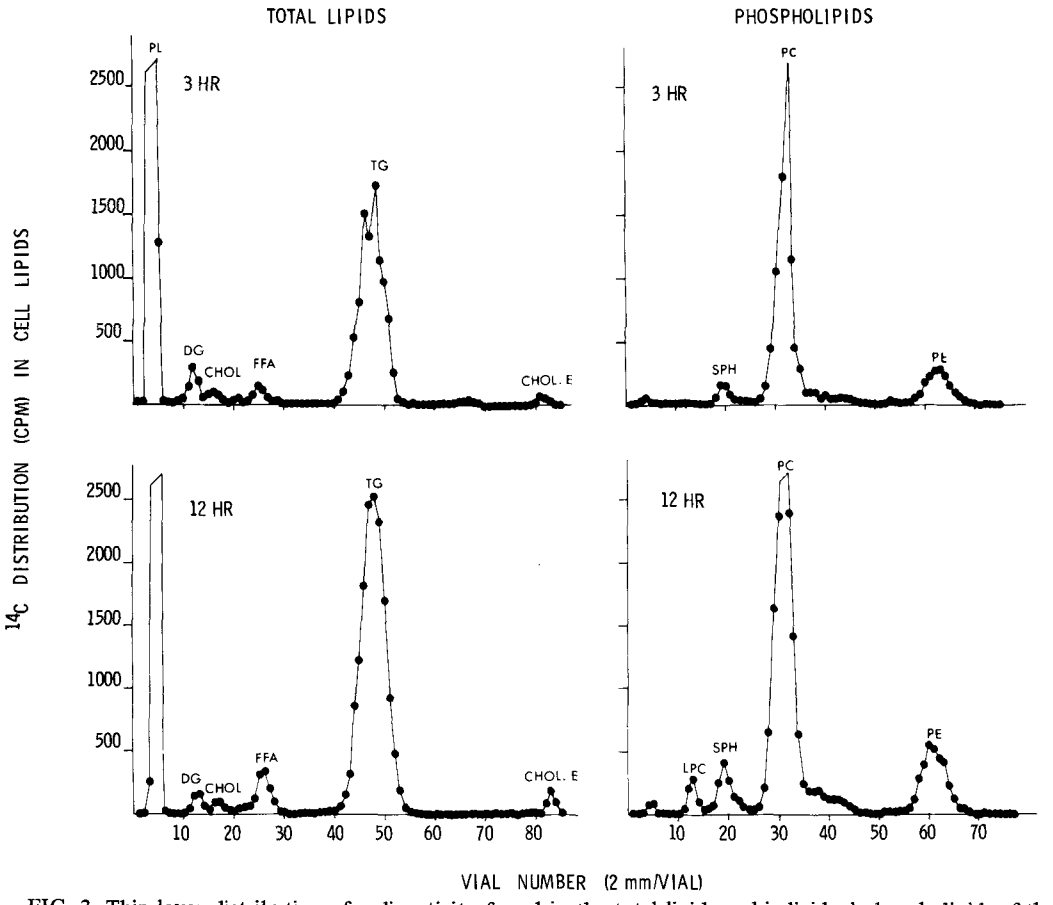

FIG. 3. Thin layer distribution of radioactivity found in the total lipids and individual phospholipids of the cell obtained from the 3 and 12 hr incubation of minimal deviation hepatoma cells with potassium-1-^{14}C-palmitate. Culture conditions, isotope administration, extraction, and purification of the lipids are given in the text. The total lipids were separated on Silica Gel G in a solvent system of hexane-diethylether-acetic acid (80:20:1 by volume). Phospholipids were separated on Silica Gel HR in a solvent system of chloroform-methanol-acetic acid-0.9% aqueous sodium chloride (50:25:8:4 by volume). Distribution of radioactivity was determined by counting successive 2 mm sections of adsorbent removed from a developed thin layer chromatoplate. Abbreviations: PL = phospholipid, DG = diglyceride, CHOL = cholesterol, FFA = free fatty acid, TG = triglyceride, CHOL.E = cholesterol ester, LPC = lysophosphatidylcholine, SPH = sphingomyelin, PC = phosphatidylcholine, and PE = phosphatidylethanolamine.

phatidylinositol, as well as sphingomyelin.

TLC distribution patterns of radioactivity in the cellular neutral lipids and phospholipids at 3 and 12 hr are given in Figure 3. Low levels of labeled free fatty acids and cholesterol esters were observed. Detectable amounts of radioactive label were not observed in the glycerol ether diester fraction, which agrees with mass data (9).

Distribution of Radioactivity in Fatty Acids of Lipid Classes

The percent of administered ^{14}C recovered in the various fatty acids of cellular phosphatidylcholine, triglyceride, and phosphatidylethanolamine is given in Figure 4. The percentage of administered radioactivity remaining as palmitic acid in phosphatidylcholine reached a maximum ca. 12 hr. Similarly, the radioactivity incorporated into stearic and oleic acids increased in a concomitant manner until 12 hr, wherein divergence occurred with an increase in oleic acid. The distribution of radioactivity in the fatty acids of triglyceride shows that during the first 6 hr, two distinct sets of incorporation curves are evident. Similarities in the percentages of palmitic and oleic acids were observed for the initial 6 hr, thereafter palmitic acid leveled off, whereas oleic acid increased for another 6 hr. As shown in Figure 4, increasing amounts of the isotope appeared in the fatty acids with retention times greater than oleic

acid. Ca. 50% radioactivity in this fraction eluted with arachidic and eicosenoic acid. The percent of administered radioactivity recovered in palmitic, stearic, and oleic acids of phosphatidylethanolamine (Fig. 4) was ca. equal up to the sixth hr, at which time stearic and oleic acids continued to rise while the activity in palmitic acid increased only slightly. The percentage of administered radioactivity recovered at 24 hr in fatty acids with retention times greater than oleic acid was 0.7%.

We also investigated the positional distribution of radioactivity in the fatty acids of cellular phosphatidylcholine, triglyceride, and phosphatidylethanolamine isolated from the six incubation periods (Fig. 5). During the initial 3 hr, 50-70% ^{14}C radioactivity in either acyl position of phosphatidylcholine was palmitic acid. Furthermore the ^{14}C activity remaining as palmitic was reduced from both acyl positions at the same rate. Throughout the entier incubation period ca. 20% of the ^{14}C activity in the 1 acyl position was stearic acid whereas stearic acid in the 2 acyl position was insignificant. Only after 6 hr was there a significant increase in oleic acid activity appearing in the 2 acyl position. Through the first 6 hr, a similar distribution of ^{14}C activity in palmitic and oleic acids at the 1 and 3 positions of triglycerides was observed. Thereafter, a divergence occurred wherein palmitic decreased and oleic increased. Palmitic acid was the predominate fatty acid in the 2 acyl position of triglycerides during the initial time periods and was reduced from that position throughout the 24 hr period in a rate similar to that found with palmitic acid in phosphatidylcholine. An increase in oleic acid in the 2 acyl position of triglyceride occurred during the latter incubation periods which was also similar to that observed with phosphatidylcholine. The relative distribution of the ^{14}C radioactivity in the fatty acids of phosphatidylethanolamine did not change significantly during the entire incubation period. Palmitic and oleic acids predominate in the 1 and 2 acyl positions respectively.

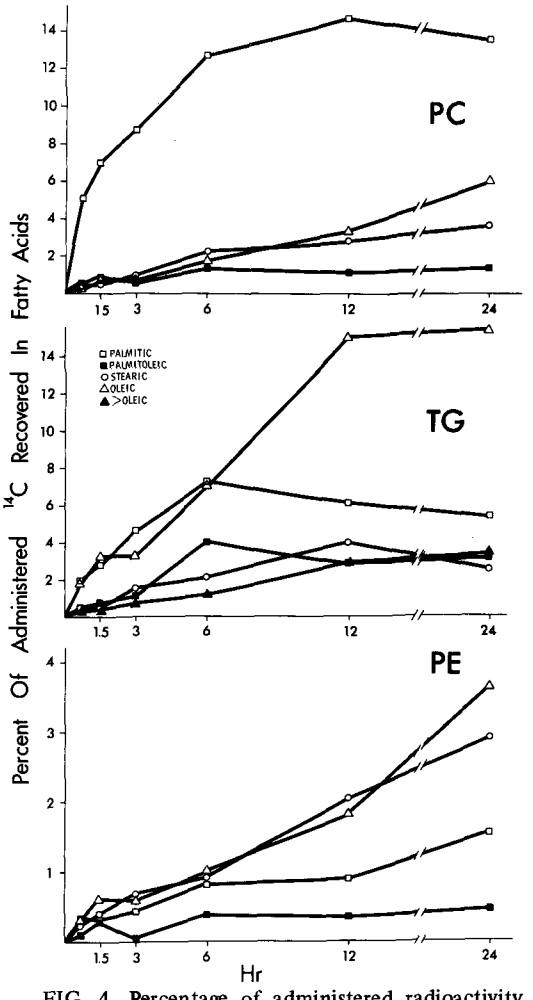

FIG. 4. Percentage of administered radioactivity recovered in the fatty acids of minimal deviation hepatoma cellular phosphatidylcholine (PC), triglyceride (TG), and phosphatidylethanolamine (PE). Culture conditions, isotope administration, extraction, and purification of these glycerides, preparation of methyl esters, and quantification are given in the text. The maximum percentage of administered radioactivity appearing in fatty acids other than those listed in this figure at 24 hr was 1.3% for PC, 0.7% for TG, and less than 0.1% for PE.

DISCUSSION

The techniques of mammalian cell cultivation have been used in the present study to investigate the biosynthetic transformations of palmitic acid and its incorporation into esterified lipids by HTC cells. The distribution of radioactivity in the glycerolipid classes, as well as radiogaschromatographic analysis of the fatty acids derived from individual lipid classes and specific positions of classes, has been employed to study the lipid metabolism in a cultured neoplastic hepatoma cell.

The high recoveries of the administered ^{14}C (Table I) at the various incubation times would suggest that the HTC cells preferentially incorporated media fatty acid into glyceride ester instead of oxidizing the carboxyl labeled carbon to CO_2. Reports from several laboratories have shown that cells cultured in medium containing animal serum incorporate free fatty acids from the serum and subsequently convert them into cellular lipid (16-21). The cellular energy requirements of the HTC cell probably

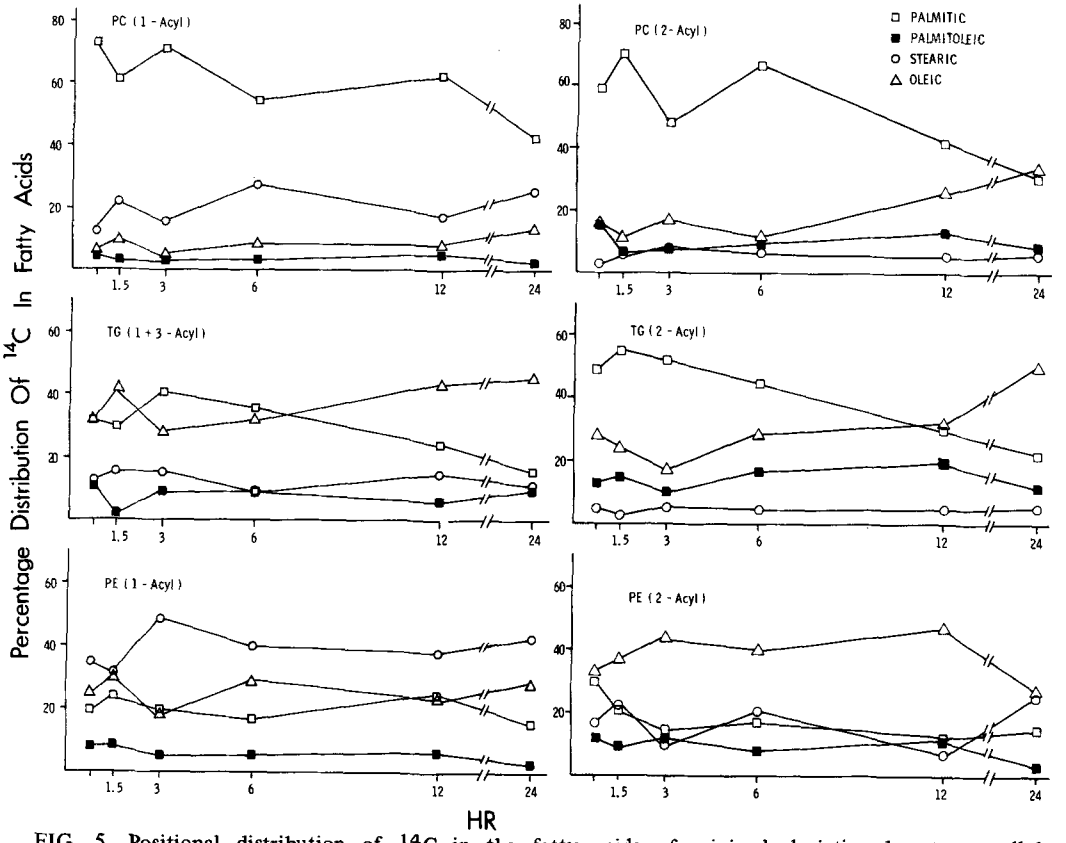

FIG. 5. Positional distribution of ^{14}C in the fatty acids of minimal deviation hepatoma cellular phosphatidylcholine, triglyceride, and phosphatidylethanolamine at various incubation times. Culture conditions, extraction, and purification of the glycerides, enzymatic hydrolysis of acyl positions, preparation of methyl esters, and quantification are given in the text. Triglyceride (TG) (1 + 3-Acyl) was calculated in the manner according to Anderson, et al., (35) by the equation: $1,3 = \frac{(3TG-MG)}{2}$ where TG equals the percentage of the particular fatty acid in the unfractionated triglycerides, and MG equals the percentage of the particular fatty acid in the 2-monoglyceride isolated after pancreatic lipase hydrolysis.

are satisfied by catabolizing glucose from the medium in preference to oxidizing the exogenous tracer fatty acid or endogenous cellular lipid. Our findings are not surprising in light of the results presented by Spector and Steinberg (22), who demonstrated that the addition of glucose to the incubation media reduced the oxidation of exogenous fatty acid in Ehrlich Ascites cells. Furthermore, they found that the presence of glucose increased the incorporation of fatty acid into cellular phospholipid and triglyceride.

The presence of labeled glycerides in the culture media at all time periods (Table II) shows that HTC cells esterify exogenous fatty acids into glycerolipids and subsequently excrete a portion of them back into the culture media, possibly in the form of a lipoprotein complex. Hruban, et al., (23) concluded from histological examination of a series of Morris hepatoma cells that the tumor cells form and excrete lipoproteins. Cell viability, which was greater than 95% in these studies as measured by trypan blue and erythrosin B, suggests that the presence of radioactivity in media lipid did not result from degradation of dead cells. Results from this laboratory have shown that HTC cells cultured on a variety of growth media excrete both neutral lipids and phospholipid into the media (9).

The reduction in the incorporation rate of the isotope into triglyceride between the twelfth and twenty-fourth hr would suggest that intermolecular changes do in fact take place in these cells (Fig. 1). The accumulation of triglyceride allows these cells to cope with and metabolize an excess of unesterified fatty acid. Furthermore, rather than being primarily a source of energy, the triglyceride may furnish material for phospholipid biosynthesis.

Throughout the 24 hr culture period the levels of labeled free fatty acids remained low. Howard and Kritchevsky (24) have shown that the actual level of free fatty acid in cultured cells is generally low and is not readily influenced by varying conditions of culture.

The phospholipid incorporating the largest amount of radioactivity throughout the 24 hr was phosphatidylcholine. Studies using several different cell lines have demonstrated that the phospholipid patterns of cultured cells are similar to those obtained from most intact animal tissue (25-28). The leveling off in the incorporation rate of the isotope into phosphatidylcholine after 6 hr, plus the steady incorporation of the isotope into phosphatidylethanolamine during 24 hr may indicate that these two phospholipids possess different turnover rates.

Radioactivity remaining as palmitic acid in triglycerides and phospholipids leveled off or decreased as the rate of interconversion into other acids by elongation and desaturation increased (Fig. 4). Stoffel and Scheid (20) reported that HeLa cells are able to carry out chain elongation and desaturation reactions in an efficient manner. These investigators showed an asymmetric incorporation of fatty acids into the 1 and 2 positions of phosphatidylcholine and phosphatidylethanolamine. It has been demonstrated that saturated fatty acids occupy the 1 position and unsaturated fatty acids are esterified at the 2 position of normal rat liver phosphatidylcholine and phosphatidylethanolamine (29). These investigators further showed that the 1,2-diglycerides derived from phosphatidylcholine were different from the 1,2-diglycerides derived from triglycerides, thus indicating a selectivity of diglycerides during biosynthesis or retailoring of glycerides later. Our results show that the percentage of the radioactive label incorporated into the 2 position of triglyceride and phosphatidylcholine during the entire incubation is similar but different from phosphatidylethanolamine (Fig. 5). These observations would suggest that either the diglycerides were selected at random during the synthesis of these lipid classes or that these tumor cells have lost the ability to perform the deacylation-acylation reactions which are shown to be highly specific in normal tissue (30). It has been shown that the 1,2-diglycerides derived from triglyceride and phosphatidylcholine of the tumor Ehrlich Ascites cells were similar but different from the diglycerides derived from phosphatidylethanolamine (31). Further studies are required to establish if the apparent loss of diglyceride selectivity or deacylation-acylation reactions in the tumor cell is an important metabolic difference in the metabolism of lipids between normal and neoplastic tissue.

The percentage of radioactive palmitic acid in both the 1,3 and 2 positions of triglyceride (Fig. 5) decreased more than 25% from the earlier time periods to the twenty-fourth hr while that of oleic acid in the 1,3 and 2 positions was increased by ca. the same amount. This decrease in palmitic acid radioactivity and a corresponding increase in oleic acid radioactivity occurred while the radioactivity in stearic acid remained unchanged and the sums of the percentages of palmitic and oleic acid at the 1,3 or 2 position also remained relatively constant. These observations would suggest that palmitic acid is incorporated into oleic acid without causing a change in stearic acid. At this point it is not understood how such a conversion could take place, but it may be important since oleic acid is known to occur at elevated levels in hepatomas. At least three separate laboratories (32-34) in addition to this one, have shown that phosphatidylcholine contained elevated levels of oleic acid relative to host liver and normal liver.

ACKNOWLEDGMENTS

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SYMPOSIUM: MICROBIAL LIPOLYTIC ENZYMES

Presented at the AOCS 64th Spring Meeting, New Orleans

ROBERT G. JENSEN, Chairman

Characteristics of the Lipase from the Mold, *Geotrichum candidum*: A Review[1,2]

ROBERT G. JENSEN, Department of Nutritional Sciences, University of Connecticut, Storrs, Connecticut 06268

ABSTRACT

The mold *Geotrichum candidum* produces an extracellular lipase, readily concentrated by removal of the culture medium in which the microorganism is grown. The lipase is characterized by a unique, but not absolute, specificity for fatty acids containing *cis*-9 or *cis,cis*-9, 12 unsaturation, hydrolyzing both regardless of position within the triglyceride molecule. The enzyme also hydrolyzes *cis*-9-16:1, *cis,trans*-9,12-18:2, *trans,cis*-9,12-18:2, palmitoyl oleate and cholesteryl oleate. Digested at comparatively slow rates are: *trans,trans*-9,12-18:2, double bond positional isomers of 18:1 (other than *cis*-9), stearolic acid, oleoyl-palmitate, dilinoleoyl phosphatidyl choline, and saturated acids. The enzyme has an optimum pH of 8.2, and the lyophilized powder is extremely stable, retaining activity for at least eight years when stored at -20 C. A purification of 81-fold has been achieved.

[1]One of five papers presented in the Symposium "Microbial Lipolytic Enzymes," AOCS Spring Meeting, New Orleans, April 1973.

[2]Scientific contribution 556, Agricultural Experiment Station, University of Connecticut, Storrs, Conn. 06268.

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INTRODUCTION

Geotrichum candidum is a mold with septate mycelia which has been found growing on the surfaces of sour cream and cheese as a firm white mass (1). The microorganism attracted the attention of food microbiologists because of its ability to lipolyze the fat in dairy products.

The first hint of the specificity for fatty acids containing *cis*-9-unsaturation, later found to be a characteristic of the lipase, was obtained by Wilcox, et al. (2). Their cultures readily hydrolyzed olive oil and butterfat but did not release volatile fatty acids from the latter. Being unable in 1955 to identify the free fatty acids (FFA) easily, the authors could not determine

(as we shall see later) that most of the FFA was oleic acid with little release of short chain fatty acids.

Alford and Pierce in 1961 (3), were the first investigators to report on the specificity of *G. candidum* lipase for unsaturated fatty acids in natural oils. In 1964, Alford, et al., (4) confirmed the specificity for oleic acid with synthetic triglycerides (TGs) as substrates.

The 1964 paper of Alford, et al., (4) encouraged us to conduct additional investigations with the enzyme on such questions as: are *trans* isomers digested, is there discrimination between 18:1 and 18:2, what are the conditions required for optimum activity, are unsaturated acids shorter or longer than 18:1 digested, are positional isomers of 18:1 hydrolyzed, is stearolic (octadecynoic) acid released, does location of 18:1 within the TG molecule affect rate of release, can the lipase be used for structural analysis of TGs, etc.? Many of these questions have been answered, and I shall present the results herein.

ISOLATION

The lipase is an extracellular enzyme obtained by concentration of the culture medium in which the microorganism is grown. In the first investigation (3), the microorganism was grown at 20 C in peptone broth buffered at pH 7.0 with phosphate (0.05 M). The cells and mycelia were removed from the medium by centrifugation or filtration and the supernate or filtrate used as a source of enzyme.

In a later study (4) the filtrate was dialyzed against distilled water, concentrated to ca. 5-10% by dialysis against polyethylene glycol and lyophilized to a dry powder. This preparation is stable at -20 C, retaining activity to our knowledge for at least eight years.

Improved production of the enzyme was reported in 1965 (5). Of the media tested, the best yields were obtained with a glucose-salts basal medium plus either 4% Edamin S (Sheffield) or trypticase (Baltimore Biologicals) as a source of nitrogen. The activity was ca. twice that noted with the peptone medium used earlier.

Until recently the enzyme had not been purified beyond these initial concentrations from the culture media, then Kroll, et al., (6) reported an 81-fold purification (460 specific activity) over the culture medium filtrate using an acetone powder and chromatography on Sephadex G-25 and G-200. The final preparation produced a single band by polyacrylamide gel electrophoresis and had an estimated mol wt of 32,000 ± 10%. Interestingly, the acetone powder and the high activity fraction from the Sephadex released ca. identical quantities of 16:0, 16:1, 18:1, and 18:2 from olive oil. Apparently, the same lipase hydrolyzed both saturated and unsaturated acids as purificiation did not alter the patterns.

To date there has not been a commercial source of the enzyme, but Worthington Biochemicals, Freehold, N.J., has produced the enzyme and should soon have it ready for release.

FACTORS AFFECTING ACTIVITY

Alford, et al., (4) observed optimal activity of *G. candidum* lipase at 20 C and pH 7.0. Calcium ions were added to the assay medium because lipolytic activity was increased by the presence of Ca^{++}. Later Alford and Smith (5) described a medium which employed phosphate buffer for obtaining lipase preparations with greater activity. We made a systematic study of factors affecting lipolytic activity of the enzyme during assay (7). Tris buffer promoted greater activity than phosphate buffer, with a broad pH optima of 8-9. $CaCl_2$ did not increase the rate of lipolysis nor did variations in temperature of incubation between 15-40 C. We now routinely use 37 C and pH 8.1-8.2 (Tris) for assays and add $CaCl_2$. Kroll, et al., (6) also observed, in general, the same pH and temperature optima but found that Ca ions activated the enzyme. These conflicting reports might be attributed to the use of gum arabic as an emulsion stabilizer by both groups. Krysan and Guss (8) reported that the gum arabic they used contained large amounts of Ca. It is possible that the gum arabic we employed contained Ca while the supply of Kroll, et al., (6) did not.

Alford, et al., (4) found that diisopropylfluorophosphate (DFP) (10^{-4} M) reduced the activity of *G. candidum* lipase 25-35%. Carpenter and Jensen (9) observed similar reductions when the enzyme was exposed to diethyl-p-nitrophenylphosphate and Parathion. We have noted that the products produced by the oxidation of polyunsaturated acids also inhibit the enzyme. Smith and Alford (10) reported that activity was inhibited by Na oleate. Partial inhibition by DFP (10^{-3} M) was confirmed by Kroll, et al., (6) while Fe^{++}, Hg^{++}, and Cu^{++}, all at 10^{-2} M, caused complete inhibition.

The lipase may be a histidine-serine enzyme because of the pH range of optimum activity 8-9 and because activity was inhibited by the serine hydroxyl binding compounds diisopropylfluorophosphate, Parathion, and diethyl-p-nitrophenyl phosphate. Beyond these fragmen-

TABLE I

Fatty Acid Composition (M%) of Glycerides and Free Fatty Acids Resulting from Lipolysis of Glycerol-1-elaidate-2,3-dioleate and Margarine by *Geotrichum candidum* Lipase[a]

Substrate and fatty acid composition	Intact TG[b]	Residual TG	FFA	DG	MG
Glycerol-1-elaidate-2,3-dioleate					
18:1 *cis*	70	73.5	94.5	66.0	30.0
18:1 *trans*	30	26.5	5.5	34.0	70.0
Margarine					
12:0	tr	tr	tr	tr	5.1
14:0	tr	tr	tr	tr	2.3
16:0	14.0	16.1	7.2	25.2	33.7
18:0	7.2	10.5	3.5	7.4	12.6
18:1	61.2	66.9	74.3	56.8	41.7
18:2	17.6	6.5	15.0	10.6	4.9
trans as elaidic	26.4	32.2	5.5	25.5	16.8

[a]Jensen, et al. (11).

[b]TG = triglyceride, FFA = free fatty acid, DG = diglyceride, DG = diglyceride, MG = monoglyceride, and tr = trace.

tary data, no information is available upon the active site of the enzyme, except that the specificity of the enzyme suggests a rate limiting binding site for double bonds at a relatively long distance from the active site. Thus, the acyl chains of the substrate apparently must be oriented specifically for hydrolysis to occur.

SPECIFICITY

Alford and Pierce (3) reported that the enzyme hydrolyzed mainly unsaturated acids from several fats. For example, the FFA from the hydrolysis of lard contained 73% 18:1 and 19% 18:2, while the original fat had 47% and 8% each of the two acids. Thus, the specificity of the enzyme for unsaturated fatty acids was shown, as well as a lack of differentiation between 18:1 and 18:2.

Stimulated by their observation of the unique specificity of the enzyme for 18:1 and 18:2, Alford, et al., (4) digested several synthetic TGs with the lipase. The results confirmed that the lipase had a specificity for, in this investigation, 18:1, regardless of position within the TG.

The paper of Alford, et al., (4) encouraged us to further investigate the specificity of the enzyme with the goal of using the lipase for structural analysis of natural TGs.

Our first effort compared lipolysis of oleic (*cis*) and elaidic (*trans*) acids (11). With glycerol-1-elaidate-2, 3-dioleate and margarine as the substrates, we obtained the results in Table I. Discrimination between the *cis*- and *trans*-isomers is indicated clearly by the high *cis*-18:1 content of FFA. The margarine, a partially hardened cottonseed and soybean oil product, yielded similar results. With both substrates the *trans*-isomer content was measured by IR spectrophotometry; therefore, we did not determine if *trans*-isomers other than elaidate were released from the margarine. In addition, cod liver, Macadamia nut, peanut, safflower, and palm oils were tested as substrates. Again 18:1 was preferentially hydrolyzed from all oils. Kroll, et al., (6) and Franzke (12) have presented confirmation with some of these and several additional oils. Palmitoleic acid (16:1) was present in the FFA in excess of its content in the original oils, indicating that removal of two terminal carbons did not affect specificity.

At ca. the same time, we (13) digested milk fat and a mixture of glycerol-1-oleate-2,3-dicaproate and glycerol-1-palmitate-2,3-dibutyrate with the enzyme. The FFA from the milk fat contained 61.8 M% oleic acid with much smaller quantities of the whole range of fatty acids found in milk fat. Conversely the monoglycerides (MGs) had more than 90 M% saturated acids, with 8.3 M% 18:1. Some of this was the *trans*-isomer. However, we were unaware at the time that the enzyme discriminated against positional isomers of 18:1, other than *cis*-9; hence, some of these were probably also present. The oleic acid content of the FFA from the mixed synthetic TGs was 81.6 M%. There was no preferential release of either 4:0 or 6:0.

Encouraged by the results from the two prior studies, we initiated what has become a continuing investigation on the specificity of the enzyme. To confirm the lack of positional specificity noted by Alford, et al., (4) we synthesized and digested glycerol-1-oleate-2,3-

TABLE II

Oleic Acid (M%) Found after Lipolysis of Oleate-Saturated Glycerides by *Geotrichum candidum* Lipase

Substrate	Residual TGs[b]	FFA	DGs	MGs
18:1–16:0–16:0[a]	32.6	89.7	1.2	tr
16:0–18:1–18:1	64.4	96.1	48.0	tr
16:0–18:1–18:1	31.4	94.3	tr	tr
18:1–16:0–18:1	66.1	97.7	47.7	tr
18:1–18:0–18:0	26.3	82.7	3.2	tr
18:1– 6:0– 6:0	31.6	99.0	0.0	0.0
18:1– 8:0– 8:0	38.1	89.1	4.4	tr
18:1–10:0–10:0	31.3	89.8	2.4	tr
18:1–12:0–12:0	31.0	86.0	2.0	tr

[a]18:1–16:0–16:0 is racemic glycerol-1-oleate-2,3-dipalmitate, Marks, et al. (7).
[b]See Table I for definitions of abbreviations.

dipalmitate, glycerol-1-palmitate-2,3-dioleate, glycerol-2-oleate-1,3-dipalmitate, and glycerol-2-palmitate-1,3-dioleate (7). The data at the top of Table II confirm that the position of oleate within the TGs did not affect the amount of 18:1 in the FFA. The rates of digestion of dioleate substrates were ca. twice that of the monooleate compounds. Therefore, the extent of hydrolysis was related to the quantity of oleate present; and, at least in this respect, the lipase was suitable for structural analysis of glycerides. There was no particular tendency toward increased rates of lipolysis of short chain, as compared to long-chain, fatty acids. Also worth noting is the oleate content, ca. 50 M%, of the diglycerides (DGs) from the two dioleoyl substrates. The accumulated DGs were of the 16:0-18:1 type. Apparently, these substrates were not as attractive to the enzyme as the original TGs. We did some work on the effects upon specificity of altering the geometry of the substrate with resulting data in Table III. When we used mixed triolein and trilinolein as substrates, the lipase did not discriminate between 18:1 and 18:2, as all the digestion products contained the same amount of oleate as the control. We attempted to check this with TGs, such as glycerol-1-oleate-2,3-dilinoleate, but had difficulties with lack of activity of the enzyme. We did not know, at the time, that oxidation products, probably from 18:2, were inhibiting the enzyme. The enzyme did differentiate between double bond positional isomers of 18:1 and *cis*-9-16:1 preferentially hydrolyzing *cis*-9-16:1 as compared to pestroselinate (*cis*-6) or vaccenate (*cis*-11) (Table III). The other positional isomers of 18:1 were not available at the time, nor did the enzyme distinguish between oleate and palmitoleate (*cis*-9-16:1) (Table III). Chain shortening to the extent of one methylene and one methyl group beyond C-16 did not alter the specificity. Further research with myristoleic acid (*cis*-9-14:1), etc., should be done.

Our research on the specificity of the lipase has continued with the following observations: (A) stearolic acid (octadecynoic acid) from glycerol-1-stearolate-2,3-dioleate, dilinoleoyl phosphatidylcholine, and oleoylpalmitate were not hydrolyzed and (B) palmitoyloleate and

TABLE III

Oleic or Palmitoleic[a] Acids Found after Lipolysis of Synthetic Triglycerides by *Geotrichum candidum* Lipase

Substrate	Control TG[b]	Residual TG	FFA	DGs	MGs
Triolein-trilinolein	58.5	58.4	62.2	56.0	55.0
Glycerol-1-palmitoleate-2,3-dioleate	66.6	67.4	65.2	71.7	68.9[c]
Glycerol-1-*cis*-vaccenate-2,3-dioleate	74.6	61.6	92.8	37.6	4.4[c]
Glycerol-1-palmitoleate[a] -2,3-dipetroselinate	33.6	32.4	94.6	0.9	1.4

[a]Values for glycerol-1-palmitoleate-2,3-dipetroselinate are palmitoleic; all others are oleic.
[b]See Table I for definitions of abbreviations.
[c]Little MG was formed, Marks, et al. (7).

TABLE IV

Fatty Acid Composition of Products from the Hydrolysis of Two Triglycerides Containing Geometric Isomers of Linoleate by *Geotrichum candidum* Lipase

	9,12–18:2 isomer, wt %			
	TG 1[b]		TG 2	
Product	*cis,cis*	*trans,trans*	*cis,trans*	*trans,cis*
Original TG	47.6	52.4	46.3	53.7
Residual TG	35.2	64.8	48.5	51.5
FFA	84.5	15.5	44.8	55.2
DGs	29.2	70.8	49.5	51.5
MGs	tr[a]	90+	43.5	56.5

[a]Peaks were small, but only a trace of *cis,cis* isomer was observed. Jensen et al. (14).
[b]See Table I for definitions of abbreviations.

cholesterol oleate were digested (R.G. Jensen, R.E. Pitas, and D.T. Gordon, unpublished results). Apparently nonglycerol oleate esters can be hydrolyzed with the exception of the phosphatidylcholine. The charged phosphoryl and nitrogenous moieties in the latter may be responsible.

In another study (14) we observed the hydrolysis of 9,12-*cis*, *trans*-18:2, and 9,12-*trans,cis*-18:2 from synthetic TGs, while 9,12-*trans,trans* was poorly digested (Table IV). We found the hydrolysis of the two *cis,trans*-isomers most astonishing, as both elaidate (*trans*-18:1) and linolelaidate (*trans,trans*-18:2) are digested at comparatively slow rates. At the moment we have no explanation for this puzzling phenomenon, assuming that the 18:2 isomers were pure but offer the following suggestions. In the 18:2 isomers that were hydrolyzed, *cis,cis*-9,12-18:2; *cis,trans*-9,12-18:2, and *trans,cis*-9,12-18:2, there were at least three hydrogens on one side of the pentadiene system and one hydrogen on the other. The *cis,cis*-isomer had four hydrogens on one side. The poorly digested *trans,trans*-9,12-18:2 isomer had two hydrogens on one side of the double bonds and two on the other. Apparently the rate limiting site which binds double bonds requires at least three hydrogens on one side of the two double bonds and a *cis*-kink. Another possibility is that mentioned by Okuyama, et al., (15) in their study of positional isomeric discrimination of 18:1s by rat liver acyltransferase, binding to specific π bond configurations. This also could explain the overall specificity of *G. candidum* lipase for *cis*-9-unsaturation, although Okuyama, et al., used *trans*-isomers. Nevertheless, the concept is still valid.

We further delineated the specificity of the enzyme for *cis*-9-unsaturation (16). We were provided with TGs containing 18:2, 12:0, 14:0, 16:0, and one of each of the positional isomers of 18:1. These were digested as usual, and the fatty acid compositions of all the involved glycerides and FFA are presented in Tables V-VII. The data reveal that the enzyme preferentially hydrolyzed the *cis*-9-isomer. Comparatively small quantities of the 18:1 isomers other than *cis*-9-18:1 were digested (Table V). Relatively large amounts of 18:2 were released from all substrates except the one containing 9-18:1. In this case there was no preference between 9-18:1 and 18:2. The accumulation of positional isomers, other than 9-18:1, in the DGs and MGs (Table VI) suggests that the lipase could be used to help isolate the positional isomers for further identification. The trend is also evident in the relatively small accumulations of the 18:1 isomers, other than 9-18:1, in the residual TGs as compared to the initial intact substrate (Table VII). Worth noting are the similar ratios of *cis*-9-18:1 and 18:2, before

TABLE V

Composition of the Fatty Acids (M%) Released by Hydrolysis with *Geotrichum* Lipase from Triglycerides Containing Isomeric *cis*-18:1 Acids[a]

	Fatty acids				
18:1 isomer	12:0	14:0	16:0	18:1	18:2
Δ2	16.5	10.3	14.6	0	58.6
Δ3	9.0	8.6	13.9	3.1	64.8
Δ4	7.8	5.4	7.3	0	79.5
Δ5	8.3	5.0	8.2	2.7	75.8
Δ6	9.4	6.8	14.4	2.0	67.4
Δ7	10.2	5.5	6.8	6.6	70.9
Δ8	8.0	3.7	9.2	4.3	74.8
Δ9	3.5	9.1	17.5	50.6	15.3
Δ10	7.9	4.6	6.6	6.9	74.0
Δ11	7.7	6.7	7.8	4.3	73.5
Δ12	22.8	11.3	15.4	6.7	43.8
Δ13	11.7	8.2	17.6	6.1	56.4
Δ14	17.2	8.6	12.4	5.1	56.7
Δ15	6.5	4.2	10.3	3.9	75.1
Δ16	10.1	8.3	12.0	1.9	67.5

[a]Jensen, et al. (16).

TABLE VI

Fatty Acid M% Composition of Residual Monoglycerides and Diglycerides after Hydrolysis by *Geotrichum* Lipase of Triglycerides Containing Isomeric *cis*-18:1 Acids[a]

	12:0		14:0		16:0		18:1		18:2	
18:1 isomer	M[b]	D	M	D	M	D	M	D	M	D
Δ2	24.7	31.0	33.9	30.3	20.5	28.4	10.0	2.8	3.8	4.4
Δ3	34.4	15.1	41.0	30.8	16.3	33.7	8.3	7.5	0	6.0
Δ4	17.9	20.5	26.6	26.2	17.1	22.3	33.4	27.0	3.3	4.0
Δ5	24.2	24.4	26.9	25.2	16.5	21.6	28.5	22.6	3.9	6.2
Δ6	22.0	27.6	30.4	28.2	12.8	23.1	34.8	18.3	0	2.8
Δ7	28.6	27.0	34.2	24.4	18.4	21.6	18.8	23.1	0	3.9
Δ8	0	18.4	20.9	26.3	22.5	2.42	54.9	27.0	1.7	4.1
Δ9	2.4	25.2	33.7	33.7	33.7	57.4	0	4.7	0	1.7
Δ10	23.9	24.8	31.7	23.2	19.3	22.1	22.4	22.5	2.7	7.4
Δ11	26.5	19.9	31.6	26.6	18.0	22.9	20.7	26.5	3.2	4.1
Δ12	14.3	24.4	20.5	27.0	8.0	23.3	49.0	23.8	6.6	1.5
Δ13	32.4	30.5	33.5	25.6	15.7	22.0	18.4	19.1	0	2.8
Δ14	30.6	24.9	39.0	27.8	11.0	21.4	19.4	22.9	0	3.0
Δ15	7.6	20.3	28.4	24.9	23.1	23.4	40.9	27.9	0	3.5
Δ16	0	31.1	0	28.9	0	27.7	0	8.0	0	4.3

[a]Jensen, et al. (16).

[b]M = monoglycerides and D = diglycerides.

and after lipolysis, further evidence that there was no differentiation between the two acids.

Our original reason for investigating *G. candidum* lipase was to determine whether or not it might prove useful for structural analysis of glycerides. The impetus to test our supposition came about in a serendipitious fashion. Brockerhoff (17) discussed the problem of resolving a mixture of triacid TGs and concluded that with the techniques available, resolution into individual isomers was impossible. In a mixture containing palmitic, oleic, and stearic acids, for example, use of all preparative techniques would still leave the possibility of the presence of six isomers: sn-glycerol-1-palmitate-2-oleate-3-stearate (sn-POS), sn glycerol-1-stearate-2-oleate-3-palmitate (sn-SOP), sn-glycerol-1-oleate-2-palmitate-3-stearate (sn-OPS), sn-glycerol-1-stearate-2-palmitate-3-oleate (sn-SPO), sn-glycerol-1-palmitate-2-stearate-3-oleate (sn-OSP), and sn-glycerol-1-oleate-2-stearate-3-palmitate (sn-SPO). Data from the stereospecific analysis of Brockerhoff (18) would give the amounts of each acid in each sn position but could not assign the quantities to individual enantiomers. We were discussing the

TABLE VII

Fatty Acid (M%) Composition of Triglycerides before and after Hydrolysis with *Geotrichum* Lipase

	12:0		14:0		16:0		18:1		18:2	
18:1 isomer	B[b]	A	B	A	B	A	B	A	B	A
Δ2	21.7	29.5	27.9	29.1	27.7	28.4	3.6	3.5	16.9	7.0
Δ3	22.8	25.6	25.3	27.3	27.8	26.1	9.2	11.4	14.2	7.8
Δ4	19.1	22.6	20.4	21.2	17.9	21.0	23.4	26.7	19.2	8.5
Δ5	12.8	23.5	21.8	21.9	23.0	19.5	23.9	22.3	18.5	12.8
Δ6	19.6	23.8	21.3	24.5	19.9	22.4	22.2	21.1	17.0	8.2
Δ7	12.6	25.3	21.4	24.1	20.6	21.1	22.5	20.4	22.6	9.1
Δ8	16.2	23.4	20.9	24.3	18.7	18.9	22.8	21.3	21.3	12.1
Δ9	20.7	21.8	20.9	28.4	29.8	31.2	22.1	14.1	6.5	4.5
Δ10	14.3	26.1	18.7	23.9	20.4	20.3	26.4	22.8	20.2	7.0
Δ11	20.7	22.2	24.9	32.3	19.8	19.0	22.5	19.8	12.1	6.7
Δ12	22.1	26.6	24.3	24.5	21.6	23.2	22.6	21.2	9.4	4.5
Δ13	21.7	22.0	23.4	24.8	21.6	25.1	21.9	21.2	11.4	6.9
Δ14	20.8	25.2	22.9	25.2	20.8	22.8	18.9	18.9	16.6	7.9
Δ15	17.6	21.9	21.5	22.0	18.3	22.3	25.1	27.1	17.5	6.7
Δ16	22.8	23.7	22.7	24.4	22.1	21.7	14.9	23.7	17.3	6.5

[a]Jensen, et al. (16).

[b]B = before and A = after.

TABLE VIII

Comparison of Triacid Triglyceride Isomers Observed to Those Actually Present in a Mixture Subjected to Enzymatic Analysis[a,b,c]

Isomer	Calculation	Actual observed mole %		Standard deviation
sn-POS	(A) x (B)	12.5	12.39	1.44
sn-SOP	(A) x (C)	12.5	13.51	1.44
sn-PSO	(D) x (E)	37.5	35.62	2.53
sn-SPO	(D) x (F)	12.5	12.58	2.53
sn-OPS	(G) – % sn-SPO	12.5	12.82	2.53
sn-OSP	(H) – % sn-PSO	12.5	13.08	2.53

[a]Analyzed as described in text.

[b]Values were derived from the results shown in Table IX as discussed in the text: A = % 18:1 in 2 position, B = % 18:0 in β-lysophosphatide, C = % 16:0 in β-lysophosphatide, D = % 18:1 in 3 position, E = % 16:0 in α-lysophosphatide, F = % 18:0 in α-lysophosphatide, G = % 16:0 in 2 position, and H = % 18:0 in 2 position.

[c]Sampugna and Jensen (20).

problem, had drawn the six possible isomers on a blackboard, and agreed that the problem could not be solved. Upon being challenged, a graduate student solved the problem on the spot by proposing first a digestion with *G. candidum* lipase to form sets of DGs which subsequently could be resolved as depicted in Figure 1. After our initial embarrassment, we lost little time in publishing our hypothesis (19). To explain Figure 1, after digestion to obtain sets of diacylglycerols, the 1,2 + 2,3- and 1,3 isomers are separated by boric acid thin layer chromatography (TLC), converted to phosphatidyl phenols, and digested by phospholipase A_2. This enzyme, which hydrolyzes the sn-2-acid from the sn-3-substrate and the sn-1-acid from the sn-2-substrate, leaves monoacylphosphatides and untouched sn-1-phenylphosphatide, which are separable by TLC. Thus, on paper, the six isomers could be resolved. To test our hypothesis rac-PPO, rac-POS, rac-PSO, rac-SPO, sn-OSP, sn-PSO, and sn-OOP were synthesized and hydrolyzed separately and in a mixture (20). The subsequent steps are those illustrated in Figure 1, with pancreatic lipolysis to confirm the identity of the 2 position acid. The data from lipolysis of the mixture listed in Table VIII supported our hypothesis that *G. candidum* lipase could be employed to resolve enantiomeric TGs containing oleate. Encouraged by these results, we tested our technique with the monounsaturated fraction from cocoa butter (21). Cocoa butter was selected because it consists largely of oleate, stearate, and palmitate in racemic glycerol-1-palmitate-2-oleate-3-stearate (22). The monounsaturated fraction (ca. 75%) was isolated from cocoa butter by $AgNo_3$-TLC with ca. 100 plates required to obtain enough material for analysis. This material was analyzed as shown in Figure 1 and discussed above with an additional step. Since two saturated acids, palmitate and stearate, were present in the fraction, it was necessary to determine the carbon number distribution of the DGs derived from lipolysis by *G. candidum*. The DGs were converted to acetates and analyzed by gas liquid chromatography. We were thus able to determine for the first time the individual TG isomers in a fraction from a natural fat. The composition, listed in Table IX, confirms the observation of Schlenk (22) that a major TG of cocoa butter is rac-POS. Our procedure should be applicable to any fat containing 16:1, 18:1,

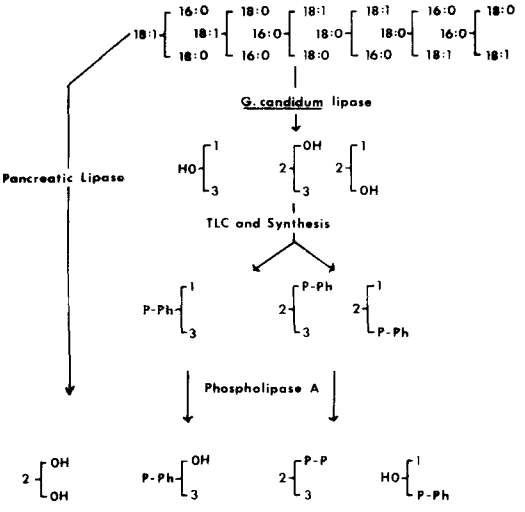

FIG. 1. Stereospecific analysis of a triacid triglyceride mixture. 16:0, 18:1, and 18:0 represent palmitic, oleic, and stearic acids esterified to glycerol. P-Ph represents phosphoryl phenol. The numbers 1, 2, and 3 represent fatty acids esterified to the glycerol molecule at those positions (20).

TABLE IX

Estimated Isomer Proportions in the Monounsaturated Triglyceride Fraction of Cocoa Butter[a]

Glyceride	Mole % Based upon diglyceride acetates[b]	Based upon phosphatides[b]	Calculated	Total glycerides
POP	17.5	41.0[c]	17.5	12.1
sn-SOP	47.5		23.5	16.3
sn-POS		59.0[c]	24.0	16.5
SOS	35.0		35.0	24.2
sn-OPP	21.3	0.0[d]	0.0	0.0
sn-PPO		27.3[e]	21.3	1.1
sn-PSO	48.1		6.0	0.3
sn-OSP		0.0[d]	0.0	0.0
sn-OPS		0.0[d]	0.0	0.0
sn-SPO		59.1[e]	42.1	2.1
sn-SSO	30.6		17.0	0.8
sn-OSS		13.6[f]	13.6	0.7

[a]Sampugna and Jensen (21).

[b]The first four glycerides are calculated as a percentage of the β-unsaturated α,α-disaturated fraction while the rest of the glycerides are pereentages of the α-unsaturated-α,β-disaturated triglycerides.

[c]Based upon the percentage of palmitate and stearate in the 3-mono-acyl-2-*sn*-phosphatides.

[d]Based upon the absence of palmitate in the 2,3-diacyl-1-*sn*-phosphatides.

[e]Based upon the amounts of palmitate and stearate found in the 1-mono-acyl-3-*sn*-phosphatides and the pcrcent hydrolysis of α phosphatides.

[f]Based upon the amount of stearate found in the 2,3-diacyl-1-*sn*-phosphatides and the percent hydrolysis of the α phosphatides.

TABLE X

Specificity of the Lipase from *Geotrichum candidum*[a]

Compounds hydrolyzed	Compounds hydrolyzed slowly[b]
cis-9-18:1[c]	*trans*-9-18:1
cis-9-16:1	*trans,trans*-9,12-18:2
cis,cis-9,12-18:2	Positional isomers of *cis* 18:1 other than Δ-9
cis,trans-9,12-18:2	Octadecynoic acid
trans,cis-9,12-18:2	Erucic acid
Palmitoyloleate	Oleoylpalmitate
Cholesteryl oleate	Dilinoleoyl phosphatidyl[d] choline

[a]Substrates were triglycerides with obvious exceptions.

[b]Relative to *cis*-9-18:1 or *cis,cis*-9,12-18:2.

[c]Hydrolyzed regardless of location at positions *sn* 1, 2, or 3.

[d]Not hydrolyzed.

or 18:2 with suitable prior fractionation. Also, since the enzyme largely ignores most *trans*-isomers and positional isomers other than *cis*-9-, it could be useful for the structural analysis of partially hydrogenated food fats. The data we obtained in our study on the positional isomers of 18:1 (Table VI) indicates that the odd isomers accumulated in the MG and DG fractions could be utilized for further characterization of the acids.

Since the enzyme hydrolyzes *cis*-9-18:1 or *cis,cis*-9, 12-18:2 from either the 1 or 2 position in a TG, the course of lipolysis depends upon the location of these acids in the substrate. Therefore, either sn-1,2-, sn-2,3- or 1,3-DGs, and sn-1- 2- or sn-3-MGs can be produced by lipolysis. As mentioned, hydrolysis of mono 18:1 TGs will produce DGs suitable for stereospecific analysis. Indeed, Gurr, et al., (23) utilized the enzyme to help determine the structure of *Crambe abyssinica* TGs. When

these TGs were hydrolyzed 1,3-DGs resulted indicating that the 2 positions largely were occupied by oleate; the FFA contained 75 M% 18:1. Erucic acid (13-22:1) was not hydrolyzed.

With a substrate containing both saturated and unsaturated fatty acids, small quantities of the former are hydrolyzed by the lipase, the amount increasing with the length of the digestion. We recently digested glycerol-1 palmitate-2,3-dioleate with the enzyme and analyzed the FFA after 5 and 15 min of incubation. At 5 min, the FFA contained 18.2 M% 16:0 and 81.9 M% 18:1, after 15 min, 24.6 M% 16:0 and 75.4 M% 18:1.

CONCLUSIONS

The mold, *G. candidum*, secretes an extracellular lipase that hydrolyzes mostly fatty acids containing *cis*-9 and *cis,cis*-9,12-18:2 from natural and synthetic glycerides regardless of sn location; the enzyme is not stereospecific. The specificity of the enzyme is summarized in Table X.

The enzyme has been purified (81-fold, 460 specific activity) with the final preparation showing one band by polyacrylamide gel electrophoresis. The pattern of acids released from olive oil by both an acetone powder and the purified lipase were almost identical. The tendency to hydrolyze small quantities of saturated acids was not removed by purification.

The lipase is probably a histidine-serine enzyme with a rate controlling binding site for *cis*-9- double bonds some distance away from the active site. Specific π bond configuration may account for the specificity of the enzyme as suggested by Okuyama, et al., (13) in his study of acyltransferases.

The enzyme has been used to determine the strucutre of both synthetic and natural glycerides containing oleate and can, in conjunction with a subsequent stereospecific analysis, resolve enantiomeric TGs. The TG species in the monounsaturated fraction of cocoa butter were identified in this manner.

Since the specificity of the enzyme results in the accumulation of 18:1 isomers, other than *cis*-9-, in the DGs and MGs resulting from a digestion, these fractions could be used to isolate and help identify these isomers from a partially hydrogenated food fat. Conversely, the enzyme releases an essential fatty acid, *cis,cis*-9,12-18:2, from TGs containing the acid; and this could prove useful.

The *G. candidum* enzyme is the only known example of a lipase with a definite specificity for a few fatty acids. The structure of the binding site for double bonds and its relation to the active site present provocative challenges to enzymologists.

It should be noted that Tsujisaka, et al., (24) also recently purified the lipase from *G. candidum* about 44-fold obtaining crystals that were homogeneous by electrophoresis. The crystals contained ca. 7% carbohydrate and some lipid with an estimated mol wt for the enzyme of 53,000-55,000. An amino acid analysis revealed that the enzyme did not contain cysteine and methionine and accordingly could not have S-S bridges. Serine and histidine were present, as well as relatively large quantities of aspartic and glutamic acids. The pH optima was 5.6-7.0.

ACKNOWLEDGMENTS

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Staphylococcal Lipases[1]

D.V. VADEHRA,[2] Food Science and Microbiology, Cornell University, Ithaca, New York 14850

ABSTRACT

A lipase rich fraction was isolated from the cell free supernatant of 24 hr broth culture of *Staphylococcus aureus* B-120, grown in trypticase soy broth at 37 C. Lipase from the cell free supernatant was precipitated with equal volumes of absolute ethanol. This fraction was purified further by differential precipitation at pH 8.6 and 4.3. Subsequent purification, using Sephadex G-200 and BioGel 300, yielded a preparation with 350-450-fold increase in specific activity. The purified lipase had an optimum pH of 8.5 at 37 C. The electrophoretic mobility was -7.78×10^{-5} cm^2/volt/sec. The sedimentation coefficient for the two peaks was 2.85 and 8.5, respectively, and the mol wt was 100,000. The purified lipase hydrolyzed a variety of natural oils and fats. The amount of free fatty acids liberated from hydrogenated soybean oil (iodine value <3) was one-third compared to natural oils and fats. Gas chromatographic analysis of hydrolyzed synthetic triglyceride, with palmitic, stearic, and oleic acids at the rac 1, 2, and 3 positions, respectively, indicated that the enzyme was capable of hydrolyzing the glycerol-fatty acid bonds at all three positions. The yield was 40% palmitic, 20% stearic, and 39% oleic acids. Formaldehyde, mercaptoethanol, cysteine, glutathione, and terramycin had inhibitory effects upon lipase activity while hydrogen peroxide, streptomycin, and sodium taurocholate had a stimulatory effect upon the activity.

INTRODUCTION

Microbial lipases have received a great deal of attention during the past several years because of their possible applications in industry and medicine. Another aspect which has drawn considerable attention is their specificity or lack thereof depending upon the microorganism under study (1,2). The association of microorganisms with foods is inevitable, and invariably the breakdown of lipids results in either desirable flavor in certain cheese or undesirable off flavors in meats. Except for the liberation of volatile fatty acids in a few products like milk, the production of off flavor by lipolytic organisms cannot be directly correlated to lipases as most of the odoriferous compounds are produced as a result of further oxidation of the fatty acids. These free fatty acids (FFA) are important contributing factors to autoxidation as they are more prone to oxidation than the triglycerides. There are several excellent reviews in the area of lipases (3-5) and a few recent articles on microbial lipases (6-8). Lipases are produced by wide variety of microorganisms, including bacteria, yeast, and molds, and a few protozoa. Most of the lipase producing strains are saprophytic with the exception of staphylococci, clostridia, letospira, and certain pathogenic mycoplasma. The possibility of using lipase as a diagnostic tool for pathogenic organisms has been advocated (9) on the premise that there is a relationship between the production of this enzyme and pathogenecity. However, the inconsistency of the production of this enzyme among various strains of the same species makes it rather useless as a taxonomic tool.

The interest in staphylococcal lipase stems from the early work of Gillespie and Alder (10) and Alder, et al., (11) who reported that the egg yolk opacity factor was a lipase and it was related to the virulence of the organism. An exolipase from the cell free filtrates of *Staphylococcus aureus* also was reported (9) and characterized for its immunological properties. The relationship between lipase production and virulence or pathogenicity has been supported (12,13), as well as refuted (14,15). The wide distribution of this enzyme among various strains of staphylococci is indicated by the fact that in one survey 95% strains tested were lipase positive (16); and, in a second survey involving over 600 strains, 84% were positive for lipase (17). However, there is still some doubt if the egg yolk factor is a lipase per se; it may be a lipoprotein lipase or even a phospholipase. The lipolytic activity of staphylococci could have a large role in pathogenesis, since it has been reported that the amount of oleic acid in blood plasma of patients suffering from staphylococcal infections is considerably higher than the normal subjects (9,18). This may have serious consequences, as oleic acid can inhibit

[1]One of five papers presented in the symposium "Microbial Lipolytic Enzymes," AOCS Spring Meeting, New Orleans, April 1973.

[2]Present address: Department of Microbiology, Panjab University, Chandigarh 160014, India.

TABLE I

Effect of Age and Conditions of Growth upon Production of Staphylococcal Lipase[a]

Incubation time (days) at 37 C	Conditions of growth		
	No agitation	Agitation for first 24 hr	Continuous agitation
	Lipase activity[b]		
Uninoculated control	0.0	0.0	0.0
1	0.0	1.3	1.3
2	0.0	1.8	2.8
3	0.0	1.1	3.8
5	0.0	1.3	4.5
7	0.0	1.4	4.1

[a]*Staphylococcus aureus* B-120, trypticase soy broth medium.

[b]Milliliters of 0.01 N NaOH required to titrate free fatty acids produced from olive oil substrate in 10 min at 37 C.

oxidative phosphorylation (19,20).

Our interest in staphylococcal lipase developed from the report (21) that whole milk was not as good a substrate for *S. aureus* as skim milk. It became apparent that this difference was connected with the lipid fraction of the milk.

This report discusses the isolation, purification, and characterization of the staphylococcal lipase and its activity on milk fat and other lipids.

In all the studies, unless otherwise specified, a 24 hr old inoculum of *S. aureus*, B-120 was used. The growth medium was trypticase soy broth (TSB), and the cells were grown for 24 hr at 37 C on a reciprocating shaker (180 rev/min) when desired. The cell free supernatant was prepared by centrifuging in a refrigerated centrifuge at 8000 x g for 30 min. The enzyme assay was performed essentially according to the procedure outlined in the Worthington manual (22) and according to its modifications (23) which use olive oil emulsion. The protein concentration was determined according to the procedure of Lowry, et al. (24).

FACTORS AFFECTING PRODUCTION OF LIPASE

The effect of extent and conditions of incubation were studied, and the data are summarized in Table I. The production of lipase appears to be a time dependent phenomenon; however, a peak is reached in ca. 5 days of incubation under constant agitation, and further incubation did not increase the activity or amount of the enzyme. The stationary culture, despite a population of 8.4 x 10^6 cells/ml, did not produce any lipase. An experiment was designed to determine if the lipase production actually was related to the presence of oxygen or if mechanical agitation merely released the lipase, which might actually be an exoenzyme loosely bound to the cell surface. The data presented in Table II support the hypothesis that oxygen is required and it somehow affects the synthesis of the enzyme rather than its release from the surface. Small amounts of enzyme produced when agitation was performed under vacuum may be due to the lack of complete absence of oxygen due to mechanical reasons. However, agitation is not

TABLE II

Effect of Air and Agitation upon Production of Staphylococcal Lipase[a]

Incubation time (days) at 37 C	Conditions of growth		
	Mechanical agitation under vacuum	Continuous agitation	No mechanical agitation but bubbled with filtered air
	Lipase activity[b]		
1	0.3	1.3	1.0
2	0.5	2.7	2.7
3	0.6	3.6	3.2

[a]*Staphylococcus aureus* B-120, trypticase soy broth medium.

[b]Milliliters of 0.01 N NaOH required to titrate the free fatty acids produced by enzyme action from the olive oil substrate.

TABLE III

Effect of pH upon the Production of Staphylococcal Lipase[a]

pH of the medium			
Initial	Final	Lipase activity[b]	Cell wt[c] (mg)
4.0	4.05	0.20	8.60
6.0	5.50	1.25	53.50
7.0	6.70	5.90	77.70
8.0	7.25	7.30	84.10
10.0	7.95	0.90	20.20

[a]*Staphylococcus aureus* B-120, trypticase soy broth medium.

[b]Milliliters of 0.01 N NaOH required to titrate the free fatty acids by the enzyme action from olive oil emulsion.

[c]Cell wt/50 ml growth medium.

essential for the production of lipase by all strains or on all media. Large amounts of FFA were produced by this strain in milk (25).

The effect of pH upon the production of lipase in TSB was determined, and the data are presented in Table III. The initial pH of 8.0-9.0 gave optimal results for the production of lipase in TSB. The lipase production was related to the cell wt; however, the optima of the two, wt of the cells produced and the amount of lipase produced, were slightly different.

An intensive search for an intracellular lipase in this strain was futile, and it appears that *S. aureus* B-120, when grown, does not produce an intracellular lipase.

ACTION ON MILK FAT

The occurrence of *S. aureus* in various dairy products has been reported (24), and the deteriorative changes, as well as the safety of the foods, have been discussed. Walker (21) pointed out that skim milk was a better substrate than whole milk for *S. aureus*. In these experiments sterilized cereal milk (10% milk fat) was inoculated with an 18 hr old culture, and the milk was incubated at 22 and 30 C. The gross lipolytic changes were determined by acid degree value (ADV) according to the modified procedure (26) of Thomas, et al. (27). The results are summarized in Table IV. The extracted lipids were percolated through Amberlite IR 400, and the adsorbed fatty acids were methylated (28) and analyzed by gas liquid chromatography (GLC) (26). The results are shown in Table V. The neutral lipids were analyzed for various lipid classes according to the procedure of Carroll (29), using Florisil chromatography. The data are presented in Table VI. The extensive lipolysis of milk was

TABLE IV

Acid Degree Values of Fat Extracted from Milk Inoculated with *Staphylococcus aureus* S-1 and B-120 and Incubated as Indicated

Inoculum	Incubation temperature, C	Days of incubation							
		0[a]	1	2	3	4	5	6	7	8
		Acid degree value								
S-1 grown in milk	22	2.51	5.10	—[b]	—[b]	10.20	—[b]	—[b]	—[b]	14.20
	30	2.35	18.20	—[b]	—[b]	46.42	—[b]	—[b]	—[b]	78.52
B-120 grown in milk	22	3.37	5.81	7.42	9.72	12.78	14.53	15.54	16.94	18.34
	30	3.60	25.97	51.39	78.36	(lost)	102.78	112.22	116.94	118.19
B-120 grown in trypticase soy broth	30	3.60	11.25	33.61	48.89	54.17	58.33	73.61	90.47	90.58

[a]Noninoculated control.

[b]Not determined.

[c]Milliliters of 1 N methanolic KOH required to titrate 100 g of fat.

TABLE V

Analysis of Lipids Extracted from Milk Inoculated with *Staphylococcus aureus* S-1 and Incubated as Indicated

Compound	Noninoculated control (0 days)[a]	22 C			30 C		
		1 day	4 days	8 days	1 day	4 days	8 days
Free fatty acids	50	170	230	330	255	380	480
Monoglycerides	0	5	5	5	10	40	5
Diglycerides	0	50	5	5	50	10	10
Triglycerides[b]	910	700	615	540	615	490	445
Cholesterol	40	30	50	40	50	40	40
Percentage recovered	100.0	95.5	90.5	92.0	98.0	96.0	98.0

[a]Results are expressed as mg/g fat.

[b]Includes cholesterol esters.

TABLE VI

Amount of Free Fatty Acids Liberated from Milk Inoculated with *Staphylococcus aureus* S-1 and Incubated under the Conditions Indicated

Acid	Noninoculated control (0 days)[a]	22 C			30 C		
		1 day	4 days	8 days	1 day	4 days	8 days
Capric	0.335	0.785	1.776	4.142	1.856	4.644	6.166
Lauric	0.517	1.024	2.261	5.515	2.587	6.053	8.222
Myristic	0.391	3.162	6.489	14.684	7.944	19.297	24.379
Palmitic	2.997	7.534	16.403	44.803	21.128	49.672	53.835
Stearic	1.495	1.982	4.578	13.140	7.997	17.489	26.966
Oleic	2.233	4.896	9.326	22.766	12.178	27.929	42.966

[a]Results expressed as mg/g fat.

apparent, based upon extremely high ADV's. Calculations based upon GLC data indicate that, at the end of 8 days of incubation at 30 C, ca. 56.4% of the fat was hydrolyzed. The milk fat contains on the average ca. 1.5 times more oleic acid than palmitic acid, but the GLC analysis showed that lipolyzed samples contained palmitic acid in greatest amount, which is indicative of a preference for the palmitic ester bond by this lipase. Any contribution by the bacterial lipids due to autolysis over an extended period of incubation is discounted because of the small amount of lipids in *S. aureus* cells. The fatty acids liberated had a substantial effect upon the growth and proteo-

TABLE VII

Activity of Staphylococcal Lipase upon Several Natural Substrates[a]

Lipid source	Activity (percent of olive oil)
Soybean oil	82.9
Safflower oil	103.6
Corn oil	102.4
Cottonseed oil	80.5
Butter oil	97.5
Lard	80.5
Turkey fat	92.7
Beef tallow	100.0
Hydrogenated soybean oil	34.1

[a]All substrates were in emulsion form and the reaction performed at 37 C for 10 min at pH 8.0.

TABLE VIII

Effect of Hydrogen Peroxide, Formaldehyde, and Mercaptoethanol upon the Activity of Staphylococcal Lipase on Olive Oil Emulsion

Chemical	Quantities of the chemical used (ml)						
	0.1	0.2	0.3	0.4	0.5	0.8	1.0
	Percentage of activity as compared to control						
Formaldehyde	7	47.7	40.9	36.4	27.3	18.2	2.3
Hydrogen peroxide[a]	120	141	161	159	134	102	95
2-Mercaptoethanol	80	60	40	22	0.0	0.0	0.0

[a]A 30% hydrogen peroxide solution.

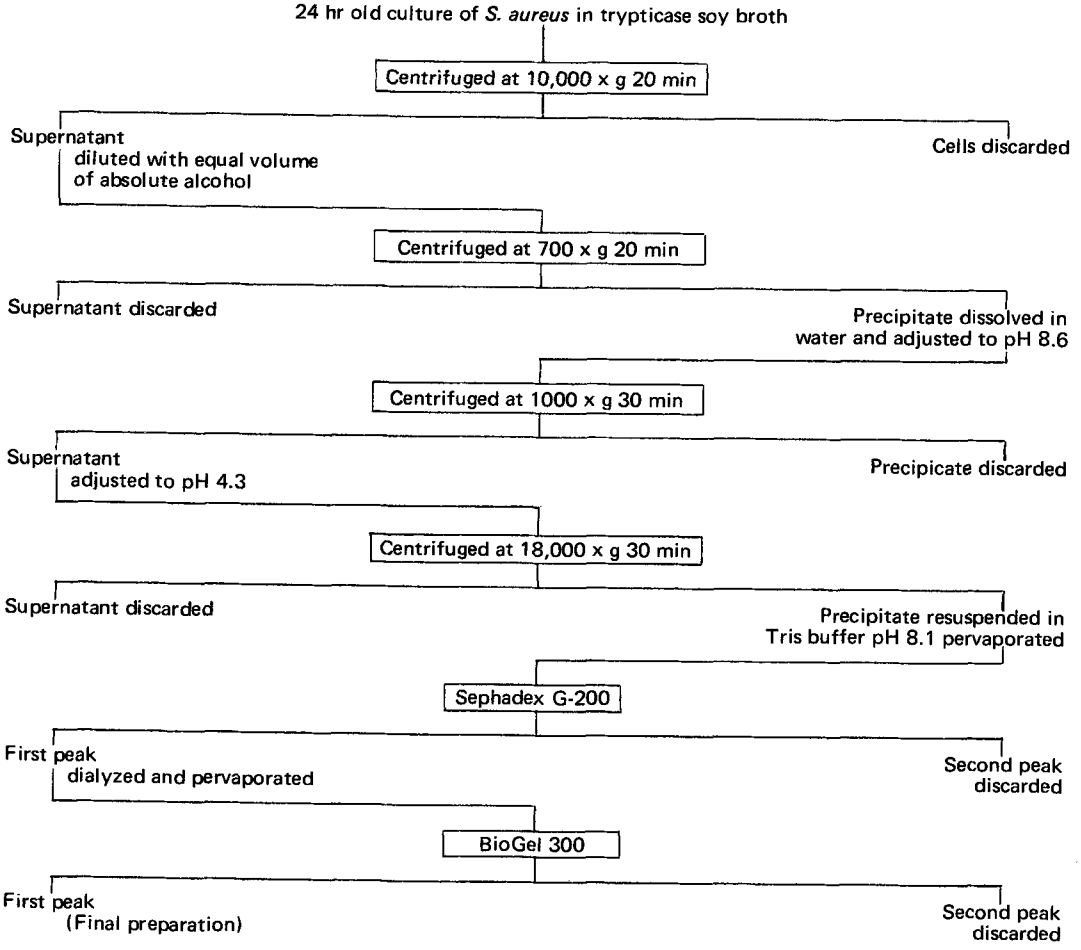

FIG. 1. Flow chart for the isolation and purification of staphylococcal lipase.

lytic activity of *S. aureus*, and caprylic and capric acids were the only fatty acids to show inhibitory properties (25).

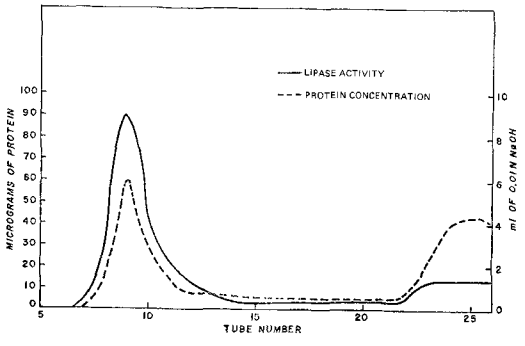

FIG. 2. An elution diagram of a lipase preparation on BioGel 300 using Tris buffer pH 8.1, $\Gamma/2 = 0.1$. Lipase concentration is represented as micrograms of protein and the lipase activity as ml of 0.01 N NaOH required to titrate free fatty acids liberated by the lipase in olive oil emulsion for 10 min at 37 C and pH 8.0.

PURIFICATION OF THE LIPASE

Zolli and SanClemente (30), while trying to purify coagulase, reported that lipase was precipitated when the cell free supernatant was dialyzed against a pH 4.3 acetate buffer, containing 10% v/v of ethyl alcohol. Direct precipitation of cell free supernatant was tried with various concentrations of ethyl alcohol, and 50% concentration gave the best results. The schematic used for obtaining a purified fraction is shown in Figure 1, and the details of the procedure have been reported elsewhere (31). Renshaw and SanClemente (32) also obtained a highly purified preparation of *S. aureus* lipase using the procedure of Zolli and SanClemente (30) for initial concentration. The purified lipase appears to be a high mol wt moiety, as it is eluted soon after the void volume on Sephadex G200, as well as BioGel 300 (Fig. 2). This

TABLE IX

Effect of Various Chemicals upon the Activity of Staphylococcal Lipase on Olive Oil Emulsion

Chemical	Amount (ml) of a 5.0% solution of chemical agent				
	0.5	1.0	2.0	3.0	5.0
	Percentage of activity as compared to control				
Glutathione	76	13.3	0.0	0.0	0.0
Cysteine	43.3	13.3	0.0	---	---
Ascorbic acid	—[a]	66.6	50.0	---	30
Iodoacetic acid	78.8	51.5	---	---	0.0
N-ethylmaleimide	106	96.7	100	106	100
P-chloromercuribenzoate	81.2	46.8	---	---	0.0

[a]Not determined.

preparation was homogenous in a moving boundary analysis at pH 8.6 in vernol buffer pH 8.6, $\Gamma/2 = 0.1$, and had a mobility of -7.78 x 15^5 Cm^2 $volt^{-1}$ sec^{-1} (31). The enzyme shows a major and a minor component on ultracentrifugal analysis, with $S_{20,w}$ values of 2.85 and 8.50. The preliminary mol wt value by ultracentrifugal analysis of the major component was ca. 100,000. The isoelectric point of this purified enzyme appears to be pH 4.3 as the enzyme could almost always be precipitated at this pH value.

CHARACTERIZATION OF THE PURIFIED ENZYME

The optimum pH was determined by quantitating lipase activity at 37 C over a pH range of 5.0-12.0; the optimum temperature at pH 8.0 was determined by assaying at 22, 30, 37, 40, 45, 50, 55, 60, and 70 C; these data are presented in Figures 3 and 4. The storage stability of the purified enzyme over a 21 day period, and temperatures ranging from -23-37 C also was studied. The data are presented in Figure 5. The thermal stability to various processing temperatures (50-70 C) and various lengths of time (5-30 min) was studied. The details of the experimental procedures have been discussed in another paper (33). The enzyme activity on several natural substrates and a synthetic substrate, rac-glycdryl 1-palmitate, 2-oleate, and 3-stearate was tested; and the results are summarized in Table VII. The effect of several chemical agents upon the activity of lipase was studied, and the results are summarized in Tables VIII and IX.

The purified enzyme had an optimum pH in the range of 8.0-8.5 with a peak at ca. pH 8.3. These values agree closely with those reported by other authors (32) but differ from the value of 7.8 reported (34,35) for purified egg yolk factor. The optimum temperature of this purified preparation was 45 C, while Renshaw and SanClemente (32) reported an optimum temperature of 40 C. These differences could be due to the degree of purification, as well as the source of the enzyme. The purified enzyme is extremely stable at 4 C or frozen storage at -23 C, and the loss of activity is less than 10% over a 21 day storage period. In another report (32) almost no loss of activity of purified enzyme was seen over a 2 year period at -20 C or 13 months at 7 C. These authors also report that the crude preparation was stable for several months at room temperature. This storage stability is in contrast to milk lipase (36) which is almost completely inactivated within a few days at room temperature.

The extreme thermal stability of purified staphylococcal lipase was surprising, since only 6% of the activity was lost after 30 min of heating at 50 C. However, the inactivation was ca. complete at 70 C for 30 min. This raises an

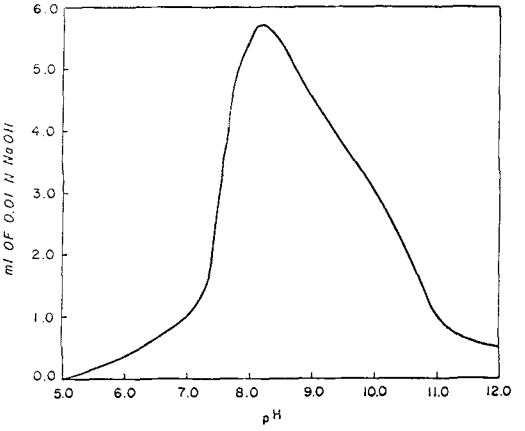

FIG. 3. Milliliters of 0.01 N NaOH used to titrate the fatty acids liberated by the action of staphylococcal lipase in olive oil emulsion for 10 min at 37 C at various pH values.

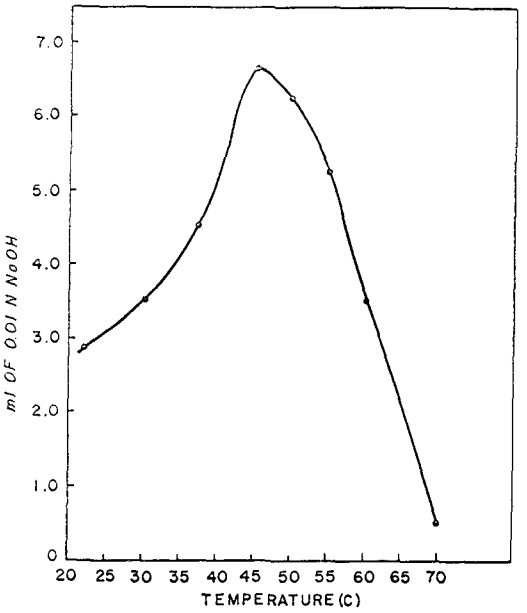

FIG. 4. Milliliters of 0.01 N NaOH required to titrate the free fatty acids liberated from olive oil by staphylococcal lipase after 10 min at pH 8.0 at various incubation temperatures.

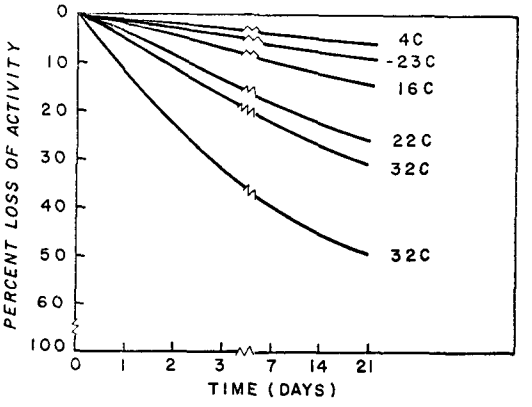

FIG. 5. Percent loss of activity of staphylococcal lipase when stored at various temperatures for the times indicated.

interesting question about the possibility that flavor deterioration of some pasteurized dairy products may be due to lipases from staphylococci or other organisms. The enzyme did not show any particular preference for different substrates. All the natural oils and fats tested were hydrolyzed to a similar extent, with the exception of hydrogenated soybean oil (iodine value <3) in which case the activity was reduced by two-thirds. The synthetic triglyceride was found to be hydrolyzed in all three positions. Similar results were reported by Alford, et al. (1). Even though the lipase attacks all three positions, there appears to be slight preference for 1 and 3 positions as compared to 2 positions as the ratios of fatty acids were 40:21:39, respectively. Another important fact about the enzyme appears to be the all or none phenomenon, i.e. if the enzyme attacks a triglyceride, it has the capability to digest it completely into constituent fatty acids and glycerol.

The enzyme activity was inhibited by reducing agents while oxidizing agents stimulated the activity. Mercaptoethanol, cysteine, and glutathione inhibited the activity, while H_2O_2 stimulated the activity. Perhaps -SH groups are not involved for the enzyme activity or counterwise the -S-S are required.

The presence of antibiotics had a varying effect. Penicillin and terramycin inhibited the activity, while streptomycin and aureomycin had a stimulating effect. Similar results have been reported for milk lipase (37), but the exact mode of action is not clear. The immunological properties of the purified lipase were studied (32) and two precipitation zones were noticed: one large heavy zone indicating a probable lipase-antilipase interaction and a small area of weak precipitation indicating a contaminating fraction.

Davies (9) reported that lipases were antigenically homogenous and did not find any evidence of naturally occurring lipase antibodies. Renshaw and SanClemente (32) reported that staphylococcal lipase was fully antigenic and produced agglutinating precipitating and neutralizing antibodies in cows, rabbits, and mice. The possible use of this enzyme as a part of vaccine for controlling mastitis has been implicated (38), but no definite proof is available about its protective role and needs further investigation.

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Some Properties of an Exocellular Lipase from *Rhizopus arrhizus*[1]

GILBERT BENZONANA,[2] Department of Biochemistry, University of Geneva, 1211 Geneva 4, Switzerland

ABSTRACT

Rhizopus arrhizus, a mold of the mucor family, excretes an active lipase when cultured properly. This lipase has a mol wt of 43,000 and a high carbohydrate content, Upon storage at 4 C in aqueous solution, lipase I is slowly converted by proteolysis to a more cationic form, lipase II, which has a lower mol wt (32,000) and no carbohydrate. *Rhizopus* lipase shows the same positional specificity on long chain triglycerides as pancreatic lipase; it has no preferential side chain specificity against oleic vs. palmitic acid. Like pancreatic lipase, *Rhizopus* lipase acts on micelles of short chain triglycerides and is inhibited by high concentrations of bile acids; however, in the presence of deoxycholate, *Rhizopus* lipase does not require added Ca^{++} for full activity.

INTRODUCTION

Numerous investigators have shown that microorganisms display lipolytic activity against long chain triglycerides (1-4). Alford, et al., (5) classified these enzymes into three large groups according to specificity. The most important group, corresponding to the specificity of pancreatic lipase, hydrolyzes only the external ester bonds of triglycerides. The second group is composed of enzymes which have no positional specificity and hydrolyze both types of glycerol ester bonds. The last and least common class is represented by lipases which have a high affinity for esters of unsaturated fatty acids regardless of position.

Since microorganism lipases frequently can be produced on an industrial scale using relatively simple conditions (5,6), there is a growing interest in their study, especially in view of possible applications in industry and medicine. Pancreatic lipase (EC 3.1.1.3), on the other hand, has been studied for many decades in numerous laboratories (7,8) and can be used as standard reference for comparison purposes.

It is the purpose of this paper to review the properties of an exocellular lipase from *Rhizopus arrhizus* and compare them to those of pancreatic and microbial lipases.

[1]One of five papers presented in the symposium "Microbial Lipolytic Enzymes," AOCS Spring Meeting, New Orleans, April 1973.

[2]Chargé de Recherche on leave from the Centre National de la Recherche Scientifique, Paris, France.

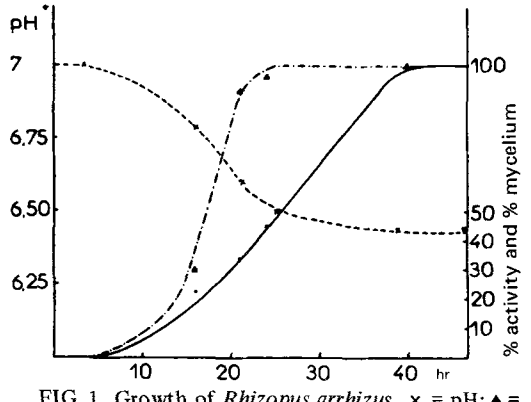

FIG. 1. Growth of *Rhizopus arrhizus*. x = pH; ▲ = mycelium growth; ● = lipase activity on the filtrate free of mycelium (From ref. 9).

PREPARATION

Industrial scale culture conditions for induction of the *R. arrhizus* lipase are given by Laboureur and Labrousse (9). The culture medium contains pancreatin hydrolyzed lactic casein, hydrolyzed corn flour, calcium carbonate, and ammonium sulfate; growth was carried out at 29 C with stirring and aeration for 40-45 hr. As can be seen from Figure 1, growth stops after 20-25 hr, but maximum lipase activity is not observed until 40 hr. These conditions differ from those used for *Candida lipolytica* (10), for example. This microorganism excretes a lipase in direct proportion to its growth; when exponential growth ceases, the total lipase activity begins to decrease (10). Furthermore, *R. arrhizus* does not seem to require any inducer for biosynthesis and excretion of lipase; most of the other lipase producing microorganisms do (11,12).

Once the maximum lipase activity is reached, the mycelium is filtered; and the filtrate is concentrated under vacuum, fractionated with acetone and then barium chloride, and lyophilized. The powder has an activity of 40-50,000 lipase units/g (one lipase unit is the amount of enzyme able to liberate one μmole of fatty acid/min in the assay conditions. The specific activity corresponds to

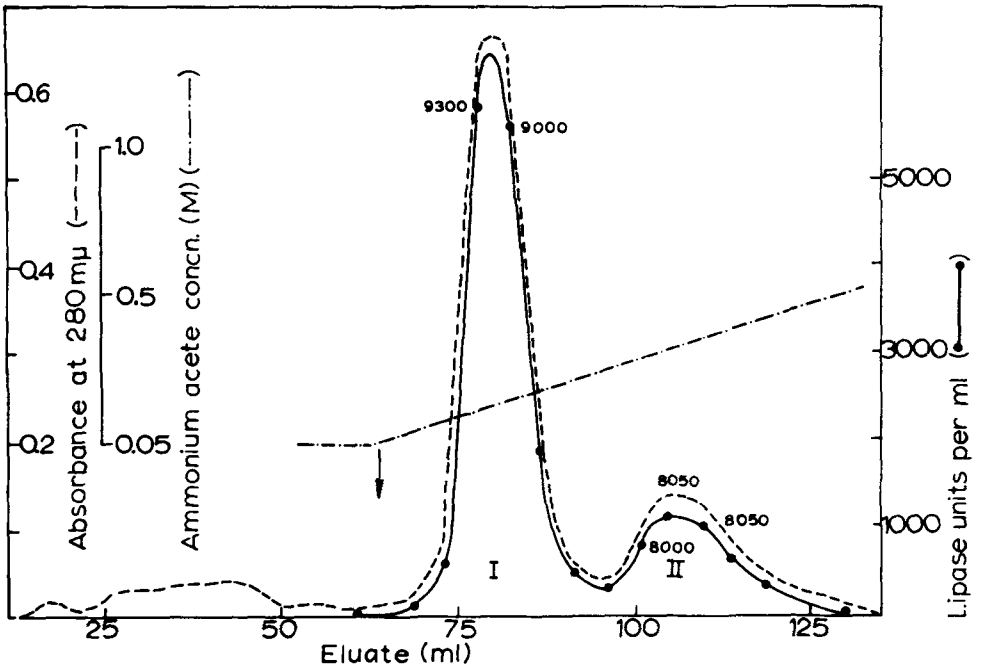

FIG. 2. Chromatography of *Rhizopus* lipase on Amberlite IRC 50, eluted with an ammonium acetate gradient. Specific activities of certain fractions are indicated on the figure (From ref. 13).

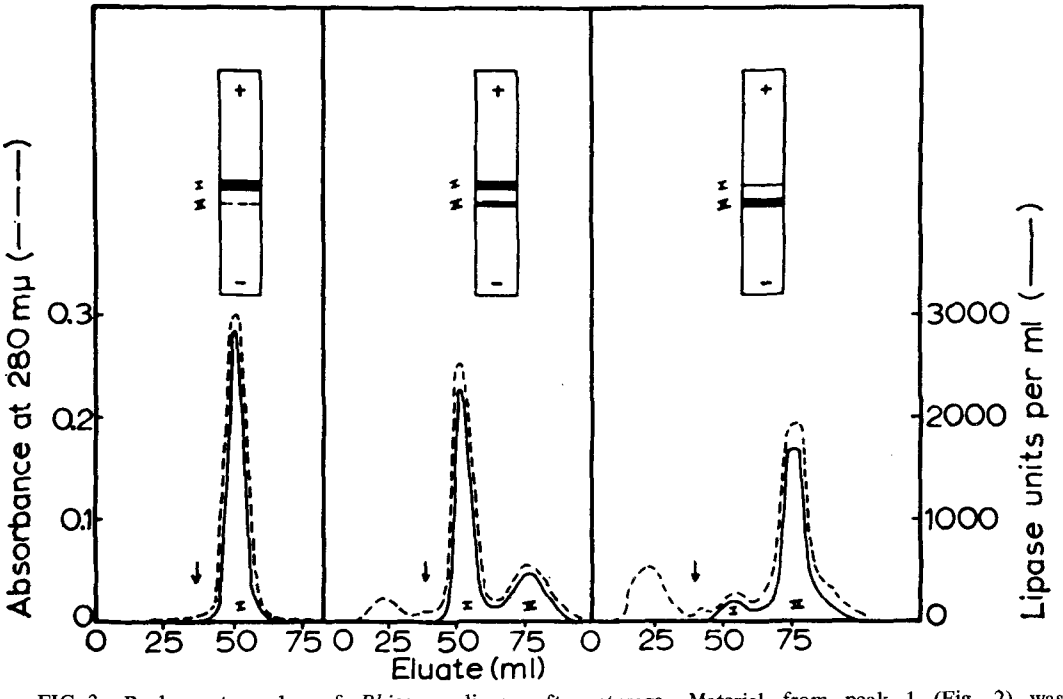

FIG. 3. Rechromatography of *Rhizopus* lipase after storage. Material from peak 1 (Fig. 2) was rechromatographed on Amberlite IRC 50 either at once (left) or after storage for 16 days (center) or 92 days (right). The conditions are the same as in Figure 2. The upper drawings show the results of disc electrophoresis at pH 4.5 of the solutions applied to each column (From ref. 13).

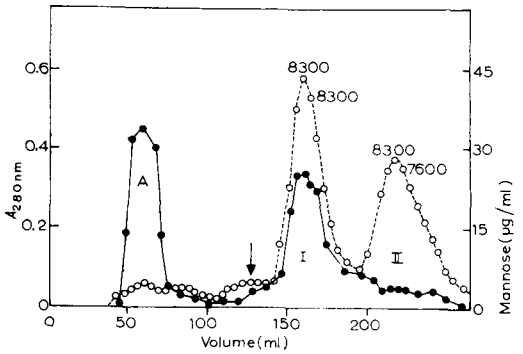

FIG. 4. Determination of neutral sugars in the chromatographic eluate of a mixture of lipases I and II. Lipase I was stored 4 days at 22 C in buffer (pH 5.7) before chromatography on Amberlite IRC 50. Solid line = neutral sugars and dotted line = absorbance at 280 nm (From ref. 14).

the activity reported/mg protein) or a specific activity of ca. 400 (based solely upon the protein content). It is dissolved in water, adsorbed to a column of Amberlite IRC 50, and eluted with 1 M ammonium acetate; 95% of the activity is recovered with a specific activity of 5000.

After filtration through Sephadex G 75, the lipase is rechromatographed on Amberlite IRC 50 as shown in Figure 2 (13). Lipase activity emerges in two peaks (lipase I and lipase II), the ratio of lipase I to lipase II is not constant from one preparation to another. The specific activities of lipase I and II are ca. 9000 and 8000, respectively. If, as shown in Figure 3 (13), lipase I is rechromatographed immediately on Amberlite IRC 50, it gives a single active peak in the same place. However, if stored in

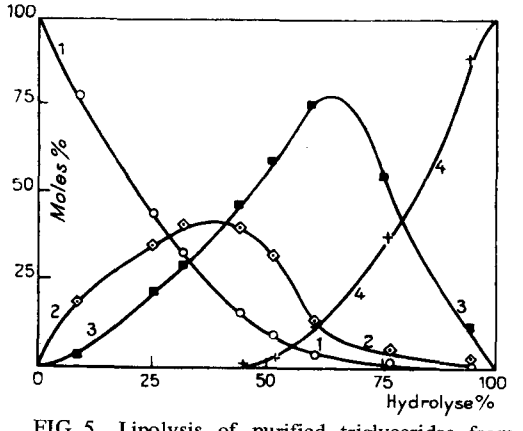

FIG. 5. Lipolysis of purified triglycerides from olive oil by *Rhizopus* lipase. Lipolysis was performed at 37 C, pH 8.0, in .02 M NH_4Cl-NH_4OH buffer in the presence of 0.22% bovine serum albumin and 5.5 mM Cl_2Ca. Curves 1, 2, 3, and 4 refer respectively to triglycerides, diglycerides, monoglycerides, and glycerol (From ref. 25).

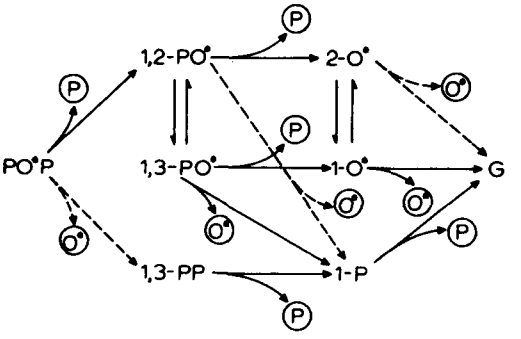

FIG. 6. Possible transformations of dipalmitylglycerol during lipolysis. → Normal cleavage of external chains; ⇢ abnormal cleavage of internal chains; ⇌ spontaneous acyl migrations. Abbreviations: P*OP: 1,3-dipalmityl-2-[9,10^{3}H] oleylglycerol; 1,3 PP: 1,3 dipalmityl-glycerol; 1,2 or 1,3 P*O = 1 palmityl-2-oleylglycerol or 1 palmityl-3-oleylglycerol; 1-P = 1 palmitylglycerol; 1 or 2 *O = 1 or 2 oleylglycerol; and P, *O, G = palmitic acid, oleic acid, and glycerol respectively (From ref. 25).

aqueous solution at 4 C, lipase I disappears; and the more cationic lipase II appears in parallel. In contrast, lipase II always appears at the same place when rechromatographed after various periods of storage in the cold.

Lipase II, therefore, is the result of proteolytic degradation of lipase I and is not excreted by the mold as lipase II. The addition of 2 mM diisopropylfluorophosphate (DFP) (14) to an incubation medium containing lipase I stops the formation of lipase II. On the other hand, storage of lipase I at 20 C in the presence of 1 mM NaN_3, to inhibit bacterial growth, promotes the rapid formation of lipase II at the expense of lipase I.

MOLECULAR PROPERTIES OF LIPASES I AND II

The mol wt, determined by filtration through Sephadex G 100 of lipase I is 40,000 ± 2000, while that of lipase II is only 30,000 ± 1500. Figure 4 shows the chromatography of a mixture of lipases I and II resulting from the incubation of pure lipase I. In addition to the two lipases, some material with an absorbance at 280 nm appears in front of lipase I. Titration of neutral sugars on all the column fractions shows that lipase I is rich in carbohydrates while lipase II has lost most of them; the liberated carbohydrates appear early in the chromatography (fraction A), coincident with some absorbance at 280 nm.

The carbohydrate moiety can be liberated from lipase I as a glycopeptide of mol wt 8500 by several methods: adding 5% trichloroacetic acid to a solution of lipase I at 0 C, boiling for 3 min, or adding 6 M HCl in the cold until the

TABLE I

Amino Acid Composition of Lipase I, of Lipase I after Separation of the Glycoprotein Fraction, and of Glycoprotein Fraction[a]

	Number of residues in 1 mole of						
	Lipase I		Lipase I after separation of the glycoprotein fraction		Glycoprotein fraction		Glycoprotein fraction + precipitated lipase I
Amino acid	Experimental value	Nearest whole number	Experimental value	Nearest whole number	Experimental value	Nearest whole number	Integral value
Alanine	23.4	23	18.66	19	5.00	5	24
Arginine	10.08	10	8.72	9	0.98	1	10
Aspartic acid or asparagine	39.80	40	28.67	29	7.67	8	37
Cystine	5.93	6	5.30	5	1.03	1	6
Glutamic acid or glutamine	29.50	29-30	26.26	26	5.54	5-6	31-32
Glycine	28.08	28	25.65	26	4.45	4-5	30-31
Histidine	8.38	8	7.15	7	1.06	1	8
Isoleucine	19.67	20	17.97	18	2.04	2	20
Leucine	24.54	24-25	19.49	19-20	4.40	4-5	24-25
Lysine	20.04	20	16.17	16	4.18	4	20
Methionine	2.94	3	0.92	1	0.80	1	2
Phenylalanine	15.25	15	16.93	17	0	0	17
Proline	23.71	24	16.77	17	5.25	5	22
Serine	40.00	40	30.59	31	8.72	9	40
Threonine	28.17	28	27.79	28	2.58	2-3	30-31
Tryptophan	6.00	6					
Tyrosine	14.63	15	14.05	14	1.09	1	15
Valine	28.96	29	28.37	28	0.94	1	29
Mol wt	40,281 ± 750		34,270 ± 1000		6100 ± 300		

[a]From ref. 14.

TABLE II

Amino Acid Composition of Lipase II and Lipase I after Separation of the Glycoprotein Fraction[a]

Amino acid	Lipase II	Lipase I after separation of the glycoprotein fraction
Alanine	18	19
Arginine	9	9
Aspartic acid or asparagine	27-28	29
Cystine	5	5
Glutamic acid or glutamine	25	26
Glycine	23-24	25-26
Histidine	7	7
Isoleucine	18	18
Leucine	18	19-20
Lysine	15-16	16
Methionine	1	1
Phenylalanine	15	17
Proline	16-17	17
Serine	27	30-31
Threonine	24	28
Tryptophan	4	
Tyrosine	12	14
Valine	28-29	28
Mol wt	32,128 ± 800	34,270 ± 1000

[a]From ref. 14.

pH is less than 1.0. Each treatment gives two fractions: a supernatant containing almost all of the glycopeptide and an inactive precipitate. The amino acid composition of the two fractions is given in Table I together with the amino acid composition of native lipase I. The amino acid composition of native lipase I agrees well with the sum of the amino acid compositions of the glycopeptide and the precipitate. Moreover, the sugar composition of lipase I, 13-14 molecules of mannose and 2 molecules of amino sugar, is similar to that of the glycopeptide fraction.

In addition, a good correlation is found between the amino acid composition of lipase II and that of lipase I separated from its glycopeptide component. However, lipase II seems to be somewhat smaller (Table II), probably as the result of a proteolytic degradation of lipase I under somewhat ill defined conditions. This hypothesis is confirmed by determinations of the N-terminal residues of lipases I and II shown in Table III. While pure lipase I shows a single N-terminal residue, two new residues appear if the enzyme is stored in the absence of DFP. Lipase II has as many as six end groups, presumably corresponding to various stages of degradation. Laboureur and Labrousse (15), who found an N-terminal serine for lipase I, were probably unable to prevent its degradation.

The role of the glycopeptide in catalysis is not apparent. Lipase I has a specific activity of ca. 9000; lipase II (8000) is not markedly different. Other lipases (16,17), among them hog pancreatic lipase (17-19), also possess carbohydrates. However, bovine pancreatic lipase, which is devoid of carbohydrates (17), has catalytic properties not markedly different from those of hog lipase. The role of the carbohydrate moiety remains obscure; perhaps, as suggested by Eylar (20), it facilitates the passage of the protein through the external membrane of the mold during excretion.

Positional and Substrate Specificities

It has been shown that the hydrolysis of triglycerides by pancreatic lipase leads first to the rapid and transitory formation of diglycerides; monoglycerides later accumulate in considerable amounts (21,22). The formation of glycerol, resulting from the hydrolysis of 1-monoglycerides formed by the isomerization of 2-monoglycerides (23,24), is slow and begins only after ca. 50% total ester bonds have been hydrolyzed (21). This process results from the absolute specificity of pancreatic lipase for primary ester bonds and from its decreasing affinity for di- and monoglycerides.

The qualitative determination of both the positional and substrate specificities of *Rhizopus* lipase, using purified triglycerides from olive oil, is shown in Figure 5 (25). The pattern of product formation resembles that observed for pancreatic lipase: glycerol does not appear, for example, until ca. half of the total ester bonds have been hydrolyzed. In comparison, the *Candida cylindracea* lipase is known to be devoid of positional specificity (26); here, glycerol appears almost immediately.

A more precise determination of substrate specificity was made with 1,3-dipalmityl 2-[9-10 ^{3}H]oleyl glycerol (P*OP). Figure 6 (25) shows that, if hydrolysis of P*OP proceeds only through the primary ester bonds, diglycerides and monoglycerides should bear the tritiated oleyl chain; their specific activity should be the same as that of the initial P*OP unless isomerization occurs. In fact the specific radioactivities of tri-, di-, and monoglycerides were 60.0, 59.7, and 56.4 x 10^{3} cpm/μmole glyceride, respectively, after 32% the total ester bonds had been hydrolyzed. The liberated fatty acids contained less than 1% of the total initial radioactivity. However, the ratio of 1,3 to 1,2 diglycerides was already 2% and that of 1 to 2 monoglycerides was 6%, indicating that isomerization, which could produce oleic acid and the decrease in monoglyceride specific activity was taking place.

Various oleo-palmitic glycerides were used

to show that *Rhizopus* lipase has no preferential specificity for oleic vs. palmitic acid chains (25). This is common to both pancreatic (7) and *Candida cylindracea* lipase (26).

Lipases can be distinguished from esterases by the fact that they act only on insoluble substrates or, at least, on substrates in a plurimolecular state. Pancreatic lipase, for instance, displays no activity on truly soluble or monodisperse substrates (27). Figure 7 (28) shows the behavior of *Rhizopus* lipase on two slightly soluble glycerides, triacetin and tripropionin. These glycerides are poor substrates for the enzyme; a small amount of activity appears only above the critical micelle concentration (CMC) of each glyceride. Above the CMC, the activity increases regularly; no jump is observed at saturation when insoluble substrate globules appear. This behavior resembles that of pancreatic lipase except that the pancreatic enzyme shows a jump in activity when the substrate begins to appear in an insoluble form. On the other hand, pancreatic lipase hydrolyzes tripropionin at 230% and triacetin at 12% rate observed for triolein, while these values are only 5% and 0.5% for the mold enzyme.

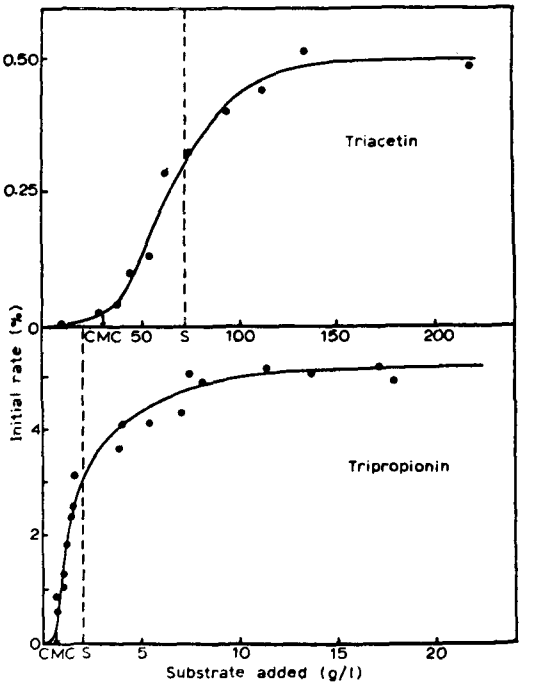

FIG. 7. Initial rate of hydrolysis as a function of the amount of added substrate. Rates are expressed in percentage of the maximal rate observed with the same amount of lipase and a triolein emulsion. The vertical dashed lines indicate saturated solutions. The critical micelle concentration was 0.7 mg/ml for tripropionin and 23 mg/ml for triacetin (From ref. 28).

Influence of Various Effectors

Studies of the influence of effectors on triglyceride hydrolysis by lipases are of particular interest since they should promote the understanding of this important interfacial phenomena. For pancreatic lipase, it has been shown that bile acids are needed to sweep free fatty acids, formed during the hydrolysis, from the lipid-water interface; these free fatty acids strongly inhibit hydrolysis (29). However, bile acids themselves are strong inhibitors of pancreatic lipase at high concentrations if the lipase cofactor is absent or present in insufficient amounts (29,30). Qualitatively, the phenomenon is similar with *Rhizopus* lipase: the mold enzyme is highly sensitive to bile acids when their concentrations reach the CMC (28). As with the substrate, it appears that the aggregation state of the effector is of great importance; when bile acid is in true molecular solution it has relatively little effect upon the catalytic properties of *Rhizopus* lipase (28). However, as soon as aggregates begin to appear, a dramatic decrease in activity occurs, indicating that micelles are the inhibitory species. This inhibition seems to result from a direct interaction between the lipase and bile acids. Recent experiments (28), showing that the inhibition is competitive, indicate that the enzyme is able to bind, at or near its catalytic

TABLE III

N-Terminal Residues of Lipases I and II[a]

Lipase	DNP[b]-amino acids (moles/mole of lipase I)					
	Asp[c]	Threonine	Serine	Glx[c]	Valine	Alanine
Lipase I, treated with DFP[b]	0.63					
Lipase I, untreated	0.62		0.16			0.11
Lipase II	0.006	0.17	0.36	0.06	0.01	0.06

[a]From ref. 14.

[b]DNP = 2,4-dinitrophenyl, DFP = diisopropylfluorophosphate.

[c]Asp = aspartic acid or asparagine. Glx = glutamic acid or glutamine.

site, micelles which have nothing in common with the substrate but their physical state. By comparison pancreatic lipase is inhibited noncompetitively by deoxycholate: direct interaction of the enzyme with the bile acids also has been shown by ultracentrifugation (G. Benzonana and B. Chorvath, unpublished experiments). A final interesting point is that *Rhizopus* lipase does not require added calcium ions (28) to hydrolyze triglycerides in the presence of bile salts as does pancreatic lipase (31).

Conclusion

This short review shows that numerous similarities can be observed between two lipases secreted by organisms far apart on the evolutionary scale. These similarities can be of great value in understanding the mechanism of action of lipases. Unfortunately, lipases are still large molecules; their complete sequence and three dimensional structure will not be easy to obtain. Physicochemical studies, as already begun on pancreatic lipase (32), are currently our greatest hope for a better understanding of these enzymes.

ACKNOWLEDGMENTS

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Sterols, Methylsterols, and Triterpene Alcohols in Three *Theaceae* and Some Other Vegetable Oils

TOSHIHIRO ITOH, TOSHITAKE TAMURA, and **TARO MATSUMOTO,** College of Science and Technology, Nihon University, 8, Kanda Surugadai, 1-chome, Chiyoda-ku, Tokyo, 101 Japan

ABSTRACT

The unsaponifiables from three *Theaceae* (*Camellia japonica* L., *Camellia Sasanqua* Thunb., and *Thea sinensis* L.) oils and alfalfa, garden balsam, and spinach seed oils and shea fat were separated into four fractions: sterols, 4-methylsterols, triterpene alcohols, and less polar compounds by thin layer chromatography. While the sterol fraction was the major one for the unsaponifiables from alfalfa and spinach seed oils, the triterpene alcohol fraction was predominant for the unsaponifiables from all other oils. The sterol, 4-methylsterol, and triterpene alcohol fractions were analyzed by gas chromatography. All the sterol fractions were alike in their compositions, consisting exclusively of Δ^7-sterols, such as α-spinasterol and Δ^7-stigmastenol as predominant components together with Δ^7-avenasterol and 24-methylcholest-7-enol. Obtusifoliol, gramisterol (occasionally accompanied with cycloeucalenol), and citrostadienol, together with several other unidentified components, were found in the 4-methylsterol fractions from all of the oils except shea fat. The 4-methylsterol fraction from shea fat showed a characteristic composition containing a large proportion of unidentified components which had relative retention time greater than that of citrostadienol, while no citrostadienol was detected. β-Amyrin, lupeol, and butyospermol were major components of the triterpene alcohol fractions from most of the oils, but the fraction from spinach seed oil contained cycloartenol and 24-methylenecycloartanol as predominant components. There is a close similarity in the compositions of unsaponifiables (sterols, 4-methylsterols, and triterpene alcohols) of the three *Theaceae* oils. Two sterols, α-spinasterol and Δ^7-stigmastenol, and five triterpene alcohols were isolated from tea seed oil. Moreover, five unidentified components beside parkeol, butyrospermol, α-amyrin, and lupeol were isolated from the triterpene alcohol fraction of shea fat.

INTRODUCTION

In the previous studies (1, 2) in this laboratory, the unsaponifiables from 19 vegetable oils were separated into 4 fractions, less polar compounds, triterpene alcohols, 4-methylsterols, and sterols; and the compositions of the triterpene alcohol, 4-methylsterol, and sterol fractions from each oil were determined. In the sterol fractions (1), Δ^7-stigmastenol and Δ^5- and Δ^7-avenasterols, in addition to three common sterols, campesterol, stigmasterol, and β-sitosterol, were found in most of the oils. Cholesterol and brassicasterol in minor amounts also were found in majority of the oils examined. Four 4-methylsterols (2), obtusifoliol, cycloeucalenol, gramisterol, and citrostadienol were identified as common constituents in many of the oils examined. Moreover, cycloartenol and 24-methylenecycloartanol were found as common triterpene alcohols in many of the oils; and the widespread occurrence of cyclobranol (24-methylcycloartenol), cycloartanol, and α- and β-amyrins was demonstrated (2).

In this investigation, three *Theaceae* oils, i.e. camellia oil from *Camellia japonica* L., sasanqua oil from *Camellia Sasanqua* Thunb., and tea seed oil from *Thea sinensis* L., and alfalfa, garden balsam, and spinach seed oils and shea fat were analyzed for the compositions of the unsaponifiables and of the sterol, 4-methylsterol, and triterpene alcohol fractions. (Designation of fractions in this stidy, sterol, 4-methylsterol, and triterpene alcohol is based upon the thin layer chromatographic [TLC] behavior of the components of each fraction. Hence, these 3 fractions contain 4-desmethylsterols, 4-monomethylsterols, and 4,4-dimethylsterols, respectively.)

According to several studies on the unsaponifiables of tea leaf wax and tea seed oil, tea leaf wax contains α-spinasterol (3-6) and β-amyrin (3, 5), while tea seed oil contains α- and β-amyrins (7, 8), cycloartenol (7, 8), 24-methylenecycloartanol (7, 8), and butyrospermol (8) as triterpene alcohols and brassicasterol (8), campesterol (7, 8), stigmasterol (7), β-sitosterol (7, 8), and Δ^7-stigmastenol (8) as sterols. However, it seems rather curious that α-spinasterol has not yet been identified in tea seed oil in spite of its presence in tea leaf wax. On the other hand, the occurrence of α-spinasterol in spinach leaves (9) and alfalfa leaves and seeds (10) and the occurrence of α-spinasterol and β-amyrin in garden balsam seeds (11) have been

TABLE I

Content of Oils in Dried Seeds, Content of Unsaponifiables in Oils, and Yields of Four Fractions from Unsaponifiables by Thin Layer Chromatography

Oil	Content of oil in dried seed, %	SV[a]	IV[b]	Unsaponifiables in oil, %	Fraction[c] from unsaponifiables, %			
					1	2	3	4
Camellia (Japan)[d]	---	192.5	81.0	0.4	19	48	7	26
Sasanqua (China)[d] I	---	190.7	85.1	0.6	22	59	1	18
Sasanqua (China)[d] II	---	191.7	84.7	0.4	18	64	tr[f]	18
Tea (Japan)[d]	---	192.0	91.5	0.6	22	59	1	18
Alfalfa (Japan)[e]	9.5	189.8	144.7	3.3	7	30	5	58
Garden balsam (Japan)[e]	13.9	189.0	192.1	5.6	43	31	4	22
Spinach (Japan)[e]	3.0	190.8	129.2	2.9	23	14	4	59
Shea fat (West Africa)[d]	---	196.7	63.9	5.1	18	75	2	5

[a]SV = saponification value.

[b]IV = iodine value, Wijs' method.

[c]Fraction 1 = less polar compounds (hydrocarbons, etc.), fraction 2 = triterpene alcohols, fraction 3 = 4-methylsterols, and fraction 4 = sterols.

[d]Commercially prepared crude oil.

[e]Laboratory extracted oil (Soxhlet extraction, methylene chloride).

[f]*tr in this and other tables = numerical values less than 0.5%.*

reported. Shea fat contains β-amyrin (12), lupeol (12), butyrospermol (13, 14), and parkeol (15, 16) as unsaponifiable components.

It would be seen from the scattered investigations described above that the compositions of the fractions of sterol, 4-methylsterol, and triterpene alcohol from *Theaceae* oils, alfalfa, garden balsam, and spinach seed oils and shea fat differ markedly from those of common vegetable oils (1, 2). This work is undertaken in an attempt to study fully the compositional patterns of the unsaponifiable fractions from these oils. It is interesting that the triterpene alcohol fractions from all oils examined, except spinach seed oil, contain scarcely any of cyclopropane containing triterpene alcohols, such as cycloartenol and 24-methylenecycloartanol, which are common constituents of many vegetable oils.

EXPERIMENTAL PROCEDURES

Materials

The seed oils of alfalfa, garden balsam and spinach were prepared from the corresponding dried seeds by Soxhlet extraction with methylene chloride. The oil contents of these dried seeds are indicated in Table I.

Camellia, sasanqua (I and II) and tea seed oils and shea fat were commercially prepared oils. Table I shows saponification and iodine values and unsaponifiables contents of these oils.

Authentic specimens of cholesterol and a sterol fraction consisting of campesterol, stigmasterol, and β-sitosterol were supplied by Riken Vitamin Oil Co., Tokyo, Japan. Δ^5- and Δ^7-Avenasterols were not prepared in the form of individual pure sterols; the fractions rich in Δ^5- and Δ^7-avenasterols were prepared from castor and safflower oils, respectively. α-Spinasterol from tea wax (3), lophenol from cholesterol (T. Iida, T. Tamura, and T. Matsumoto, unpublished), cycloeucalenol from rice bran oil (17), gramisterol (24-methylenelophenol), and citrostadienol from wheat germ oil (18), cycloartanol and cyloartenol from rapeseed oil (19), 24-methylenecycloartanol from wheat germ oil (20), and β-amyrin from tea wax (3) were prepared in this laboratory. Obtusifoliol, α-amyrin, butyrospermol and lupeol, and 24-dihydroparkeol were supplied as gifts.

Table II shows relative retention time (RRT) for these authentic specimens and the sterols identified in this work. RRT is expressed by the ratio of the retention time for the substance under examination to the retention time (30 min) for β-sitosterol throughout this article.

Saponification

The oil (100 g) in 1000 ml alcoholic 1.0 N potassium hydroxide was refluxed for 1 hr under nitrogen. The reaction mixture was diluted with 2000 ml distilled water and the unsaponifiable material was extracted with one 1000 ml portion and three 800 ml portions of isopropyl ether (IPE). The IPE extracts were combined, washed 5 times with 700 ml portions of distilled water and dried over anhydrous sodium sulfate, and the IPE was removed by evaporation.

TABLE II

Relative Retention Times of Sterols, 4α-Methylsterols, and Triterpene Alcohols (4,4-Dimethylsterols)

Compounds	Position of double bond	Other structural characteristics	RRT[a]
Sterols (cholestane series)			
Cholesterol	5	—	0.61
Brassicasterol	5, 22	24R-CH_3	0.70
Campesterol	5	24R-CH_3	0.81
Stigmasterol	5, 22	24S-C_2H_5	0.88
24-Methylcholest-7-enol	7	24-CH_3	0.95
β-Sitosterol	5	24R-C_2H_5	1.00
α-Spinasterol	7, 22	24S-C_2H_5	1.03
Δ^5-Avenasterol	5, 24(28)	24Z-C_2H_4	1.12
Δ^7-Stigmastenol	7	24R-C_2H_5	1.18
Δ^7-Avenasterol	7, 24(28)	24Z-C_2H_4	1.32
4α-Methylsterols			
Lophenol	7	—	0.83
Obtusifoliol	8, 24(28)	14α-CH_3, 24-CH_2	0.94
Cycloeucalenol	24(28)	14α-CH_3, 24-CH_2, 9:19-cyclo[b]	1.11
Gramisterol	7, 24(28)	24-CH_2	1.13
Citrostadienol	7, 24(28)	24Z-C_2H_4	1.52
4,4-Dimethylsterols			
Lanostane series			
24-Dihydroparkeol	9(11)	—	1.01
Cycloartanol	—	9:19-cyclo	1.02
Parkeol	9(11), 24	—	1.22
Cycloartenol	24	9:19-cyclo	1.24
24-Methylenelanost-9(11)-enol?	9(11), 24(28)	24-CH_2	1.37
24-Methylenecycloartanol	24(28)	24-CH_2, 9:19-cyclo	1.38
Euphane (20-epi-Tirucallane) series			
Butyrospermol	7, 24	—	1.17
Pentacyclic Triterpene Alcohols			
β-Amyrin			1.13
α-Amyrin			1.28
Lupeol			1.33

[a]RRT = relative retention time. Retention time for β-sitosterol (30 min) is taken as 1.00. See text for operating conditions of gas liquid chromatography.

[b]9:19-cyclo = 9:19-cyclopropane ring.

Hydrogenation of Side Chain Double Bond of Sterols

Platinum oxide (10 mg) was added to the solution of sterol or sterol acetate (10-30 mg) in anhydrous ether (20 ml), and hydrogenation was achieved with stirring for 3 hr at room temperature at a slight positive pressure of hydrogen.

TLC

Unsaponifiable material was fractionated on 20 x 20 cm plates coated with a 0.5 mm layer of Wakogel B-10 (Wako Pure Chemical Industries Ltd., Osaka, Japan). The sample (30 mg) was applied uniformly along a line 1.5 cm from one edge of the plate and developed with hexane-ether (8:2) for 1 hr with a Toyo continuous flow development preparative TLC. The plate was sprayed with a 0.01% rhodamine-6G solution in ethanol and observed under UV light (3600 A). Separate zones (4 zones) containing less polar compounds, triterpene alcohols, 4-methylsterols, and sterols, respectively, were cut off and quantitatively extracted with ether. The ether extracts from the zones containing triterpene alcohol, 4-methylsterol, and sterol were dessicated for subsequent gas liquid chromatography (GLC).

Argentation TLC

TLC plates (20 x 20 cm) coated with a 0.5 mm layer of 20% silver nitrate impregnated Silica Gel HF254 (E. Merck, Darmstadt, Germany) were used for a further fractionation of sterol acetate mixtures of triterpene alcohol acetate mixtures.

Argentation Column Chromatography

The argentation column was prepared in a

TABLE III

Compositions of Sterol Fractions of *Theaceae* and Some Other Vegetable Oils Determined by Gas Liquid Chromatography

	Percent composition									
	I	II		III	IV	V	VI	VII		
RRT[a] of individual sterols[b]	0.61	0.70	0.82	0.88	0.95	1.03	1.18	1.32	1.41	Others
Oil, camellia	tr[c]			1	3	45	45	6		
Sasanqua I			tr	tr	2	30	62	6		
Sasanqua II	tr	tr	tr		1	27	58	7	2	
Tea		tr			4	59	33	2	tr	2
Alfalfa			tr	2	7	46	40	5	tr	
Garden balsam				tr	6	59	27	8		
Spinach	tr	tr	2	2	8	35	45	8		
Shea fat			2		6	43	37	11	tr	1

[a]RRT = relative retention time. Retention time for β-sitosterol (30 min) is taken as 1.00.

[b]I = cholesterol, II = brassicasterol, III = stigmasterol, IV = 24-*methylcholest-7-enol*, V = α-spinasterol, VI = Δ^7-stigmastenol, and VII = Δ^7-avenasterol.

[c]See Table I.

similar manner as proposed by Vroman and Cohen (21). The aqueous solution of silver nitrate (7.5 g/150 ml water) was shaken with silicic acid (30 g, Mallinkrodt, 100 mesh) to a homogeneous suspension. The suspension was dried in vacuo and then activated at 110 C for 3 hr in vacuo. The activated material was mixed thoroughly with one-half its wt of diatomaceous earth, Hyflo Super-Cel (Johns-Manville Product Corp., New York, N.Y.) and then packed into the column with hexane.

GLC

Triterpene alcohol, 4-methylsterol, and sterol fractions were analyzed with a Shimadzu GC-5A gas chromatograph equipped with a flame ionization detector. The chromatograph was fitted with a 2 m glass column, 3 mm inside diameter, packed with 1.5% OV-17 on Gas Chrom-Z, 80-100 mesh. The column was operated generally at 250 C with nitrogen at 50 ml/min as carrier gas. Detector temperature was 280 C. Under these conditions, the retention time of β-sitosterol was 30 min.

Combined GC-Mass Spectrometry

Analyses were performed on a Shimadzu LKB-9000 gas chromatograph-mass spectrometer (GC-MS). The chromatograph was fitted with a 2 m glass column, 3 mm inside diameter, packed with 1.5% OV-17 on Gas Chrom-Z, 80-100 mesh. Operating conditions: column 246 C, helium carrier gas at 30 ml/min, molecular separator 290 C, ion source 310 C, ionizing voltage 70 eV, trap current 60 μA, and accerelated high voltage 3500 V.

NMR spectra were measured with a JNM-C-60 HL (60 MHz, Japan Electron Optics Laboratory Co., Tokyo, Japan) in deuterochloroform. The spectra were calibrated against internal tetramethylsilane as 0 ppm. IR spectra were taken in KBr tablets on a Type IRA-2, diffraction grating IR spectrophotometer (Japan Spectroscopic Co., Tokyo, Japan).

All mp were determined on a Micro mp apparatus (Yanagimoto Seisakusho Ltd., Kyoto, Japan) and indicated as uncorrected values. Recrystallizations were carried out in acetone-methanol unless otherwise stated.

RESULTS

Unsaponifiables

The unsaponifiables from the vegetable oils were separated into four fractions, less polar compounds (hydrocarbons, aliphatic alcohols, etc.) (fraction 1), triterpene alcohols (fraction 2), 4-methylsterols (fraction 3), and sterols (fraction 4) by TLC, with fraction 1 closest to the solvent front and fraction 4 to the starting line. Table I shows the content of unsaponifiables in the oils and the percentage yield of four fractions from the unsaponifiables by TLC. Fraction 2 (triterpene alcohol fraction) was a major one for most of the oils except alfalfa and spinach seed oils which yielded fraction 4 as a predominant one. The yield of fraction 3 was the smallest of all oils examined.

Sterols

As shown in Table III, the qualitative and quantitative compositions determined by GLC of the sterol fractions from each oil were similar. These fractions consisted of Δ^7-sterols, such as 24-methylcholest-7-enol (IV), α-spinasterol (V), Δ^7-stigmastenol (VI), and Δ^7-avenasterol (VII), among which α-spinasterol (V) and Δ^7-stigmastenol (VI) are the most predominant

in the fractions from all oils. Cholesterol (I), brassicasterol (II), stigmasterol (III), and Δ^7-avenasterol (VII) in Table III were identified by comparing their RRT with those of the reference specimens. Identification of sterols IV, V, and VI was carried out as described below.

Sterol-IV (24-methylcholest-7-enol): Sterol-IV is presumed to consist of a Δ^7-isomer of campesterol on the analogy of the separation factors of Δ^7/Δ^5 double bond by GLC: 1.18 for Δ^7-stigmastenol/ β-sitosterol; 1.17 for α-spinasterol/stigmasterol; 1.17 for sterol-IV/ campesterol (Table II). Mass spectrum of sterol-IV in the sterol fraction from spinach seed oil gave large molecular ion (M^+) at m/e 400 (relative intensity: 56%) and other ions at m/e 385 (M-CH_3) (26%), 382 (M-H_2O) (8%), and 367 (M-CH_3-H_2O) (10%). Peaks also were found at m/e 273 (30%) and 255 (100%), corresponding to loss of side chain alone and with loss of water. The peak at m/e 246 (19%) involves loss of side chain and 27 extra mass units (22). The strong peak at m/e 229 (49%) results from a net loss of OH from the ion m/e 246. These fragmentation patterns are characteristic for Δ^7-sterols (22). Consequently, sterol-IV is considered to consist of 24-methylcholest-7-enol. However, a weak ion at m/e 414 (6%) was observed in the mass spectrum of sterol-IV, indicating the possible presence of β-sitosterol (mol wt 414) in the sterol fraction of spinach seed oil, though the peak corresponding to β-sitosterol could not be observed on the GLC curve.

Sterol-V (α-spinasterol) from tea seed oil: Unsaponifiable material (2280 mg) of tea seed oil was fractionated by preparative TLC to yield the sterol fraction (390 mg). Sterol acetate (206 mg) prepared from the TLC refined sterol fraction (273 mg) was separated into two zones by argentation TLC on plates of 20% silver nitrate impregnated Silica Gel HF254 (eluent, hexane-benzene 9:1, time 80 min). The zone closer to the starting line was rich in sterol-V acetate and the zone closer to the solvent front was rich in sterol-VI acetate. The zone rich in sterol-V acetate was cut off from the plates and refined by repeated argentation TLC to give sterol-V acetate: 28 mg, 96% pure by GLC, mp 182-183 C, RRT 1.38. IR spectrum (KBr) of the acetate provided 1731 and 1245 cm^{-1} (acetate); 1368 cm^{-1} (geminal dimethyl) (23); 845, 830, and 798 cm^{-1} (trisubstituted double bond); and 970 cm^{-1} (*trans*-disubstituted double bond [24,25]). NMR spectrum ($CDCl_3$) (chemical shifts are given in ppm downfield from internal tetramethylsilane standard) gave singlets at 0.56 (C-18 methyl), 0.81 (C-19 methyl), and 2.00 ppm(-$OCOCH_3$); doublets centered at 0.84 (J 7.2 Hz) (C-26 and C-27 dimethyl), and 1.02 ppm (J 6.6 Hz) (C-21 methyl ?); and multiplets at 4.33-4.93 (>CHOAc) and 4.93-5.17 ppm (-CH=CH- and >C=CH-). The values of the chemical shift of singlet signals of C-18 and C-19 methyl groups were in good agreement with those of Δ^7-sterols (18, 26-28). The pattern of the spectrum was in accord with that of α-spinasterol acetate reported by Sucrow (24). The mass spectrum of the free sterol (RRT 1.03) derived from the acetate by hydrolysis showed M^+ at m/e 412 (21%), with other ions at m/e 397 (M-CH_3) (13%) and 379 (M-CH_3-H_2O) (3%). The ion at m/e 369 (17%) involving loss of 43 mass units from the M^+ and m/e 351 (9%) derived from m/e 369 with loss of H_2O. This fragmentation was suggested by Knights (22) to involve the isopropyl group at the end of the side chain and appears to be characteristic for Δ^{22}-sterols. The peak at m/e 271 (M-side chain-2H) (100%) formed based peak with other ions at m/e 255 (M-side chain-H_2O) (63%), 246 (M-side chain-27) (27%) and 229 (M-side chain-27-OH) (28%). The fragmentation pattern of the mass spectrum of sterol-V was basically similar to that of authentic α-spinasterol (mol wt 412). Hence, sterol-V is recognized as α-spinasterol (24S-ethylcholesta-7, 22-dienol).

Sterol-VI (Δ^7-stigmastenol) from tea seed oil: The zone rich in sterol-VI acetate was refined further by argentation TLC to give sterol-VI acetate: 22 mg, 95% pure by GLC, mp 158.5-159.5 C, and RRT 1.58. IR spectrum showed 1731 and 1249 cm^{-1} (acetate); 1368 cm^{-1} (geminal dimethyl) (23); and 845, 830, and 798 cm^{-1} (trisubstituted double bond) (29). NMR spectrum gave singlets at 0.54 (C-18 methyl), 0.82 (C-19 methyl), and 2.00 ppm (-$OCOCH_3$); doublet centered at 0.84 ppm (J 6.0 Hz) (C-29 methyl) and multiplets at 4.44-4.80 (>CHOAc) and 4.93-5.17 ppm (>C=CH-). The values of the chemical shift of singlet signals of C-18 and C-19 methyl groups were in accord with those of α-spinasterol (V) and Δ^7-sterols (18, 26-28). Sterol-VI acetate was converted into the free sterol-VI (RRT 1.18) and analyzed by GC-MS. A large M^+ appeared at m/e 414 (88%) and other ions at m/e 339 (M-CH_3) (32%), 396 (M-H_2O) (3%), and 381 (M-CH_3-H_2O) (8%). Peaks also were found at m/e 273 (M-side chain) (29%) and 255 (M-side chain-H_2O) (100%). Moreover, peaks were observed at m/e 246 (M-side chain-27) (17%) and 229 (M-side chain-27-OH) (41%). These fragmentation patterns are quite similar to those for Δ^7-stigmastenol identified in the sterol fraction of sunflower oil (1). Consequently, sterol-VI is recognized as Δ^7-stig-

TABLE IV

Compositions of 4-Methylsterol Fractions of *Theaceae* and Some Other Vegetable Oils Determined by Gas Liquid Chromatography

	Percent composition												
					I		II				III		
RRT[a] of individual 4-methylsterols[b]	0.64	0.81	0.85	0.91	0.94	0.99	1.13	1.28	1.36	1.46	1.52	1.62	Others
Oil, camellia	5		tr[c]	2	13		23	3	16	14	20		4
Sasanqua I	tr	tr		6	15	3	8	12	9	11	33	2	1
Sasanqua II	tr	tr		5	12	3	7	6	12	12	29	5	9
Tea	tr	tr		11	5	4	8	6	10	16	35	5	
Alfalfa					10	tr	26	9	12	14	29	tr	
Garden balsam			2		44	6	16	4	8		20	tr	
Spinach	1		tr	2	24	7	46[d]	tr	2	1	17		
Shea fat	1				22		4	5	3	tr			65[e]

[a]RRT = relative retention time. Retention time for β-sitosterol (30 min) is taken as 1.00.

[b]I = Obtusifoliol, II = gramisterol and/or cycloeucalenol, and III = citrostadienol.

[c]See Table I.

[d]RRT 1.11 (mainly cycloeucalenol).

[e]RRT 1.57, 4%; 2.02, 2%; 2.39, 39%; and 2.79, 20%.

mastenol (24R-ethylcholest-7-enol).

4-Methylsterols

The compositions of the 4-methylsterol fractions from individual oils determined by GLC are shown in Table IV. Obtusifolio l (I) gramisterol (II), cycloeucalenol (II, from spinach seed oil), and citrostadienol (III) were identified by comparing their RRT with those for the corresponding authentic specimens. The 4-methylsterol fraction of shea fat gave a characteristic composition, in which citrostadienol was not detected under the conditions of GLC, while a large quantity of some unidentifed components eluting after citrostadienol was observed. 4-Methylsterols from spinach seed oil gave a large quantity of a fraction with RRT 1.11 (Table IV) which coincides with that of cycloeucalenol rather than that of gramisterol. Hence, this fraction is considered to consist largely of cycloeucalenol accompanied with a small quantity of gramisterol.

Triterpene Alcohols

Table V shows the compositions of the triterpene alcohol fractions from the vegetable oils analyzed by GLC. As pointed out in the previous paper (2), the compositions of the triterpene alcohol fractions from most of the oils are complicated, and it is sometimes difficult to determine precisely the peak area of individual GLC peaks. The GLC peak-i is presumed to be cycloartanol (RRT 1.02) and 24-dihydroparkeol (RRT 1.01).

Triterpene alcohol-ii (β-amyrin) from tea seed oil: The acetate (616 mg) of the triterpene alcohol fraction of tea seed oil was fractionated by argentation TLC on the plates of 20% silver nitrate impregnated Silica Gel HF254 (eluent, hexane-benzene 2:3, time 60 min) to give the 4 principal zones: 1, 2, 3 and 4 in the order of decreasing distance from starting line. Zone 1 (107 mg) was refined by repeated argentation TLC yielding the triterpene acetate-ii:22 mg, 100% pure by GLC, mp 243-245 C, and RRT 1.34. IR spectrum of the acetate gave 1730 and 1249 cm^{-1} (acetate); 1390 and 1366 cm^{-1} (geminal dimethyl); and 822, 810, and 796 cm^{-1} (trisubstituted double bond). NMR spectrum of the acetate gave the signals at 0.75, 0.86, 0.88, 0.93, 0.95, 1.04, 1.10, and 1.43 ppm; singlet at 2.05 ppm (-$OCOCH_3$); and multiplets at 4.27-4.69 (>CHOAc) and 4.78-4.91 ppm (>C=CH-). Mass spectrum of the free triterpene alcohol-ii (mp 195-197 C, RRT 1.13) showed M^+ at m/e 426 (6%) with other ions at m/e 411 (M-CH_3) (6%) and 393 (M-CH_3-H_2O) (1%). Peak also was found at m/e 218. This might be furnished by retro Diels-Alder fragmentation at

the $\Delta^{12(13)}$-bond of molecular ion and characteristic for the α-(ursene) or β-amyrin (oleanene) series (30). The RRT and the basic fragmentation patterns of the triterpene alcohol-ii agree well with those of the authentic specimen of β-amyrin (mol wt 426). The triterpene alcohol-ii (RRT 1.13) is, therefore, regarded as β-amyrin.

Triterpene alcohol-iii (butyrospermol) from tea seed oil: Zone 2 (145 mg) was purified by repeated argentation-TLC giving the triterpene acetate-iii (40 mg, 94% pure by GLC), mp 146-147 C and RRT 1.38. IR spectrum of the acetate gave the bands at 1729 and 1245 cm^{-1} (acetate); 1386 and 1366 cm^{-1} (geminal dimethyl); and 841, 832, and 816 cm^{-1} (trisubstituted double bond). NMR spectrum of the acetate-iii showed singlets at 0.77 (C-18 methyl) (31), 0.82 (C-19 methyl) (31), 0.85 (C-14β methyl ?), and 2.02 ppm (-$OCOCH_3$); doublet centered at 0.90 ppm (J 5.4 Hz) (C-21 methyl ?), 0.94 and 0.98 ppm (C-4α and C-4β dimethyl) (31), 1.61 and 1.67 ppm (C-26 and C-27 dimethyl), and multiplets at 4.31-4.66 (>CHOAc) and 4.85-5.31 ppm (>C=CH-). The mass spectrum of the acetate gave M^+ at m/e 468 (10%) and other peaks at m/e 453 ($M-CH_3$) (69%), 408 ($M-CH_3COOH$) (2%), 393 ($M-CH_3COOH-CH_3$) (74%), and base peak at m/e 69. The mass spectrum of the free triterpene alcohol-iii (RRT 1.17, mp 107-110 C) indicated M^+ at m/e 426 (9%) with other ions at m/e 411 ($M-CH_3$) (67%) and 393 ($M-CH_3-H_2O$) (22%). The peaks also were found at m/e 313 (M-side chain-2H) (7%), 297 (M-side chain-H_2O) (4%), 273 (M-side chain-42 [part of ring D]) (7%), 271 (M-side chain-42-2H) (9%), 259 (M-side chain-42-14 [C-14 methyl]) (15%), 255 (M-side chain-42-H_2O) (9%), and 241 (M-side chain-42-14-H_2O) (12%) and base peak at m/e 69. The RRT and the fragmentation patterns of the triterpene alcohol-iii were identical with those of the authentic specimen of butyrospermol (mol wt 426, mp 105-108 C). Hence, the triterpene alcohol-iii is recognized as butyrospermol. Dihydrobutyrospermol acetate (RRT 1.13, mp 138-140 C) was prepared from the triterpene acetate-iii by hydrogenation. Dihydrobutyrospermol (RRT 0.95) obtained by hydrolysis of the acetate gave M^+ at m/e 428 (8%) with other ions at m/e 413 ($M-CH_3$) (100%), 410 ($M-H_2O$) (2%) and 395 ($M-CH_3-H_2O$) (71%). Moreover, peaks were observed at m/e 297 (4%), 273 (20%), 259 (14%), 255 (6%), and 241 (10%).

Triterpene alcobol-vi (lupeol) from tea seed oil: Zone 4 (42 mg) was refined by repeated argentation TLC giving triterpene alcohol-vi acetate (23 mg, GLC purity 100%) with mp

TABLE V

Compositions of Triterpene Alcohol Fractions of *Theaceae* and Some Other Vegetable Oils Determined by Gas Liquid Chromatography

RRT[a] of individual triterpene alcohols[b]	Percent composition													
	0.73	0.80	0.86	0.90	i 1.02	1.06	ii 1.13	iii 1.17	iv 1.24	v 1.28	vi 1.33	vii 1.38	1.44	1.65	Others
Oil, camellia		tr[c]	2	1	tr	3[d]	41	15[d]			33		3	2	
Sasanqua I	tr	tr	2	2	tr	4[d]	44	12[d]	tr		32		3	1	
Sasanqua II			tr	2		8[d]	37		3		37		8	5	
Tea		tr	3	2	tr	5[d]	36	24[d]			24		5	1	
Alfalfa	tr		tr				53			10[d]	36			tr	
Garden balsam						2[e]	43	+[d]		52	2		1[d]		
Spinach	tr		1		tr	2		11[d]	25		4[d]	56			1
Shea fat					tr		8	26	+	46	16[d]	+	1	1	2

[a]RRT = relative retention time. Retention time for β-sitosterol (30 min) is taken as 1.00.

[b]i = cycloartanol (RRT 1.02) and 24-dihydroparkeol (RRT 1.01), ii = β-amyrin, iii = butyrospermol, iv = cycloartenol (RRT 1.24) and parkeol (RRT 1.22), v = α-amyrin, vi = lupeol (RRT 1.33) and the unknown triterpene alcohol (RRT 1.32), vii = 24-methylenecycloartanol (RRT 1.38) and 24-methylenelanost-9(11)-enol ? (RRT 1.37).

[c]See Table I.

[d]Roughly calculated values.

[e]+ = positive, uncalculable.

218-219 C and RRT 1.57. IR spectrum of the acetate showed the bands at 1730 and 1245 cm^{-1} (acetate); 3080, 1640, and 876 cm^{-1} (terminal methylene); and 1393 and 1366 cm^{-1} (geminal dimethyl). NMR spectrum gave the signals at 0.82, 0.87, 0.96, 1.04, 1.27, 1.41, and 1.46 ppm; singlets at 1.70 (CH_2=C-CH_3) and 2.03 ppm (-OCOCH_3); and multiplets at 4.28-4.77 (>CHOAc), and 4.59-4.67 ppm (>C=CH_2). The free triterpene alcohol-vi (RRT 1.33) showed IR bands at 3370 and 1032 cm^{-1} (-OH); 3080, 1645, and 883 cm^{-1} (terminal methylene); and 1383 cm^{-1} (geminal dimethyl). Mass spectrum of the triterpene alcohol gave M^+ at m/e 426 (11%) with other principal ions at m/e 411 (M-CH_3) (6%), 207 (35%), 189 (77%), and 95 (100%). The strong m/e 189 and 207 ions are most characteristic for the spectrum of lupeol (30). The RRT and the basic fragmentation patterns agreed with those of authentic lupeol (mol wt 426, mp 213.5-216.5 C). Consequently, the triterpene alcohol-vi is recognized as luepol.

Triterpene alcohol-vi (unknown) from tea seed oil: An unknown triterpene acetate (50 mg, 96% pure by GLC, RRT 1.58), mp 124-125 C (methanol), also was isolated by repeated argentation TLC of zone 3 (163 mg). The acetate gave IR bands at 1733 and 1250 cm^{-1} (acetate), 1371 and 1381 cm^{-1} (geminal dimethyl), and 845 and 826 cm^{-1} (trisubstituted double bond). NMR spectrum showed singlets at 0.78 (C-18 methyl) and 0.81 ppm (C-19 methyl), and 1.61 and 1.68 ppm. Since two peaks similar to those for triterpene alcohol-vi acetate (1.61 and 1.68 ppm) has been observed for butyrospermol acetate from tea seed oil described above and also for cycloartenol reported by Tamura, et al. (20), the peaks 1.61 and 1.68 ppm in this case are reasonably ascribed to the C-26 and C-27 dimethyl protons associated with C-24 (25) double bond, as in the case of butyrospermol acetate and cycloartenol. The NMR spectrum pattern of the triterpene alcobol-vi acetate is almost identical with that of butyrospermol acetate. Mass spectrum of the free triterpene alcohol (RRT 1.32) showed M^+ at m/e 426 (11%) with other principal ions at m/e 411 (M-CH_3) (60%), 393 (M-CH_3-H_2O) (4%), 297 (4%), 273 (6%), 271 (10%), 259 (16%), and 255 (7%). The hydrogenated triterpene alcohol (M^+ m/e 428, RRT 1.09) still showed the absorbance of trisubstituted double bond at 1665, 841, 825, 815, and 808 cm^{-1} on the IR spectrum. Consequently, the triterpene alcohol-vi has two double bonds in the molecule, one at $\Delta^{24(25)}$, and the other trisubstituted one in the ring system and may be regarded as an isomer of butyrospermol.

Moreover, two acetate fractions obtained in the course of repeated argentation TLC were found to be uniform individual acetates by GLC analyses, one having mp 242-243 C and RRT 1.98 (RRT for free terpene alcohol 1.65) and the other having RRT 1.28 (RRT for free terpene alcohol 1.06).

Triterpene alcohol-v (α-amyrin) from shea fat: The triterpene alcohol fraction of shea fat was acetylated, and the acetate was treated with acetone to remove the acetone insoluble material (presumably polyisoprenes). The acetone-soluble material (1500 mg) was recrystallized from acetone-methanol (1:2). The crystalline solids obtained were purified by argentation TLC to give the triterpene alcohol-v acetate (103 mg), mp 221-223 C, RRT 1.51. IR spectrum showed the bands at 1734 and 1243 cm^{-1} (acetate); 1388 and 1367 cm^{-1} (geminal dimethyl); and 826, 817, and 803 cm^{-1} (trisubstituted double bond). NMR spectrum gave the signals at 0.81, 0.88, 0.99, 1.02, and 1.08 ppm; singlet at 2.04 ppm (-OCOCH_3); and multiplets at 4.33-4.73 (>CHOAc) and 5.05-5.21 ppm (>C=CH-). The mass spectrum of the free triterpene alcohol-v (RRT 1.28) showed M^+ at m/e 426 (5%) and other ions at m/e 411 (M-CH_3) (3%), 218 (M-$C_{14}H_{24}O$) (100%), 203 (M-$C_{14}H_{24}O$-CH_3) (34%), and 189 (28%). The strong peak at m/e 218 is characteristic for the α- or β-amyrin series (30). RRT and the basic fragmentation patterns of this compound were essentially similar to those for authentic α-amyrin (mol wt 426). Hence the triterpene alcohol-v is identifed as α-amyrin.

The material (1079 mg) recovered from the acetone-methanol filtrate was fractionated to ten fractions (1-10) on argentation column chromatography (packing 120 g). Fraction 1 (eluted with 1500 ml hexane, 300 mg) was a mixture of α- and β-amyrin acetates, and fraction 2 (600 ml hexane-benzene [H-B] 95:5, 23 mg) also was a mixture of several unknown components. Fraction 3 (200 ml H-B 90:10, 40 mg) gave a component (RRT 1.96) with mp 240.5-243 C. IR spectrum of this triterpene acetate showed the bands at 1734 and 1244 cm^{-1} (trisubstituted double bond). NMR spectrum gave the signals at 0.74, 0.85, 0.89, 0.97, 1.06, 1.17, and 1.65 ppm; singlet at 2.04 ppm (-OCOCH_3); and multiplets at 4.19-4.63 (>CHOAc) and 5.09-5.37 ppm (>C=CH-). Mass spectrum of the free triterpene alcohol (RRT 1.65) showed M^+ at m/e 426 (14%); other ions at m/e 411 (M-CH_3) (5%), 408 (M-H_2O) (5%), and 393 (M-CH_3-H_2O) (1%); and base peak at m/e 189. This triterpene alcohol might be regarded as identical compound with that isolated from tea seed oil (acetate RRT 1.96, mp

242-243 C) described above. Fraction 4 (700 ml H-B 90:10, 97 mg) was a mixture of several components, and fraction 5 (700 ml H-B 90:10, 321 mg) was also a mixture of butyrospermol (iii) acetate and lupeol (vi) acetate. The mixture was fractionated to two zones by argentation TLC (eluent H-B 6:4, 35 min). A zone closer to the solvent front was refined by repeated argentation TLC to give butyrospermol (iii) acetate (59 mg, 96% pure by GLC), mp 147-148 C, and RRT 1.38. The other zone after refining gave lupeol (vi) acetate (72 mg), mp 221-221.5 C, and RRT 1.57.

Triterpene alcohol-iv (parkeol) from shea fat: Fraction 6 (600 ml H-B 80:20, 50 mg) provided a uniform component (RRT 1.46), mp 170-171 C. IR spectrum indicated the bands at 1734 and 1241 cm^{-1} (acetate); 1390 and 1371 cm^{-1} (geminal dimethyl); and 820, 811, 800, and 792 cm^{-1} (trisubstituted double bond). NMR spectrum of the acetate showed singlets at 0.65 (C-18 methyl), 0.74 (C-19 methyl), 1.08 (C-14 methyl ?) and 2.04 ppm ($-OCOCH_3$); 0.87 and 0.89 ppm (C-4α and C-4β dimethyl); 1.60 and 1.68 ppm (C-26 and C-27 dimethyl); and multiplets at 4.19-4.80 (>CHOAc) and 4.80-5.30 ppm (>C=CH-). Mass spectrum of the free triterpene alcohol-iv (RRT 1.22) showed M^+ at m/e 426 (10%) with other ions at m/e 411 ($M-CH_3$) (41%) and 393 ($M-CH_3-H_2O$) (22%). Peaks also were observed at m/e 313 (M-side chain-2H) (28%), 297 (M-side chain-H_2O) (5%), 273 (M-side chain-42) (9%), 259 (M-side chain-42-14) (12%), and 255 (M-side chain-42-H_2O) (6%) and base peak at m/e 69.

Triterpene alcohol-i (24-dihydroparkeol) derived from parkeol (iv): The dihydrotriterpene acetate-i (RRT 1.21) derived from the triterpene acetate-iv by hydrogenation showed mp 174.5-176 C. IR spectrum indicated the bands at 1733 and 1243 cm^{-1} (acetate); 1392 and 1370 cm^{-1} (geminal dimethyl); and 822, 811, and 792 cm^{-1} (trisubstituted double bond). NMR spectrum showed singlets at 0.66 (C-18 methyl), 0.75 (C-19 methyl), 1.08 (C-14 methyl ?), and 2.05 ppm ($-OCOCH_3$); doublet centered at 0.88 ppm (J 5.4 Hz) (C-26 and C-27 dimethyl); 0.90 ppm (C-4α and C-4β dimethyl); and multiplets at 4.27-4.69 (>CHOAc) and 5.12-5.35 ppm (>C=CH-). Mass spectrum showed M^+ at m/e 428 (13%) and other principal ions at m/e 412 (100%), 395 (82%), 297 (6%), 273 (21%), 259 (12%), and 255 (17%). The RRT and the spectra patterns of IR and NMR of the triterpene acetate-i and the mass spectrum of the free triterpene alcohol-i were basically similar to those for authentic lanost-9(11)-enol acetate (24-dihydroparkeol acetate, mp 178.5-179.5 C) and lanost-9(11)-enol (24-dihydroparkeol, mol wt 428), respectively. Hence, the triterpene alcohol-iv is recognized as parkeol (lanosta-9[11],24-dienol).

A triterpene acetate (RRT 1.72, mp 137-138 C) was isolated as fraction 7 (500 ml of H-B 80:20, 16 mg). The free triterpene alcohol (RRT 1.47) therefrom showed M^+ at m/e 426 on the mass spectrum. Fraction 8 (300 ml H-B 70:30, 35 mg) gave a triterpene acetate (RRT 1.64, mp 158-159.5 C). IR bands were observed at 1723 and 1250 cm^{-1} (acetate); 3085, 1642, and 890 cm^{-1} (terminal methylene); 1390 and 1372 cm^{-1} (geminal dimethyl); and 813 and 800 cm^{-1} (trisubstituted double bond). NMR spectrum of the acetate showed singlets at 0.66 (C-18 methyl), 0.75 (C-19 methyl), 1.07 (C-14 methyl ?), and 2.02 ppm ($-OCOCH_3$); doublet centered at 1.02 ppm (J 6 Hz) (C-26 and C-27 dimethyl) (20); 0.87 and 0.88 ppm (C-4α and C-4β dimethyl); and multiplets at 4.24-4.73 (>CHOAc), 4.63-4.66 (>C=CH_2), and 5.04-5.30 ppm (>C=CH-). The free triterpene alcohol (RRT 1.37) and the corresponding dihydrotriterpene alcohol (RRT 1.33) gave the M^+ at m/e 440 and 442, respectively, on the mass spectra. The free alcohol with RRT 1.37 is presumed to be 24-methylenelanost-9(11)-enol, the occurrence of which in vegetable oils has not yet been reported.

Fraction 9 (100 ml of H-B 60:40, 8.4 mg) provided a triterpene acetate (RRT 1.33, mp 154-155 C), and the triterpene alcohol (RRT 1.12) therefrom showed the M^+ at m/e 440 on the mass spectrum. Fraction 10 (300 ml H-B 50:50, 19 mg) also gave a triterpene acetate (RRT 1.46, mp 145-147 C). IR spectrum indicated the presence of acetyl, terminal methylene, and geminal dimethyl groups and trisubstituted double bond in the molecule. NMR spectrum showed the signals at 0.86, 0.98, 1.09, 1.26, 1.45, 1.60, and 2.10 ppm; singlet at 2.02 ppm ($-OCOCH_3$); and multiplets at 4.32-4.79 (>CHOAc) and 4.65-4.79 ppm. The free triterpene alcohol (RRT 1.22) therefrom showed M^+ at m/e 440 on the mass spectrum. Identification of these unknown triterpene alcohols found in this work is still going on.

Triterpene alcohols-iv (cycloartenol) and -vii (24-methylenecycloartanol) from spinach seed oil: Triterpene alcohols-iv and -vii in the triterpene alcohol fraction from the unsaponifiables of spinach seed oil showed RRT 1.24 (acetate RRT 1.52) and 1.38 (acetate RRT 1.69), respectively. The ΔR_{Ac} values (RRT of acetate vs. RRT of free alcohol) for these two alcohols are found to be 1.23 and 1.22, respectively. It has recently been found in this laboratory (T. Itoh, T. Tamura, T. Iida, and T. Matsumoto,

Steroids, in press) that the 4,4-dimethylsterols containing 9:19-cyclopropane ring or saturated ring system, such as cycloartenol and 4,4-dimethylcholestanol, give the ΔR_{Ac} values of 1.22-1.23, while the 4,4-dimethylsterols with the double bond in the ring system, such as lanosterol (Δ^8), parkeol ($\Delta^{9(11)}$), and 4,4-dimethylcholest-7-enol, show somewhat smaller values of 1.19-1.21. Both the triterpene alcohols (iv and vii) in spinach seed oil are, therefore, considered to possess 9:19-cyclopropane ring or saturated ring system from their ΔR_{Ac} values. RRT of these triterpene alcohols are identical with those of authentic cycloartenol (RRT 1.24) and 24-methylenecycloartanol (RRT 1.38), respectively. The mass spectrum of the triterpene alcohol-iv showed the M^+ at m/e 426 (6%) with other principal ions at m/e 411 ($M-CH_3$) (25%), 408 ($M-H_2O$) (8%), 393 ($M-H_2O-CH_3$) (8%), 365 ($M-H_2O-C_3H_7$) (10%), 339 ($M-H_2O-C_5H_9$) (12%), 297 (M-side chain-H_2O) (11%), 286 ($M-C_9H_{16}O$) (15%), 271 ($M-C_9H_{16}O-CH_3$) (10%), and 175 (M-$C_9H_{16}O$-side chain) (32%), and base peak at m/e 69. A fragment peak observed at m/e 286 in the spectrum corresponds to the loss of ring-A plus 1 hydrogen atom from molecular ion and is characteristic for the spectra of the alcohols possessing 9:19-cyclopropane ring (32, 33). The fragmentation pattern on the mass spectrum of this compound is basically similar to that of cycloartenol (mol wt 426). Hence, the triterpene alcohol-iv from spinach seed oil is recognized as cycloartenol.

The mass spectrum of the triterpene alcohol-vii (RRT 1.38) showed the M^+ at m/e 440 (8%) with other ions at m/e 425 (18%), 422 (19%), 407 (35%), 379 (18%), 353 (11%), 297 (11%), 300 ($M-C_9H_{16}O$) (21%), and 175 ($M-C_9H_{16}O$-side chain) (65%) and base peak at m/e 95. A fragment peak observed at m/e 300 is characteristic for the spectra of the alcohols possessing 9:19-cyclopropane ring (32, 33). The principal fragmentations are essentially similar to those for 24-methylenecycloartanol (mol wt 440). Consequently, the triterpene alcohol-vii from spinach seed oil reasonably is identified as 24-methylenecycloartanol.

DISCUSSION

As noted in the previous article (1) on the unsaponifiables of 19 vegetable oils, most of the oils gave the sterol fraction as a major one of the unsaponifiables. However, in this study, the unsaponifiables of three *Theaceae* oils, garden balsam seed oil, and shea fat provided the triterpene alcohol fraction as the major one. In particular, the composition of the unsaponifiables for the *Theaceae* oils may be regarded as a characteristic pattern.

Although it already is know that three Δ^5-sterols: campesterol, stigmasterol, and β-sitosterol form the major component sterols in many vegetable oils (1, 7, 8, 34, 35), the sterols in the three *Theaceae* and other 4 oils examined in this study consist exclusively of their Δ^7-isomers (24-methylcholest-7-enol, α-spinasterol, and Δ^7-stigmastenol) and Δ^7-avenasterol. This also may be considered as a characteristic pattern of these vegetable oils. Although the GLC curves of the sterol fractions from these oils showed no peak of β-sitosterol, which is most predominant in many vegetable oils (1, 7, 8, 34, 35), a weak molecular ion of a sterol (M^+ m/e 414), presumably β-sitosterol, was observed on the mass spectrum of the sterol-IV in the sterol fraction, suggesting the presence of β-sitosterol in spinach seed oil. The occurrence of β-sitosterol, though in minute proportions at most, in other oils examined in this study also is probable.

Although previous workers (7, 8) have indicated the occurrence of β-sitosterol in tea seed oil, this sterol was not detected in tea seed oil by GLC performed in this study. The previous authors (7, 8) identified the sterol by GLC alone, and the possibility is not excluded that the GLC peak of α-spinasterol was taken for that of β-sitosterol. In the previous paper (1), a small amount of unidentified sterol (RRT 0.95) was observed in the sterol fraction of safflower oil. This sterol (RRT 0.95) now is found to be almost undoubtedly identical with 24-methylcholest-7-enol from spinach seed oil in this study. The unknown sterol, reported by Eisner and Firestone (34) to occur in the sterol fraction of safflower oil, also might be regarded as 24-methylcholest-7-enol.

The 4-methylsterol compositions of three *Theaceae*, alfalfa, and garden balsam seed oils are similar to one another. The 4-methylsterol fraction of spinach seed oil is characterized by its high content of cycloeucalenol, a 4-methylsterol containing 9:19-cyclopropane ring. The 4-methylsterol fraction of shea fat is distinguished from others by its high content of several unknown components (65% total 4-methylsterol fraction), whereas it does not contain citrostadienol, a most common constituent in the 4-methylsterol fraction of many vegetable oils (2), in amounts detectable by GLC. It also may be noted here that while lophenol (RRT 0.83) has been reported to occur in some plant tissues (36-39) and some *Cruciferae* oils (40), it could not be detected in the oils examined in this study, as well as those previously studied (2).

The triterpene alcohol fractions from three *Theaceae* and three other oils, except spinach seed oil, contain pentacyclic triterpene alcohols, such as α- and β-amyrins and lupeol, and also butyrospermol, a euphane series alcohol, as predominant components. Spinach seed oil and most of the previously investigated common vegetable oils (2) contain cycloartenol and 24-methylenecycloartanol, two lanostanes with 9:19-cyclopropane ring, as the major components of their triterpene alcohol fractions. The occurrence of α-amyrin in the triterpene alcohol fraction of shea fat was not reported in the earlier experiments (12-16); in this work, however, this compound is proved to be the most predominant component of the triterpene alcohol fraction. The presence of parkeol and a triterpene alcohol, presumably 24-methylenelanost-9(11)-enol, in the triterpene alcohol fraction of shea fat is vertified by their isolation (from the triterpene alcohol fraction) in spite of the fact that their GLC peaks are too obscure for identification.

It is important to note that the spinach seed oil contains a large quantity of cycloartenol and 24-methylenecycloartanol in the triterpene fraction and a large amount of cycloeucalenol and obtusifoliol in the 4-methylsterol fraction. Hence the following pathway of sterol biosynthesis in the spinach plant may be suggested: squalene → cycloartenol → 24-methylenecycloartanol → cycloeucalenol → obtusifoliol → gramisterol. This is the same as a general pathway of biosynthesis of higher plant sterol proposed by Goodwin, et al. (41-45). On the other hand, the compositions of the triterpene alcohol and 4-methylsterol fractions of shea fat appear to indicate the elimination of 9:19-cyclopropane ring at the stage of triterpene alcohol giving the $\Delta^{9(11)}$-triterpene alcohols as the components of lanostane series. Hence, the sterol biosynthesis in this case appears to proceed mainly through the following pathway: squalene → (cycloartenol ?) → parkeol → 24-methylenelanost-9(11)-enol → obtusifoliol → gramisterol.

Previous authors (7, 8) detected cycloartenol and 24-methylenecycloartanol in tea seed oil by GLC; in this study, however, the occurrence of these compounds was not recognized by GLC or by other procedures. Two unidentified triterpene alcohols (RRT 1.17 and 1.32) found in the majority of the oils investigated in the previous study (2) are now identified as butyrospermol (iii) and lupeol (vi), respectively, in this study. As indicated in Table V, individual GLC peaks are not always attributed to one uniform component. For example, the GLC peak-vi of the triterpene fraction from tea seed oil is attributable to either one or both of two components, one of which is lupeol (RRT 1.33) and the other is unknown euphane series alcohol (RRT 1.32). Moreover, it may be added here that each component in the following set of two or three component triterpene alcohols has the same GLC behavior with each other: β-amyrin (RRT 1.13) and the unknown alcohol (RRT 1.12) isolated from shea fat as acetate (fraction 9); cycloartenol (RRT 1.24), parkeol (RRT 1.22), and the unknown alcohol (RRT 1.22) isolated from shea fat as acetate (fraction 10); and 24-methylenecycloartanol (RRT 1.38) and the alcohol (RRT 1.37), presumably 24-methylenelanost-9(11)-enol. Although more detailed investigations are necessary for the precise determination of the compositions of the unsaponifiables, it is concluded from results of this study that there exists a close similarity in the composition of the unsaponifiables, including sterols, 4-methylsterols, and triterpene alcohols of the three *Theaceae* oils.

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Pyrrolidides for Mass Spectrometric Determination of the Position of the Double Bond in Monounsaturated Fatty Acids

BENGT Å. ANDERSSON[1], and **RALPH T. HOLMAN**, The Hormel Institute, University of Minnesota, Austin, Minnesota 55912

ABSTRACT

Mass spectra of pyrrolidides of monounsaturated straight chain fatty acids are presented and discussed. The spectra of pyrrolidides contain mainly ions from the polar part of the molecule. This gives simple spectra from which double bond positions can be deduced directly. If an interval of 12 atomic mass units is observed between the most intense peaks of clusters of fragments containing n and n-1 carbon atoms of the acid moiety, the double bond occurs between carbons n and n+1 in the molecule This rule is valid for double bonds occurring at positions Δ^5-Δ^{15} in an 18-carbon chain and has been applied to acids having 10-24 carbon atoms.

INTRODUCTION

The location of double bonds in fatty acids by mass spectrometry has been approached in many ways which have been summarized in reviews (1, 2). Under electron impact, double bonds have a tendency to migrate (3), so it has not been possible previously to locate the unsaturation directly without chemical modification at the double bond. Vetter, et al., (4) suggested a derivatization of fatty acids with pyrrolidine as a possible solution, because the amide group has a charge stabilization effect upon the fatty acid moiety. Recently Bohlmann and Zdero (5) studied the mass spectra of piperidides to deduce the structure of naturally

[1]Hormel Fellow.

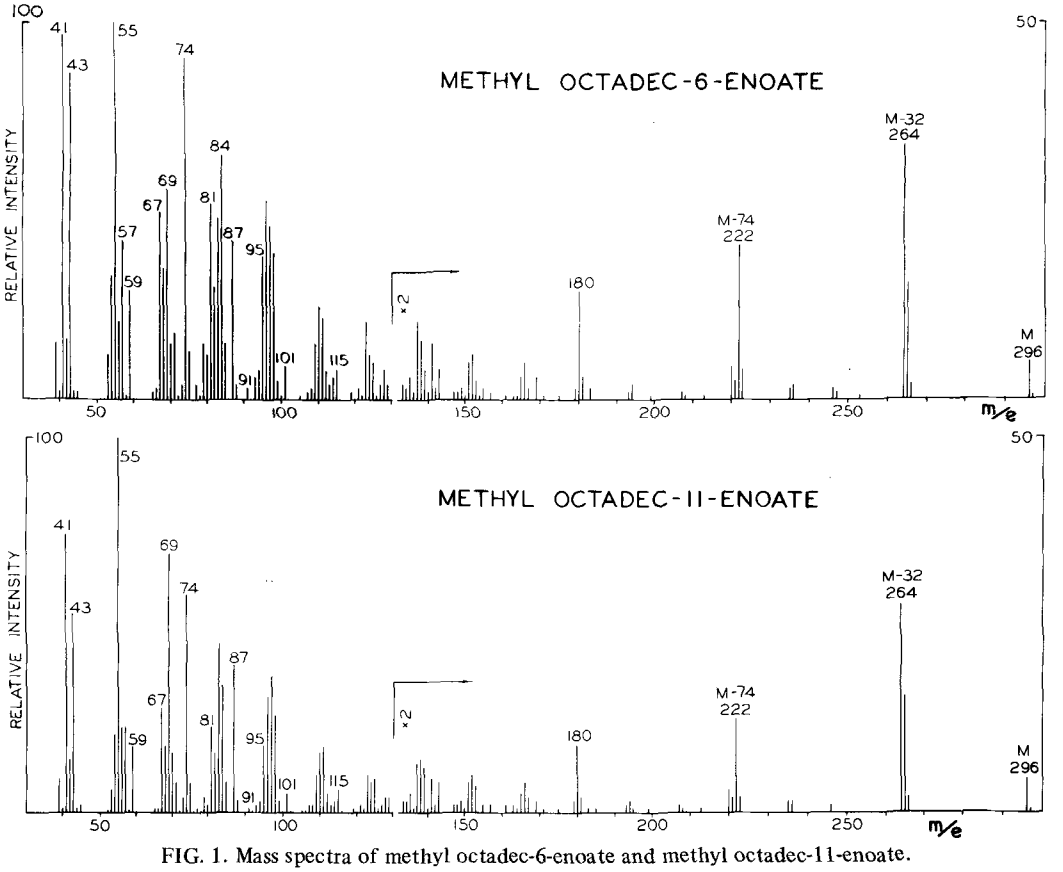


FIG. 1. Mass spectra of methyl octadec-6-enoate and methyl octadec-11-enoate.

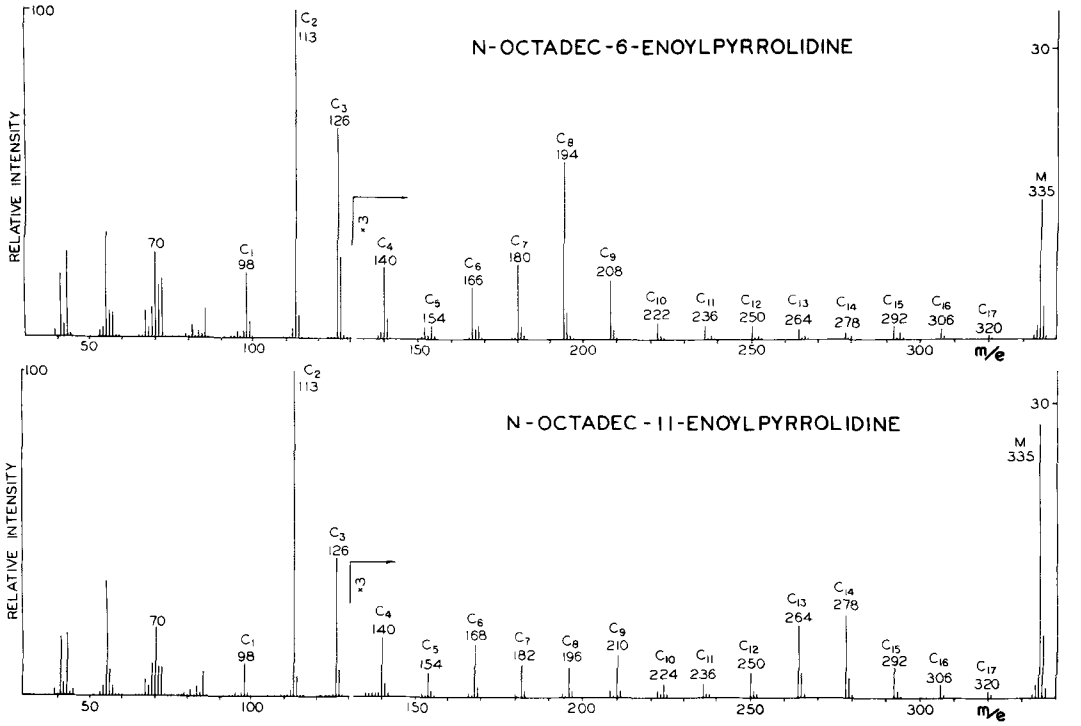


FIG. 2. Mass spectra of N-octadec-6-enoylpyrrolidine and N-octadec-11-enoylpyrrolidine.

occurring amides of conjugated unsaturated compounds. We report here a study of the low resolution mass spectra of pyrrolidine derivatives of a series of isomeric straight chain unsaturated fatty acids which confirms and implements the prediction of Vetter (4).

EXPERIMENTAL METHODS

Most of the methyl esters of the unsaturated fatty acids were supplied by the Lipids Preparation Laboratory, The Hormel Institute, Austin, Minn., although a few of the octadecenoic acid isomers were obtained from the preparations of Gunstone and Ismail (6).

The pyrrolidides were prepared in a quantitative yield on a microscale in the following way: 10 μl fatty acid methyl ester was dissolved in 1 ml freshly distilled pyrrolidine (Aldrich Chemical Co., Milwaukee, Wisc.) and 0.1 ml acetic acid. The mixture was heated to 100 C in a sealed tube for half an hr and cooled to room temperature. The conversion from methyl ester to amide is followed conveniently by gas liquid chromatography (GLC). The amide so formed is taken up in methylene chloride and washed with dilute hydrochloric acid and with water. After drying with magnesium sulfate, evaporation, and purity check by thin layer chromatography (TLC), the amide was ready for mass spectrometry (MS).

The mass spectra were obtained on a Hitachi Perkin-Elmer RMU-6D single focusing instrument operating at an ionization potential of 70 eV. The samples were introduced through an all glass heated inlet system at 175 C. The gas chromatograph was a Barber Coleman 5000 instrument equipped with an all glass 1.80 m x 2 mm column containing 3% OV-1 on Chromosorb W (HP), 80-100 mesh. Column temperature was 230 C and the flow 30 ml argon/min. The GLC-MS combination used GLC conditions the same as mentioned above. The pyrrolidine reaction mixture was injected directly onto the column and unreacted methyl ester separated readily from the pyrrolidine derivative (4).

RESULTS AND DISCUSSION

The mass spectra of the methyl ester and of the pyrrolidide of the same saturated fatty acid show similar cleavage patterns in the high mass region with peaks 14 atomic mass units apart derived from fragmentations at each bond (4). If the fatty acid is unsaturated, the spectrum of the methyl ester becomes more complicated, whereas the spectrum of the pyrrolidide remains simple.

Mass spectra of the methyl esters of petro-

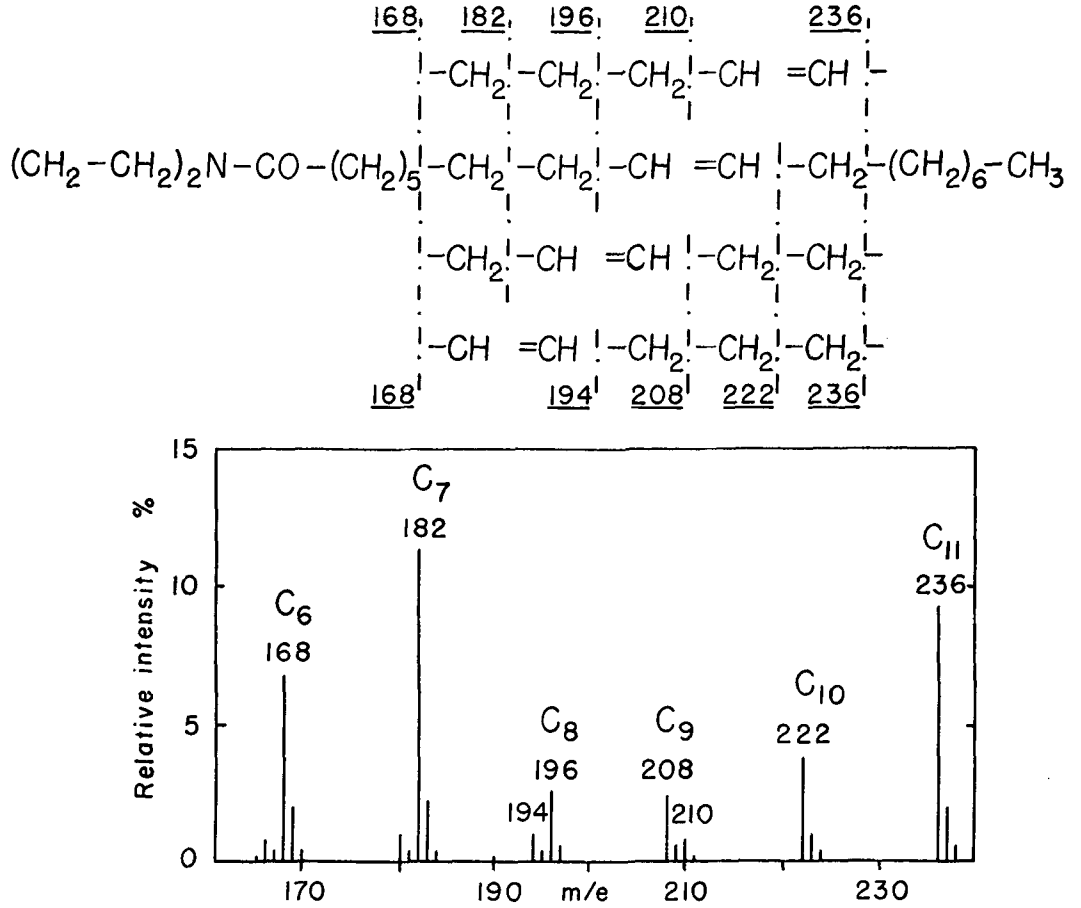


FIG. 3. Postulated fragmentations in N-octadec-9-enoylpyrrolidine and in its isomers in which the double bond has migrated after electron impact, shown above the partial spectrum obtained from N-octadec-9-enoylpyrrolidine.

selinic acid (6-18:1) and vaccenic acid (11-18:1) (7) are very much the same (Fig. 1) and very similar to the spectra of other isomeric monounsaturated fatty acid methyl esters (8-11 and B.Å. Andersson and R.T. Holman unpublished results).

If one compares the spectra of the pyrrolidide derivatives of the two acids (Fig. 2), there are clear differences. The spectra of both isomers show very pronounced fragments containing the polar part of the molecule (12, 13). No other main fragmentations disturb this pattern. In the case of the 6-18:1 isomer, the molecular ion *m/e* 335 yields a series of ions *m/e* 320, 306, 292, 278, 264, 250, 236, 222, 208, 194, 180, 166, 154, 140, 126, 113, and 98. In the case of the 11-18:1 isomer, the series is *m/e* 320, 306, 292, 278, 264, 250, 236, 224, 210, 196, 182, 168, 154, 140, 126, 113, and 98. Considering 6-18:1 and only the most prominent ion of each cluster, the interval between the fragments containing 5- and 6-carbon atoms from the fatty acid moiety (*m/e* 154 and 166) is 12 atomic mass units. For the 11-18:1 isomer, the same interval of 12 atomic mass units occurs between fragments which include carbons 10 and 11 of the fatty acid (*m/e* 224 and 236). From the mass spectra of all isomers of 18:1 from Δ^5-Δ^{15}, the following rule has been formulated: *If an interval of 12 atomic mass units, instead of the regular 14, is observed between the most intense peaks of clusters of fragments containing n and n-1 carbon atoms of the acid moiety, a double bond occurs between carbon n and n+1 in the molecule.* The 4-, 16- and 17-18:1 isomers have unique fragments that identify them. The characteristic ions are listed for each isomer in Table I. The mass spectra do not distinguish *cis*- from *trans*-isomers, exemplified in the case of the 9-18:1 isomers.

The simple cleavage pattern for the pyrro-

TABLE I

Key Fragments in the Spectra of Pyrrolidides of Monounsaturated Fatty Acids

Pyrrolidide	*m/e*	Relative intensity	*m/e*	Relative intensity	*m/e*	Relative intensity	*m/e*	Relative intensity	*m/e*	Relative intensity	*m/e*	Relative intensity	Molecular peak *m/e*	Molecular peak Relative intensity
cis-4-18:1	124	1.8	126	10.8	138	2.9	139	4.2	152	13.9	166	57.0	335	17.2
cis-5-18:1	126	6.3	138	.3	140	.6	152	.5	166	1.6	180	2.5	335	5.3
cis-6-18:1	140	7.7	152	1.5	154	1.7	166	5.6	168	1.5	180	8.1	335	14.3
cis-7-18:1	154	9.8	166	2.5	168	4.4	180	6.5	182	2.5	194	7.2	335	16.6
cis-8-18:1	168	13.2	180	2.0	182	4.5	194	2.4	196	.9	208	4.2	335	18.1
cis-9-18:1	182	11.4	194	1.0	196	2.5	208	2.3	210	.7	222	3.6	335	24.8
trans-9-18:1	182	11.0	194	1.2	196	2.4	208	2.2	210	.8	222	3.6	335	24.0
cis-10-18:1	196	7.4	208	1.0	210	2.0	222	1.8	224	.7	236	3.3	335	29.8
cis-11-18:1	210	4.9	222	1.1	224	1.6	236	1.5	238	.7	250	2.7	335	29.0
cis-12-18:1	224	4.8	236	.7	238	1.5	250	1.2	252	.8	264	3.0	335	33.8
cis-13-18:1	238	4.5	250	.6	252	1.3	264	1.1	266	.5	278	2.9	335	33.0
cis-14-18:1	252	3.9	264	.4	266	.9	278	1.2	280	.5	292	2.6	335	28.8
cis-15-18:1	266	3.1	278	.5	280	1.0	292	1.3	306	2.4	320	2.9	335	28.4
cis-16-18:1	266	2.3	278	.6	280	3.3	292	1.1	294	.7	306	1.2	335	26.0
cis-17-18:1	280	.9	292	.6	294	1.6	306	.4	308	.2	320	.4	335	8.1
cis-4-10:1	124	3.5	126	6.9	138	6.0	139	6.1	152	26.0	166	99.0	223	33.5
cis-9-14:1	182	12.0	194	1.4	196	2.7	208	2.6	210	1.0	222	4.0	279	28.0
cis-9-16:1	182	11.0	194	1.4	196	2.2	208	3.3	210	.8	222	3.8	307	26.0
cis-11-20:1	210	4.4	222	.9	224	1.8	236	1.4	238	.9	250	2.7	363	28.0
cis-13-22:1	238	4.4	250	2.1	252	2.8	264	2.0	266	1.2	278	4.2	391	37.0
cis-15-24:1	266	2.3	278	.6	280	1.1	292	1.0	294	.6	306	2.9	419	34.3

TABLE II

Metastable Peaks and Proposed Fragmentation Pathways of Pyrrolidides of 6-18:1 and 11-18:1

Petroselinic pyrrolidide		Vaccenic pyrrolidide		Fragmentation pathway
Calculated	Found	Calculated	Found	
305.7	305.8	305.7	305.8	335 → 320
279.5	279.6	279.5	279.7	335 → 306
254.5	254.6	254.5	254.6	335 → 292
230.7	230.9	230.7	230.9	335 → 278
208.1	208.2	208.1	208.3	335 → 264
186.6	186.8	186.6	186.8	335 → 250
166.3	166.5	166.3	166.4	335 → 236
		149.8	150.0	335 → 224
147.1	147.3	147.1	147.2	335 → 222
		131.6	131.8	335 → 210
129.2	129.4			335 → 208
		114.7	114.7	335 → 196
112.3	112.3			335 → 194
		98.9	99.0	335 → 182
96.7	96.9			335 → 180
		84.3	84.5	335 → 168
82.3	82.4			335 → 166
47.4	47.5	47.4	47.6	335 → 126
38.1	38.2	38.1	38.2	335 → 113

lidides can be explained in the following way: Metastable peaks (Table II) strongly indicate a direct cleavage from the molecular ion to each principal fragment in a cluster, all including the pyrrolidide group. However, no metastables were detectable for stepwise degradations. The double bond seems to move before the fragmentation occurs, preferentially towards the polar part of the molecule by one or more steps, as is shown in Figure 3 for oleoylpyrrolidide. However, a competitive fragmentation can occur if the amine group is removed (*m/e* 265) and ions of type $R\text{-}C{\equiv}O^+$ are formed. This fragmentation should be of minor influence (12) but must be investigated by high resolution MS.

If positional isomers were present in the pyrrolidide, their fragmentation patterns would contribute to these peaks in the spectrum which we interpret to be caused by isomerization under electron impact. Therefore, methyl octadec-6-enoate and the pyrrolidide derived from it were both ozonized and reduced to aldehydes (14). GLC of the products revealed dodecanal to be the only significant aldehyde product arising from the hydrocarbon end of the molecule. The two preparations had the same proportions of minor products occurring in the vicinity of possible homologous aldehydes, indicating that formation of the pyrrolidide had not measurably isomerized the acid moiety. This conclusion was confirmed by the observations that pyrrolidides synthesized via the acid chloride or via carbodiimide coupling had the same mass spectra as pyrrolidide synthesized as described above. Moreover, other tertiary amides of the same unsaturated acid have the same fragmentation pattern as shown here for pyrrolidides (B.Å. Andersson, W.H. Heimermann, and R.T. Holman, unpublished data). Thus, the conditions during the formation of the pyrrolidide do not appear to shift the double bond nor explain the ions which we believe to arise from isomerization under electron impact.

The 4-18:1 isomer has its characteristic series of fragments *m/e* 126, 139, 152. The 15- and 16-18:1 isomers have almost identical fragmentation patterns, the only difference being that in the case of the 15-18:1 isomer, the fragment, including the carbon atom in position 14 (*m/e* 280), is smaller than the fragment with 1 carbon less (*m/e* 266); and, for the 16-18:1 isomer, the intensities of the mentioned fragments are in reverse order. The 17-18:1 isomer has its series of fragments *m/e* 294, 306, and 320 which distinguishes it from the other isomers.

The rules developed on the isomers of 18:1 are true for other unsaturated acids. The spectra of the pyrrolidides of 4-10:1, 9-14:1, 9-16:1, 11-20:1, 13-22:1, and 15-24:1 were all interpretable by the same rules (Table I). The base peak in all spectra of the pyrrolidides is *m/e* 113. It is formed by a McLafferty rearrangement (Fig. 4) which was proven by Duffield and Djerassi (12) who used deuterium labeling of very short chain fatty acids. We have

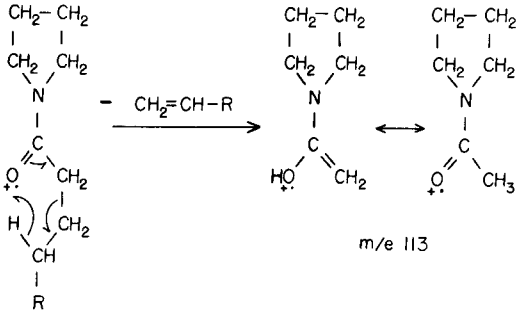
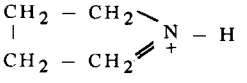
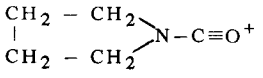


FIG. 4. McLafferty rearrangement in a fatty acid pyrrolidide.

confirmed this rearrangement by study of the spectrum of the pyrrolidide of 4,4-dideuterio octadecanoic acid. A metastable peak at *m/e* 38.1 indicates also the pathway $335^+ \rightarrow 113^+ + 222$. This peak moves to *m/e* 38.3 for 9,12-18:2, indicating the rearrangement $333^+ \rightarrow 113^+ + 220$. The fragment *m/e* 70 has been shown to have the structure (12):



and the *m/e* 98 fragment to be:



Other major peaks in the low mass region derived mainly from cleavage of the pyrrolidide ring have been discussed by other authors (12, 13).

Pyrrolidides offer several advantages for the structural analysis of fatty acids. The derivative is prepared easily quantitatively in a one step reaction on less than mg quantities. By derivatizing the carboxyl group, the reaction is equally quantitative regardless of the number of double bonds or other groups in the molecule. Solubility problems set no limits on the extent of the reaction as is the case with oxidative derivatization of polyunsaturated acids. In our hand, preparation of pyrrolidides of polyunsaturated acids having up to four double bonds offered no difficulties. The rules for interpreting mass spectra of pyrrolidides apply to a wide range of isomers and homologs of monoenoic acids. In unpublished work from this laboratory, these rules have been applicable to dienoic and trienoic acids. Mass spectra of pyrrolidides of acetylenic, cyclopropane-, branched, deuterated, and other fatty acids are more easily interpreted than are spectra of corresponding methyl esters, because only one fragmentation pattern occurs. Thus, pyrrolidides may become a general analytical tool, permitting GLC-MS structural analysis of a wide range of structures occurring in a single sample, minimizing purification steps and derivatization procedures.

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Modification of In Vitro Rat Adrenal Corticosteroidogenesis by Dietary Fat[1]

PETER O. EGWIM[2] and **F.A. KUMMEROW**,[3] The Burnsides Research Laboratory, University of Illinois, Urbana, Illinois 61801

ABSTRACT

Three groups of male weanling Holtzman rats were maintained, ad libitum, for 4 and 8 weeks on 1 of 3 diets: Purina Chow, hydrogenated soybean fat, and milk fat diets. The fats were added at the level of 36% total calories in the case of the hydrogenated soybean and milk fat diets. Adrenal homogenates prepared from tissues of each group of animals at the end of the dietary periods were used to measure the relative abilities to synthesize corticosteroids from endogenous substrates. Endogenous free cholesterol levels were found adequate to sustain the level of corticosteroids obtained. No concomitant cholesteryl ester hydrolysis was observed under the experimental conditions used. The adrenal synthetic ability for the three dietary groups was in the order milk fat > Purina Chow > hydrogenated milk fat. This order appeared not to be a reflection of the essential fatty acid status of the animals in the three dietary groups. The possible basis for this trend, and the implications of the findings for carbohydrate, protein, and lipid metabolism in the animal are indicated.

INTRODUCTION

In previous studies, we showed that the concentrations of rat adrenal-free and esterified cholesterol, as well as the fatty acid compositional pattern of the cholesteryl esters, were modified by feeding diets containing 10% and 20% partially hydrogenated soybean fat (1). Furthermore, the total ω-9 long chain fatty acids in the cholesteryl esters was significantly higher in these animals compared to animals fed similar diets but supplemented with 2% corn oil (1) or animals fed other linoleate-adequate fat diets (2).

In an attempt to find out how these modifications might affect the cholesterol and cholesteryl ester-related functional activities of the adrenals, we measured the relative abilities of adrenal homogenates from rats fed 2 different fat diets—hydrogenated fat diet (HF) and milk fat (MF) for 4 and 8 weeks—to synthesize corticosteroids from endogenous substrates in vitro. Rats fed a regular ration of Purina Chow (PC) also were studied for purposes of comparison. The results of this investigation form the subject of this article.

MATERIALS AND METHODS

Male weanling Holtzman rats (15/dietary group), maintained, ad libitum, on 1 of the 3 diets, HF, PC, and MF, for 4 and 8 weeks were used in these experiments. The hydrogenated soybean fat and milk fat have been described previously (2) and were added to the basic fat-free stock (2) at the 18.6% level by wt (36% total calories) at the expense of sucrose. The

TABLE I

Fatty Acid Composition of the Dietary Fats[a]

Fatty acid	Hydrogenated fat	Milk fat	Purina Chow[b]
	wt % of total		
10:0	0.2	2.1	—
12:0	trace	4.6	—
14:0	0.2	8.3	2.2
14:1ω5	trace	4.4	—
16:0	8.1	23.3	19.8
16:1ω7	0.2	3.7	0.2
18:0	13.2	18.4	4.9
18:1ω9	76.5[c]	30.6	31.8
18:2ω6	1.0[d]	3.1	39.5
18:3ω3	trace	—	1.5
20:0	0.4	—	—
20:1ω9	0.2	1.4	0.3

[a]Each value is the mean of three individual determinations. Fatty acids are designated by chain length: number of double bonds, the first located at the ω-position (from methyl end) indicated.

[b]Fat content 5.24% (Ralston Purina Company, Checkerboard Square, St. Louis, Mo.).

[c]Average *trans*, as elaidate, from IR and capillary gas liquid chromatographic analyses, is 48.4% of total fatty acids. The *trans*-unsaturation was found from previous degradative studies (1) to be essentially at the 9,10 position. Double bond designations of other unsaturated fatty acids based upon prior evidence (1,2).

[d]Mixture of octadecadienoates (1).

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[2]Present address: Department of Biochemistry, University of Nigeria, Enugu Campus, E.C.S., Nigeria.

[3]Person to whom requests for reprints should be directed.

mean body wt with standard errors (7 rats for each period of feeding) of the different dietary groups after the 4 week and 8 week feeding periods, respectively, were as follows: HF, 177.0 ± 7.3; MF, 197.0 ± 8.9; PC, 201.6 ± 6.6 and HF, 230.5 ± 10.8; MF, 269.2 ± 6.5; PC, 272.0 ± 6.8. However, using Student's *t* test, these differences in body wt between groups were not significant, with the possible exception of HF group vs. PC group.

The fatty acid compositions of the fats present in the three diets are given in Table I. The MF diet had the most diverse fatty acid profile (chain length C_{10}-C_{20}), followed, in that order, by the PC and HF diets. The major fatty acids common to the three dietary fats were 16:0, 18:0, and 18:1. The 18:1 fraction in the HF diet was found to consist of 26.0% as oleate and 48.5% as the *trans*-isomer, elaidate. The lipids of the PC diet had a high content of linoleate (39.5%). The MF lipids had an adequate level of linoleate (3.1%) to meet the minimum essential fatty acid (EFA) requirements. The level of 1% 18:2 in the HF diet represents a mixture of isomers (1).

Fat-trimmed adrenals pooled from 6-7 animals were used. There was little variation in the average wt of the adrenal, irrespective of the dietary group (24.5-27.5 mg/adrenal). Each adrenal in the pool was cut in half before preincubation for 1 hr at 37 C, as described by Koritz and Peron (3,4), except that the preincubation medium was 50 ml 0.154 *M* NaCl solution containing 40 μmoles $NaHCO_3$ and 8.5 μmoles glucose/ml, adjusted to the optimum pH of 7.35 (4) with 1-2 drops of 1 *N* HCl. Preincubation has been found necessary for effective steroid production in vitro in response to exogenous adrenocorticotropin (ACTH) (5). Its use in these experiments ensured that any possible effects due to ACTH responses to the dietary treatments would not complicate the results.

Following the preincubation, homogenization was done in 6.2 ml solution (pH 7.35) containing 2.0 μmoles $NaCHO_3$ and 1.6 μmoles Ca^{++}/ml 0.154 *M* KCl. The amounts of corticosteroids released into the preincubation medium were determined as described below and were found to be small in all cases (1.5-2.0 μg/100 mg adrenal tissue).

For the incubation, each tube contained 1.0 ml adrenal homogenate and 1.0 ml incubation medium—0.154 *M* KCl solution containing 5.4 μmoles glucose-6-phosphate and 2.2 μmoles nicotinamide adenine dinucleotide (NADP) or, in place of these two components, 2.2 μmoles NADPH/ml 0.154 *M* KCl. Blank (control) tubes contained 1.0 ml heat-denatured homogenate. The total volume/tube was 2.0 ml, with a depth of ca. 1 cm. The incubation was for 1 hr at 38 C (in a metabolic shaker), after saturation of the medium with 95% O_2-5% CO_2 gas mixture and capping the tubes. The incubation was terminated by adding 3.0 ml chloroform-methanol, 2:1 (v/v). Protein was determined routinely by the biuret method (6).

The total lipids were extracted at the end of the incubation, first with 3.0 ml aliquots of chloroform-methanol, 2:1 (v/v) and then with 3.0 ml aliquots of methylene chloride, 2 times in each case. The combined extracts for each tube were reduced to dryness under nitrogen and the residue dissolved in 0.5 ml methylene chloride for storage. The total lipids were fractionated by thin layer chromatography (TLC) (1) against known standards (cholesteryl esters, triglycerides, free fatty acids, cholesterol, and corticosterone). The first solvent system used was chloroform-acetone, 80:20 (v/v), after which the solvent front region (R_f 0.80-0.95) was scraped off. Lipids were recovered from the scrapings by elution with chloroform-ether, 1:1 (v/v), and then were fractionated into classes by TLC using a second solvent system, high boiling petroleum ether-diethyl ether-glacial acetic acid, 80:20:1 (v/v). The cholesteryl esters, triglycerides, and free fatty acids were recovered separately by the usual procedure (1). The rest of the TLC plate from the development in the chloroform-acetone system was exposed momentarily to iodine vapor. The corticosterone band (R_f 0.23), cholesterol band (R_f 0.54), and two plain R_f regions below (R_f 0.10-0.15) and above (R_f 0.30-0.35) the corticosterone band were scraped off and the lipids eluted once with chloroform-methanol, 2:1 (v/v) and two times with methylene chloride. The combined extracts were taken to dryness under nitrogen and the lipid material redissolved in methylene chloride.

Cholesterol and cholesteryl ester contents of the adrenals were determined routinely by the colorimetric method of Sobez and Fernandez (7). Quantification of the cholesteryl esters was done by gas liquid chromatography, as described by Walker and Carney (8). Corticosterone and lipid materials recovered from the two plain R_f regions were determined colorimetrically after reaction with 2,5-diphenyl-3-(4-styrlphenyl)-tetrazolium-chloride (K & K Laboratories, Plainview, N.Y.)—an approach commonly referred to as the blue tetrazolium (BT) procedure, first described by Elliott and coworkers (9). The reagents were scaled up to a final volume of 5.0 ml. The final yellowish-reddish color was read at 510 nm (Bausch and

TABLE II

In Vitro Corticosteroid Synthesis from Rat Adrenal Endogenous Precursors[a]

Dietary group	μg/100 mg wet tissue[b]	
	Fed for 4 weeks	Fed for 8 weeks
Hydrogenated fat	7.3 (6.8- 7.8)[c]	6.0 (4.7- 7.2)
Purina Chow	12.5 (11.2-14.7)	10.1 (9.5-11.3)
Milk fat	26.3 (24.4-29.9)	20.2 (20.0-20.7)

[a]Each value is the mean net synthesis from 3 experiments, with 5-6 incubation tubes/experiment. A pool of adrenals from 6-7 animals was used/experiment.

[b]Net new synthesis = total corticosteroid (blue tetrazolium positive) determined after incubation minus the control (heat denatured homogenate) value. Control values: hydrogenated fat, 1.7; Purina Chow, 2.3; milk fat, 2.0 μg/100 mg wet tissue.

[c]Range of values from three experiments are in parentheses.

Lomb Spectronic 20) and the steroid concentration obtained from a standard curve prepared with corticosterone (Applied Science Labs., State College, Pa.). The combined steroid levels determined in the extracts from the two plain R_f regions were designated as the non-corticosterone steroids (non-CCS). All corticosteroid concentrations were expressed as μg/100 mg wet adrenal tissue.

RESULTS

The extent of production of corticosteroids from endogenous precursors by rat adrenal homogenates from animals in the three dietary groups is shown in Table II. For the animals fed the HF diet for 4 and 8 weeks, the figures for the net new synthesis correspond, respectively, to ca. 4.3 times and 3.5 times the original endogenous level of corticosteroids in the adrenals (1.7 μg/100 mg wet tissue). With respect to the animals fed the PC diet for 4 and 8 weeks, the values for the net synthesis were 5.4 times and 4.4 times, respectively, the original endogenous concentration of 2.3 μg/100 mg wet tissue. In the case of the animals fed the MF diet for 4 and 8 weeks, the corresponding values were 13.2 times and 10.1 times, respectively, the endogenous concentration of 2.0 μg/100 mg wet tissue.

Corticosterone was found to be the predominant corticosteroid of the rat adrenals by the assay procedure employed here. However, the second predominant and, sometimes equally prevalent, steroid produced by the rat adrenal is 18-hydroxy-11-deoxycorticosterone (18-OH-DOC), which, because it exists in the 20,18-hemiketal form (10) is BT negative and so would not be detected. The production of this steroid is enhanced by ACTH stimulation. Since a preincubation step was used, it is doubtful that any significant amounts of this steroid would be produced during the incubation. With this caveat in mind, calculations based upon the data from the TLC analyses showed that corticosterone constituted 87.6%, 86.4%, and 88.7% of the total rat adrenal BT positive corticosteroids for the animals fed, respectively, the HF diet, the PC diet, and the MF diet. It was the major BT positive corticosteroid arising

TABLE III

Concentrations of Adrenal Cholesterol and Cholesteryl Esters[a]

Compound[c]	Fed for 4 weeks[b]			Fed for 8 weeks		
	HF	PC	MF	HF	PC	MF
Cholesteryl esters (endogenous)	15.4 ± 0.5	6.9 ± 0.1	9.6 ± 0.3	13.2 ± 0.4	6.8 ± 0.2	9.8 ± 0.3
Cholesteryl esters (after incubation)	15.3 ± 0.6	7.0 ± 0.0	9.5 ± 0.3	13.3 ± 0.2	6.9 ± 0.2	9.6 ± 0.5
Cholesterol (endogenous)	1.2 ± 0.0	2.0 ± 0.1	2.4 ± 0.1	1.3 ± 0.2	1.4 ± 0.1	2.5 ± 0.2
Cholesterol (after incubation)	1.0 ± 0.0	1.7 ± 0.0	2.0 ± 0.3	1.1 ± 0.0	1.1 ± 0.1	2.1 ± 0.2

[a]Values are the means of 3 separate analyses involving adrenals pooled from 6-7 rats.

[b]HF = hydrogenated fat, PC = Purina Chow, and MF = milk fat.

[c]Given as mg/g wet adrenal tissue.

TABLE IV

Cholesteryl Ester Composition of Adrenals from Rats Fed Different Fat Diets for 8 Weeks[a]

Ester	Dietary groups, mole %		
	Hydrogenated fat	Purina Chow	Milk fat
12:0	---	---	1.9 (1.8- 2.0)
14:0	5.0 (3.8- 6.3)	3.7 (3.3- 4.2)	6.3 (6.1- 6.6)
14:1ω5	1.9 (1.6- 2.6)	0.5 (0.3- 0.8)	2.9 (2.7- 3.1)
16:0	10.5 (10.0-11.0)	9.9 (9.0-11.0)	10.9 (10.5-11.2)
16:1ω7	5.6 (4.5- 6.8)	2.6 (2.1- 3.1)	5.0 (5.0- 5.1)
18:0	3.4 (2.8- 4.1)	4.0 (3.1- 5.0)	6.1 (5.9- 6.4)
18:1ω9	37.3 (34.1-41.0)	12.3 (11.5-13.1)	23.5 (22.8-24.3)
18:2ω6	0.8 (0.6- 1.1)	6.8 (5.9- 7.8)	5.1 (4.9- 5.3)
20:1ω6	---	1.7 (1.0- 2.5)	1.2 (1.0- 1.4)
20:2ω6	2.7[c] (2.0- 3.5)	1.0 (0.8- 1.3)	0.8 (0.8- 0.8)
20:3ω9	5.6 (5.0- 6.3)	0.3 (0.3- 0.3)	2.5 (2.3- 2.9)
20:3ω6	0.3 (0.3- 0.3)	0.5 (0.3- 0.8)	1.1 (1.0- 1.3)
20:4ω6	6.4 (5.6- 7.4)	8.1 (7.5- 8.7)	5.5 (5.4- 5.7)
22:1ω6	---	10.6 (9.5-11.7)	4.2 (4.0- 4.4)
22:2ω9	4.2[c] (4.1- 4.5)	trace	3.6 (3.0- 4.2)
22:2ω6	trace	2.0 (1.6- 2.5)	trace
22:3ω9	3.4 (3.1- 3.8)	0.4 (0.3- 0.5)	2.0 (2.0- 2.0)
22:3ω6	trace	1.0 (0.6- 1.5)	2.0 (2.0- 2.1)
22:4ω6	4.3 (4.0- 4.7)	14.0 (13.1-15.0)	7.8 (7.3- 8.7)
22:5ω9	0.1 (0.1- 0.1)	1.3 (0.9- 1.4)	1.9 (1.2- 2.7)
22:5ω6	1.0 (0.7- 1.5)	7.9 (7.0- 8.9)	2.0 (1.5- 2.6)
22:5ω3	1.6 (0.8- 2.4)	---	---
22:6ω6	4.2 (4.0- 4.4)	10.9 (9.6-11.4)	3.0 (2.7- 3.4)
22:6ω3	1.3 (0.9- 1.5)	0.4 (0.3- 0.7)	trace

[a]Values are the means of 3 separate analyses, as described in the text, with range of values in parentheses. The cholesteryl esters are designated by the fatty acid moiety chain length:number of double bonds, the first located at the ω-position (from methyl end of the molecule).

[b]Represent mixtures of isomers; their identifies have been described (1).

from new synthesis.

The concentrations of adrenal free cholesterol and cholesteryl esters determined after the incubation for corticosteroid synthesis are presented in Table III. The cholesteryl ester concentrations before (endogenous levels) and after the incubation were found to be practically the same. With respect to the free cholesterol fractions, decreases, although small (ca. 15-21%), were observed in each case. The smallness of these decreases in comparison with some of the standard errors (Table III) raises doubts as to the significance of the differences between groups. Thus, although there is apparently a correlation between their respective quantitative values (expressed as μg/100 mg tissue) and the amount of corticosteroids synthesized during the incubation (Tables II and III), such a conclusion would be unwarranted on the basis of the data presented.

The fatty acid composition of the adrenal cholesteryl esters from the animals fed the different fat diets is shown in Table IV. The wide range of cholesteryl esters is obvious and is consistent with published work (1,8,11). The fatty acid compositions of the dietary fats (Table I) seemed to be reflected, to some extent, in the levels of the different cholesteryl esters, especially up to cholesteryl linoleate. Dietary fat modification of the cholesteryl esters of the longer chain fatty acids (C_{20}-C_{22}) manifested itself in the differences in the relative amounts of the major cholesteryl esters (cholesteryl-20:3ω9, -20:4ω6, -22:1ω6, -22:4ω6, -22:3ω9, -22:5ω6, and -22:6ω6). These fatty acyl designations were based upon previous evidence (1,2). Furthermore, the sole occurrence of mixtures of cholesteryl-20:2 isomers, and of cholesteryl-22:2 isomers in the adrenals of the animals fed the HF diet seems noteworthy. Their identities have been published elsewhere (1).

DISCUSSION

Our results confirm previous findings by others that rat adrenal homogenates are capable of corticosteroid synthesis, in vitro, utilizing the endogenous free cholesterol present in the adrenals (12). More importantly, it was shown that the ability for corticosteroidogenesis was dependent upon the nature of the antecedent

dietary fat. This ability was found to be in the order MF > PC > HF. Following the incubation for steroidogenesis, the total cholesteryl ester concentration in the adrenal homogenate remained, in all cases, practically unchanged, indicating no net cholesteryl ester hydrolysis under the conditions of the incubation.

It has been suggested that EFA deficiency leads to a decreased ability of rat adrenals to synthesize corticosteroids (11,13). Although the HF diet was low in linoleate (18:2ω6), the growth pattern and physical well-being of the animals fed this diet compared well with the other two dietary groups. Over the relatively short feeding periods employed, no overt EFA deficiency symptoms were observed. Besides, the adrenals seem to have the ability to conserve and metabolize even minimal amounts of dietary linoleate (1,2), resulting in the synthesis of the long chain polyunsaturated fatty acids of the ω-6 family. Consequently, the characteristic adrenal cholesteryl esters of the C_{20} and C_{22} polyunsaturated fatty acids (ω-6 family) were observed in the adrenals, irrespective of the dietary fat (Table IV). However, a summation of these cholesteryl ester types for each dietary group revealed the order PC > MF > HF (ca. 55%, 29%, and 15% total, respectively), in agreement with the order of linoleate levels in the dietary fats (Table I). Furthermore, it may be noted that the PC diet, which definitely contained the highest level of linoleate (EFA), led to a lower corticosteroidogenesis than the MF diet, which had a lower EFA content. Therefore, our results were probably not influenced by the EFA status of the three groups of animals.

It is not clear from the results why the PC diet led to a lower synthetic ability than the MF diet. One can only suggest that the more complete, or better balanced, fatty acid profile of the milk fat noted earlier may have something to do with it. The conversion of cholesterol to corticosteroids involves the side chain cleavage enzymes and a series of hydroxylases. The rate limiting step in this series of complex enzymatic reactions is believed to be the conversion of cholesterol to pregnenolone, which is also the site of action of the stimulation or acceleration of adrenal steroidogenesis by Ca^{++} (4) or cyclic adenosine 5′-monophosphate (3,14). That the adrenals from the animals fed the HF diet were the least effective may be related partly to the high levels of cholesteryl elaidate in the adrenals (19-22%) under the HF regimen (1) and partly to the relatively low level of total long chain characteristic, polyenoic cholesteryl esters of the ω-6 family in connection with the composition of the lipoprotein components of the key enzymes. A decreased capacity of energy yielding processes, leading to a decreased adenosine 5′-triphosphate synthesis (15), also may be involved. However, the data in this article do not provide much evidence bearing on the possible mechanisms by which these fat diets could affect corticosteroidogenesis.

Adrenal corticosteroids are involved in carbohydrate, protein, and fat metabolism (16). Their involvement in lipid metabolism pertains to their inhibitory effect upon lipogenesis and their lipolytic action upon adipose tissue and especially the liver, resulting in lipid mobilization. If our finding that rat adrenal corticosteroid synthesis is modified by the nature of the dietary fat holds under in vivo conditions, this could have important implications for some key aspects of carbohydrate, protein, and lipid metabolism in the animal. This aspect of the study needs to be investigated.

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Lipid Products Formed during Desaturation of [1-^{14}C] Stearyl CoA by Hen Liver Microsomes

CAROLINE T. HOLLOWAY and **P.W. HOLLOWAY**, Department of Biochemistry, University of Virginia, Charlottesville, Virginia 22901

ABSTRACT

A number of lipid products are formed during the desaturation of stearyl CoA by hen liver microsomes. This article presents an analysis of the products formed when [1-^{14}C] stearyl CoA is incubated with hen liver microsomes for various time periods. [1-^{14}C] Oleyl CoA was the first radioactive unsaturated product formed. Synthesis of phospholipids containing [1-^{14}C] oleic acid occurs only after the desaturase activity has begun to decline. The specific radioactivity of [1-^{14}C] oleyl CoA was similar to the specific radioactivity of [1-^{14}C] stearyl CoA at all time periods tested. The specific radioactivities of [1-^{14}C] oleic acid and phospholipids containing [1-^{14}C] oleic acid were much lower than that of the [1-^{14}C] stearyl CoA.

INTRODUCTION

The conversion of stearic acid into oleic acid is catalyzed by a microsomal enzyme, the stearyl CoA desaturase. The enzyme has been shown to be involved intimately with the electron transport chain of microsomes (1-3), as well as with lipid synthesizing activities (4-7). The desaturase has a requirement for lipid (8), but the exact nature of this requirement is still not clear. Holloway and Katz (9) suggest that the desaturase complex has two sites which require lipid for activity: one site between reduced nicotinamide adenine dinucleotide (NADH)-cytochrome b_5 reductase and cytochrome b_5 and the other site between cytochrome b_5 and oxygen. The latter lipid requirement may be a reflection of the involvement of lipid as a substrate in a final step of the desaturation of stearyl CoA, or it may reflect a requirement for lipid in a purely physical role. To define more clearly the role of lipid in the desaturase reaction, a study was made of the products formed during the desaturation of stearyl CoA. A partially purified subfraction of microsomes, P_2, as prepared by Holloway and Wakil (3), was used in these studies. An understanding of the mechanism of stearyl CoA desaturase activity, especially with regard to the lipid requirement, is essential before further purification of this enzyme can be attempted successfully.

Previous studies of the desaturase reaction by Gurr and Robinson (4) showed that, during incubation of [1-^{14}C] stearyl CoA with hen liver microsomes, a lipid-bound [^{14}C] oleate was formed and that the oleate was distributed among phosphatidylcholine, phosphatidylethanolamine, and either triglyceride or unesterified fatty acid. While this present work was in progress, Vijay and Stumpf (5) showed that stearyl CoA served directly as a substrate for the desaturase activity and that the product of the reaction appeared to be oleyl CoA. This article presents evidence that oleyl CoA is the first product formed during the desaturation of stearyl CoA by hen liver microsomes and that the formation of phospholipids by the action of acyltransferase on oleyl CoA occurs only after the desaturase activity has begun to decline. For simplicity, the terms oleate and stearate in this paper will be used to describe the acyl groups originally attached to glycerol or to CoA and finally quantitated as their methyl esters.

EXPERIMENTAL PROCEDURES

Microsomes: Hen liver microsomes were prepared as described previously (8). The microsomes were solubilized and manipulated as described previously to yield a purified particulate fraction, P_2 (3).

Preparation of [1-^{14}C] stearyl CoA: [1-^{14}C] Stearyl CoA was prepared enzymatically by the method of Kornberg and Pricer (10).

Enzyme Assay

Stearyl CoA desaturase was assayed using the following reaction mixture: $5 \cdot 10^{-2}$M potassium phosphate pH 7.2, $2 \cdot 10^{-4}$M NADH, $5 \cdot 10^{-5}$M [1-^{14}C] stearyl CoA (3900 cpm/nmole), 1.5 mg of P_2 protein, and water to a final volume of 1.0 ml. The enzymic reaction was initiated by the addition of protein, and the incubations were at 37 C in air for the times indicated in each experiment. Aliquots of 0.2 ml were removed for each time period tested.

Total desaturase activity: For the assay of desaturase activity, a 0.2 ml aliquot of the reaction mixture was removed and the reaction terminated by adding 0.2 ml 10% ethanolic KOH to the aliquot. The desaturase activity was

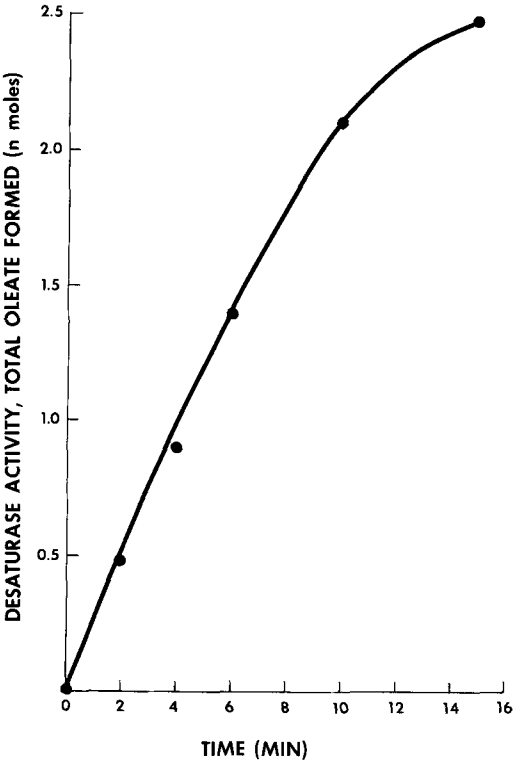

FIG. 1. Relation between time of incubation and of stearyl CoA desaturase activity. The assay conditions were those described in the text.

assayed as described previously (8).

Microsomal lipid analysis: For analysis of the microsomal lipid components labeled by [1-^{14}C] stearyl CoA during the desaturase reaction, a 0.2 ml aliquot of the incubation mixture was removed for each time period tested, and to it was added 0.6 ml chloroform-methanol (1:2). The reaction mixture was centrifuged at 1600 x g for 5 min. The protein precipitate was discarded, and all the radioactivity was associated with the supernatant. Lipids were analyzed by thin layer chromatography (TLC) on Silica Gel H-oxalate plates, using plates of 500μ thickness (11).

TLC

Resolution of saturated and unsaturated fatty acids: Fatty acid methyl esters were resolved into saturated and *cis*-monounsaturated esters by chromatography on a $AgNO_3$-impregnated Silica Gel H thin layer plate as described previously (8).

Resolution of microsomal lipids: The radioactive lipid components from the desaturase reaction mixture were resolved by chromatography on Silica Gel H-oxalate plates (11). The

TABLE I

Distribution of Radioactivity in Lipid Components of Hen Liver Microsomes Incubated with [1-^{14}C] Stearyl CoA[a]

Time (min)	Total counts per minute in each position[b] A	B	C	D
[1-^{14}C] Stearyl CoA	3900	150	78	900
2	2915	433	228	1471
4	2292	580	350	1798
6	2098	791	475	2189
10	863	982	449	2367
15	623	1061	874	2249

[a]Incubation conditions were as described in the text. Lipids were separated by thin layer chromatography and total radioactivity (cpm) determined as described in the text.

[b]R_f values for position A, 0.33; for position B, 0.47; for position C, 0.67; and for position D, 0.80.

chloroform-methanol extract was applied to the plate. Chloroform-methanol-water (45:35:10 v/v) was the developing solvent. Standards of [1-^{14}C] stearyl CoA, oleyl CoA, oleic acid, cholesterol, phosphatidylethanolamine, phosphatidylcholine, and lysophosphatidylcholine were used to characterize this system. R_fs for these compounds are: stearyl CoA, 0.29; oleyl CoA, 0.29; lysophosphatidylcholine, 0.41; phosphatidylcholine, 0.50; phosphatidylethanolamine, 0.69; oleic acid, 0.82; and cholesterol, 0.84.

For detection of [1-^{14}C] stearyl CoA, 1 cm segments were scraped from the silica gel plates, and these segments were assayed for radioactivity using a toluene scintillator. The phospholipids, fatty acids, and oleyl CoA were detected by exposing the plate to iodine vapor. Extreme care was taken to prevent the iodine vapor from contact with the material from the incubation mixture, if it was to be used for further analysis of unsaturated and saturated fatty acid compositions. Iodine vapor attacks the unsaturated fatty acids and produces derivatives which on Silica Gel $AgNO_3$ plates chromatograph with the hydroxy fatty acids (12).

Radioactive microsomal lipid components resolved on Silica Gel H-oxalate plates were fractionated further into saturated and unsaturated fatty acids. For this determination, a typical Silica Gel H-oxalate plate contained the following distinct samples: a standard lipid mixture (containing lysophosphatidylcholine, phosphatidylcholine, phosphatidylethanolamine, and cholesterol), [1-^{14}C] stearyl CoA, 100 μl aliquot of the microsomal reaction mixture, and 500 μl microsomal reaction mixture. After developing the plate in the chloroform-methanol-water (45:35:10) solvent, the

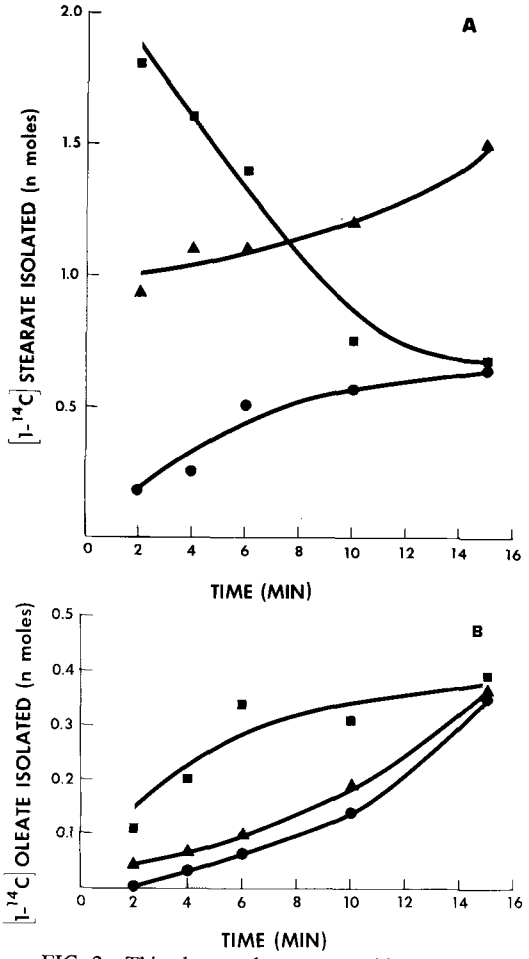

FIG. 2. Thin layer chromatographic analysis of lipid components of stearyl CoA desaturase incubation mixture. The isolation procedure and analysis are described in the text. A, [1-^{14}C] Stearate was isolated from the following lipid fractions: ■ = acyl CoA, ▲ = free fatty acid, and ● = phospholipid. B, [1-^{14}C] Oleate was isolated from the following lipid fractions: ■ = acyl CoA, ▲ = free fatty acid, and ● = phospholipid.

plate was dried and sprayed with 1% butylated hydroxytoluene. The standard lipid mixture was exposed to iodine vapor. Segments (1 cm) were scraped from the chromatogram in the area containing [1-^{14}C] stearyl CoA and these segments counted in the scintillation counter. Segments (1 cm) also were scraped from the area corresponding to the 100 μl aliquot of the reaction mixture on the chromatogram, and these segments likewise were counted. After this determination of the position of the major radioactive microsomal lipid components on the chromatogram, the material containing 500 μl aliquot of the microsomal reaction mixture was, accordingly, scraped and placed in screw cap tubes. 1.5% KOH-methanol (10 ml) was added to the dry silica gel powder and the tubes sealed with screw caps containing teflon liners. The tubes were heated at 60 C for 1 hr. After cooling to room temperature, the contents were acidified with 4N H_2SO_4 and extracted 3 times with pentane (7 ml, 7 ml, and 3 ml). The combined pentane extract was dried in a stream of nitrogen, and the residue was methylated by two or three drops of an ethereal solution of diazomethane. The residual diazomethane was removed with a stream of nitrogen. The methyl esters were dissolved in 0.1 ml chloroform and were resolved on $AgNO_3$-impregnated Silica Gel H plates as described previously or by gas liquid chromatography (GLC).

For specific radioactivity determinations, an aliquot of the chloroform solution of fatty acid methyl esters was taken to dryness in a stream of nitrogen and dissolved in 10 μl pentane. An aliquot of this pentane solution was counted in a liquid scintillation counter to determine total radioactivity in each sample, and a similar aliquot was analyzed by GLC.

GLC: A Beckman GC-4 equipped with a hydrogen flame ionization detector was used for the quantitative analysis of mixtures of fatty acid methyl esters derived from silica gel chromatography of desaturase reaction mixtures. Stainless steel columns 6 ft x 1/8 in. were packed with 15% EGSS-X on 100/120 Gas Chrom P. The column was operated at 172 C with a nitrogen gas flow of 40 ml/min at 50 lb. Standard mixtures containing 1 μg each of methyl stearate and methyl oleate were used to calibrate the machine.

RESULTS AND DISCUSSION

The stearyl CoA desaturase of hen liver microsomes has a requirement for lipid, but whether the lipid is required in a structural role or if it is, in fact, a substrate participating in the reaction is still not understood clearly. In examining the detailed mechanism of desaturation in hen liver microsomes, we attempted to characterize the product formed during the desaturation reaction. A partially purified preparation, P_2, of hen liver microsomes (3) was chosen for this study. [1-^{14}C] Stearyl CoA and NADH were incubated for various times with P_2 and the total [1-^{14}C] oleate formed was determined. The results are presented in Figure 1. The desaturase activity was linear up to 6 min and then continued to increase more slowly up to 15 min. The total desaturation after 15 min was 25% stearyl CoA added.

The distribution of radioactivity derived from [1-^{14}C] stearyl CoA among various mi-

TABLE II

Specific Radioactivities of [1-^{14}C] Stearic Acid and [1-^{14}C] Oleic Acid Methyl Esters Derived from Microsomal Lipid Fractions[a]

Time (min)		2	4	6	10
Fraction	Fatty acid methyl esters	Specific activity (cpm/nmole)[b]			
Acyl CoA	Stearic	1694	2217	1688	1364
	Oleic	850	1385	1291	1115
Free fatty acid	Stearic	430	413	530	600
	Oleic	---	42	55	330
Phospholipid[c]	Stearic	160	170	205	232
	Oleic	94	170	205	62

[a][1-^{14}C] Stearic acid methyl ester and [1-^{14}C] oleic acid methyl ester were prepared as described in the text.

[b]cpm were obtained as follows. An aliquot of the sample subjected to gas liquid chromatography was assayed for radioactivity and the total cpm obtained. This total was apportioned into cpm for stearic acid and cpm for oleic acid by assuming that the ratio of stearic acid/oleic acid in this sample was the same as that obtained for a duplicate sample analyzed on a $AgNO_3$-impregnated Silica Gel H plate.

[c]The phospholipid fraction comprised material corresponding to positions B and C in Table I.

crosomal lipid fractions during desaturation was determined. In Table I a summary of this data is presented. Radioactivity was found associated with the acyl CoA fraction, the free fatty acid fraction, and two phospholipid fractions. The amount of radioactivity present in the acyl CoA fraction decreases with time, and the amount of radioactivity in the free fatty acid and the phospholipid fractions increase with time; but no correlation can easily be made between the distribution of radioactivity in any fraction and total desaturase activity. There is an active thioester hydrolase activity present in this preparation, but there is still some acyl CoA left at the end of 15 min. Acyl transferase activity also is present in the P_2 preparation and is active throughout the incubation period tested.

To elucidate the sequence of events occurring during the desaturase reaction, it was necessary to isolate each of the radioactive lipid components of the desaturase incubation mixture to determine the total amount of radioactive saturated and unsaturated fatty acid in each component. The results are presented in Figure 2. During the course of the incubation, there is a steady decrease in the amount of stearyl CoA present with a steady increase in the amount of free stearic acid and stearate in the phospholipid fraction (Fig. 2A). In contrast, radioactive oleate appears first associated with the acyl CoA fraction (Fig. 2B). No significant amounts of radioactive oleate appear in the phospholipid and fatty acid fractions at the early times. There is no evidence that the desaturation of stearyl CoA occurs via an unsaturated phospholipid intermediate. There is radioactive oleate present in the phospholipid fraction, but this does not become significant until after the desaturase activity is beginning to taper off (after 6 min). This would suggest that it was necessary to have sufficient oleyl CoA present to act as a substrate for the acyl transferase enzyme. Radioactive oleate present as free fatty acid also makes its appearance in significant amounts only after 6 min have elapsed, presumably because of the necessity for sufficient oleyl CoA to be present for the thioester hydrolase activity to be effective.

To establish firmly that [1-^{14}C] stearyl CoA was directly converted to [1-^{14}C] oleyl CoA in our preparations, we determined the specific radioactivities of each of the lipid components isolated from the microsomal desaturase incubation mixture. If the specific radioactivity of the [1-^{14}C] oleyl CoA was the same as that for the [1-^{14}C] stearyl CoA, then this would suggest that [1-^{14}C] oleyl CoA was formed directly from [1-^{14}C] stearyl CoA without being diluted by a pool of phospholipid or acyl-acyl carrier proteins (ACP) or other derivatives. The result of this experiment is shown in Table II. Each of the fractions obtained from the silica gel fractionation procedure was subjected to GLC, as well as analysis for total radioactivity in unsaturated and saturated fatty acid. [1-^{14}C] Stearyl CoA and [1-^{14}C] oleyl CoA have similar specific radioactivities throughout the time course of this experiment. The specific radioactivities of the free fatty acid and the phospholipid derivatives are lower than that of [1-^{14}C] stearyl CoA and increase with time. They do not resemble that of [1-^{14}C] stearyl CoA or [1-^{14}C] oleyl CoA. These

results strongly suggest that [1-^{14}C] oleyl CoA was formed directly from [1-^{14}C] stearyl CoA without being diluted by a pool of phospholipid, acyl-ACP, or other derivatives. The data indicate that the first step in the desaturation reaction is to convert stearyl CoA directly to oleyl CoA. This confirms the conclusion reached by Vijay and Stumpf (5) who showed that radioactivity from [1-^{14}C] stearyl CoA was found in material which had the chemical properties of a thioester of [1-^{14}C] oleate. Although this product was not characterized further with the hen liver microsomal system, studies with the safflower desaturase suggested the product of this latter system was not an ACP derivative (5). Vijay and Stumpf (5) did not, however, obtain kinetic evidence that this material was an initial product of the hen liver desaturase, nor were the specific radioactivities of the substrate and products compared.

Lipid synthesizing enzymes, which use oleyl CoA as a substrate, have been found to be associated with the desaturase activity (4-7). The significant conversion of [1-^{14}C] oleyl CoA into radioactive phospholipid occurs at a time well beyond the initial desaturation. This evidence suggests that phospholipid is not a substrate in the desaturation reaction; however, phospholipid still may be intimately involved in this reaction. The data of Schultz and Lynen (13), have demonstrated that oleyl CoA is a competitive inhibitor of the desaturase reaction in yeast and oleyl CoA also inhibits the desaturase reaction in liver (2); hence, the removal of oleyl CoA by the phospholipid synthesizing enzymes may provide a method of control for this reaction. It also has been demonstrated that sn-glycero-3-phosphate stimulates desaturation in mouse liver microsomes (1). It was found that 98% oleyl CoA formed could be accounted for as glycerides under these conditions. This is further evidence that the removal of oleyl CoA by the microsomes may be the controlling step for the desaturase reaction. It will be interesting to use more purified preparations of the desaturase enzyme to resolve this question. If the phospholipid synthesizing activity is purified along with the desaturase activity, this would provide evidence for the intimate nature of these reactions and the interaction of these two systems.

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Effect of Dietary Saturated Fatty Acids and Linoleic Acid upon the Structures of Triglycerides in Rabbit Tissues

W.W. CHRISTIE and **J.H. MOORE**, The Hannah Research Institute, Ayr, Scotland KA6 5HL, and **J.J. GOTTENBOS**, Unilever Research Laboratories, Vlaardingen, The Netherlands

ABSTRACT

Five groups of rabbits were given a diet supplemented with safflower seed oil and safflower seed oil partially replaced by lauric, myristic, palmitic, and stearic acids respectively. After 10 weeks, plasma samples were taken from the animals in the fasted and nonfasted state; the animals then were killed, and the livers and samples of adipose tissue were removed. Lipids were extracted from the tissues, then separated into classes; and the fatty acid composition of each class was determined. In addition, the triglycerides were isolated and the structures determined by stereospecific analysis. There were marked differences in the compositions and structures of the triglycerides in the plasma from fasted and nonfasted animals. Feeding specific dietary fatty acids also greatly changed the metabolism of linoleic acid by the animals. The results are discussed in terms of the biosynthesis of triglycerides in plasma, liver, and adipose tissue.

INTRODUCTION

Although the structures of triglycerides from a wide variety of animal species have been determined by stereospecific analysis procedures that distinguish between the fatty acid compositions of positions 1, 2, and 3 of *L*-glycerol, the results were obtained invariably from animals that had been given diets of unspecified composition. There have been many reports on the effects of different dietary treatments upon the gross fatty acid compositions of triglycerides in animal tissues, but there is little information available on the effects of different dietary fatty acids upon tissue triglyceride structure. For instance, myristic acid, which is usually a minor component of most dietary fats, is found predominantly in position 2 of mammalian adipose tissue triglycerides (1,2), but it is not known whether this positional distribution is maintained when the diet contains larger proportions of myristic acid. In addition to gross fatty acid composition, the distribution of fatty acids between the three positions of *L*-glycerol is an important factor in determining the physical properties (mp) of adipose tissue triglycerides (3). It was of interest, therefore, to determine the effects of different dietary fatty acids upon the nature and structures of triglycerides of the plasma, liver, and adipose tissues of rabbits. (The structure of adipose tissue triglycerides in the rabbit is typical of most mammalian fats [1,2].) The four fatty acids chosen for this investigation were lauric, myristic, palmitic, and stearic acids since these are the most common saturated fatty acids found in dietary fats and oils and these were fed with high levels of linoleic acid.

EXPERIMENTAL PROCEDURES

Animals, Diets, and Procedure

The rabbits were 15 males of the Dutch belted strain that had been reared on a commercial pelleted diet. When the rabbits were 4 months old, olive oil was incorporated into the commercial diet in increasing proportions over a period of 3 weeks so that the animals became accustomed to a diet in which fat contributed 40% of the total energy value; the rabbits were maintained on this high fat diet until they were 10 months old. The animals then were divided into 5 groups of 3 each, and each group was given one of the experimental diets ad libitum for a further period of 10 weeks. Drinking water was available ad libitum throughout the experiment. The composition of the basal experiment diet (wt %) given to all rabbits was as follows: maize starch, 16.0; casein, 12.8; skim-milk powder, 20.1; dextrose, 10.3; sawdust, 21.2; fat, 15.6; salt mixture (4), 3.5; and vitamin mixture (4), 0.44. In the experimental diet given to the rabbits in group 1, the fat component was safflower seed oil. Lauric, myristic, palmitic, and stearic acids were incorporated into the diets given to the rabbits in groups 2, 3, 4, and 5 respectively. So that the linoleic acid content was ca. the same for each experimental diet, dietary fats given to the rabbits in groups 2-5 were prepared as follows. Methyl esters of safflower seed oil fatty acids were prepared by transmethylation with methanolic HCl; and, from these, a linoleic acid-rich fraction was prepared by fractional crystallization of the urea adducts. The more satu-

TABLE I

Fatty Acid Compositions (mole %) of the Fats in Experimental Rabbit Diets

Dietary fat	Safflower seed oil	18:2-Rich concentrate from safflower seed oil together with saturated fatty acids			
		12:0	14:0	16:0	18:0
Rabbit group no.	1	2	3	4	5
Fatty acids					
12:0	---	36.7	---	---	---
14:0	---	---	31.2	---	---
16:0	8.7	---	---	28.3	---
18:0	2.5	---	---	---	23.2
18:1	13.9	1.8	1.9	4.0	3.1
18:2	74.9	61.5	66.9	67.7	73.7

rated methyl esters crystallized first, leaving a liquid fraction containing more than 95% linoleic acid. This latter fraction was saponified; and, after acidification, the free fatty acids were extracted. To portions of this free fatty acid mixture were added the appropriate amounts of either lauric, myristic, palmitic, or stearic acid; and the mixtures were esterified with glycerol. The fatty acid compositions of the five dietary fats are given in Table I.

When the rabbits had been given the experimental diets for 3 weeks, a blood sample was taken from the marginal ear vein of each animal in the nonfasted state, i.e. during the morning when food was available. After the rabbits had been given the experimental diets for 6 weeks, another blood sample was taken from the marginal ear vein of each animal in the fasted state, i.e. food containers had been removed from the rabbit cages 20 hr before the blood sample was taken. After 10 weeks, the animals had all increased in wt by similar amounts and had consumed similar amounts of the various diets. A final blood sample then was taken when the rabbits were in the nonfasted state. This final blood sample was taken by aortic cannulation, and the rabbits were immediately killed. The liver and a sample of perinephric adipose tissue were removed from each animal. All samples of blood serum, liver, and adipose tissue were freeze dried and stored at -20 C before extraction and analysis of the lipids.

Lipid Analysis

Samples of individual tissues were pooled for each group, lipids were extracted from the pooled samples by the procedure of Folch, et al. (5); and various lipid classes were separated by preparative thin layer chromatography on 200 mm x 200 mm glass plates coated with a 0.5 mm layer of Kieselgel G (E. Merck, A.G., Darmstadt, Germany), using a solvent system of hexane-diethyl ether-formic acid (80:20:2 by volume). Plates were sprayed with 2,7-dichlorofluorescein in methanol (1 g/liter) and bands were located with UV light and were identified by comparison with standards chromatographed on the same plates. For analysis by gas liquid chromatography, cholesteryl esters, triglycerides, and phospholipids were transesterified with sodium methoxide in methanol, and free fatty acids were methylated with boron trifluoride in methanol (6). The analyses were made with 2000 mm columns EGSS-X on Gas Chrom P (150 g/Kg, 100-120 mesh; Applied Science Laboratories, State College, Pa.). The amount of each ester was calculated from the product of the peak height, and retention time and results were converted to molar percentages by multiplying the detector responses by the appropriate correction factors. Component fatty acids were identified by their retention times relative to standards on several liquid phases.

Stereospecific Analysis of Triglycerides

The stereospecific analysis of triglycerides was performed by the procedure of Christie and Moore (7,8). Briefly, α,β-diglycerides were prepared from triglycerides by reaction with ethyl magnesium bromide and these were converted synthetically into phospholipids which were hydrolyzed with the stereospecific phospholipase A of snake venom. The resulting lysophosphatide contained only the fatty acids of position 1 of the original triglyceride, and the fatty acid composition of position 2 was obtained by analysis of the free fatty acids released or by pancreatic lipase hydrolysis of the triglycerides. The fatty acids of position 3 could be determined by difference from the composition of the original triglyceride. Checks were possible on the results for positions 2 and 3, and analyses were accepted only when they conformed to the standards of accuracy described by Christie and Moore (7,8).

TABLE II

Fatty Acid Composition (Molar Percentage) of Triglycerides in Lipid Extracts from Plasma, Livers, and Adipose Tissue of Five Groups of Fasted and Nonfasted Rabbits, Each Containing Three Males Fed Diets Containing Different Amounts of Fatty Acids[a]

Tissue	Nutritional status	Group no.	Fatty acid composition						
			12:0	14:0	16:0	16:1	18:0	18:1	18:2
Plasma	Nonfasted	1	---	0.9	19.6	1.1	2.8	17.9	57.7
		2	4.9	2.6	19.1	1.4	1.8	16.0	54.2
		3	---	18.8	12.7	0.9	0.9	6.7	60.0
		4	---	1.7	29.4	0.7	2.4	8.1	57.7
		5	---	1.5	17.7	1.2	10.2	16.6	52.8
	Fasted	1	---	1.3	30.5	4.5	6.1	41.1	16.5
		2	0.7	1.4	26.5	1.6	2.3	29.1	38.4
		3	---	2.8	31.0	2.4	8.3	36.4	19.1
		4	---	0.6	28.7	5.1	3.4	46.9	15.3
		5	---	0.9	31.0	1.4	4.9	26.5	35.3
Liver	Nonfasted	1	---	1.5	21.3	1.3	4.3	29.4	42.2
		2	3.8	5.4	25.4	1.4	8.4	27.6	28.0
		3	---	14.1	23.4	1.7	4.0	22.8	34.0
		4	---	2.2	42.9	1.3	3.8	15.5	34.3
		5	---	1.9	19.6	1.4	8.4	23.9	44.8
Adipose	Nonfasted	1	---	1.1	13.2	1.0	3.9	26.6	54.2
		2	2.7	2.1	14.2	1.7	3.0	39.2	37.1
		3	---	13.7	12.9	1.2	2.7	23.8	45.7
		4	---	1.6	23.5	1.8	3.9	25.0	44.2
		5	---	0.9	10.6	0.9	10.5	30.9	46.2

[a]Compositions of fats fed to the five groups of rabbits are given in Table I.

RESULTS AND DISCUSSION

Four main lipid classes were detected in the plasma and liver lipids: cholesteryl esters, triglycerides, free fatty acids, and phospholipids. Triglycerides accounted for more than 99% of the lipids of the adipose tissue. Six fatty acids, myristic (14:0), palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), and linoleic (18:2) were found in most of the lipid classes; and they were accompanied by eicosadienoic acid (20:2) and arachidonic acid (20:4) in the phospholipids. Lauric acid (12:0) was found only in the tissue lipids of animals given appreciable amounts of this fatty acid in the diet (group 2).

The fatty acid compositions of the triglyceride fractions from the plasma, liver, and adipose tissue of the rabbits on each dietary treatment when they were in the nonfasted state and from the plasma of the same animals when they were in the fasted state are given in Table II. Results from the two plasma samples taken when the rabbits were in the nonfasted state were similar, and only one set of results (those obtained when the animals had been given the experimental diets for 10 weeks) is included in Table II. As might be expected, the inclusion of a particular saturated fatty acid in the diet resulted in increased incorporation of that acid in the plasma and tissue triglycerides. When the diet was supplemented with 12:0 (group 2), there was also evidence of an increased incorporation of 14:0, formed presumably by chain elongation of 12:0, in the triglycerides of the liver and adipose tissues; in the plasma triglycerides, this effect was only observed when the rabbits were in the nonfasted state. In addition to these predictable effects, the various supplementary saturated fatty acids in the diets affected the incorporation of 18:2 into the triglycerides of the tissues in markedly different ways. Dietary treatment had little effect upon the concentration of 18:2 in the plasma triglycerides of the rabbits in the nonfasted state. However, when compared with the results obtained for the rabbits in group 1, the inclusion of 12:0 (group 2) or 18:0 (group 5) in the diet resulted in a twofold increase in the concentration of 18:2 in the plasma triglycerides of the animals in the fasted state. However, the inclusion of 12:0 in the diet resulted in marked decreases in the concentrations of 18:2 in the liver and adipose tissue triglycerides. The concentrations of 18:2 in the triglycerides of liver and adipose tissue also were decreased by supplementing the diet with 14:0 and 16:0. The addition of 18:0 to the diet decreased the concentration of 18:2 in the adipose tissue triglycerides but had little effect upon the 18:2 content of the liver triglycerides. Although diets 2, 3, and 4 contained slightly less 18:2 than diets 1 and 5, the observed effects could only be partly accounted for by this.

TABLE III

Stereospecific Analysis of Triglycerides in Lipid Extracts from Livers and Adipose Tissue from Five Groups of Rabbits (Nonfasted), Each Containing Three Males, Fed Diets Containing Different Amounts of Fatty Acids[a]

Tissue	Group	Position of fatty acid in *L*-glycerol moiety	Fatty acid composition (molar %)						
			12:0	14:0	16:0	16:1	18:0	18:1	18:2
Liver	1	1	---	2.3	32.8	2.2	8.1	24.7	29.9
		2	---	1.7	14.2	1.7	1.0	28.1	53.3
		3	---	0.5	16.9	---	3.8	35.4	43.4
	2	1	4.9	5.2	43.2	1.8	10.4	18.5	16.0
		2	2.0	7.1	17.2	2.0	2.9	29.1	39.7
		3	4.5	3.9	15.8	0.4	11.9	35.2	28.3
	3	1	---	17.5	37.0	1.9	6.8	17.5	19.3
		2	---	17.8	13.3	2.3	0.8	21.7	44.1
		3	---	7.0	19.9	0.9	4.4	29.2	38.6
	4	1	---	2.6	65.6	1.5	5.1	11.5	13.7
		2	---	2.1	24.0	1.9	1.8	17.8	52.4
		3	---	1.9	39.1	0.5	4.5	17.2	36.8
	5	1	---	3.7	43.3	2.9	12.4	15.6	22.1
		2	---	1.0	6.1	1.1	5.4	22.9	63.5
		3	---	1.0	9.4	0.2	7.4	33.2	48.8
Adipose	1	1	---	1.0	24.3	1.2	9.3	24.8	39.4
		2	---	0.9	11.1	1.4	0.5	26.0	60.1
		3	---	1.4	4.2	0.4	1.9	29.0	63.1
	2	1	3.8	2.8	25.8	1.9	6.9	34.6	24.2
		2	2.5	3.3	10.0	2.1	0.5	39.9	41.7
		3	1.8	0.2	6.8	1.1	1.6	43.1	45.4
	3	1	---	17.4	24.6	1.4	6.7	20.7	29.2
		2	---	18.2	8.9	1.6	0.3	23.5	47.5
		3	---	5.5	5.2	0.6	1.1	27.2	60.4
	4	1	---	2.0	30.1	1.7	8.9	19.8	28.5
		2	---	2.4	18.4	2.8	0.8	25.2	50.4
		3	---	0.4	13.0	0.9	2.0	30.0	53.7
	5	1	---	1.0	18.2	1.2	24.8	26.1	28.7
		2	---	1.6	9.4	1.4	2.8	32.4	52.4
		3	---	0.1	4.2	0.1	3.9	34.2	57.5

[a]Composition of fats fed to the five groups of rabbits are given in Table I.

The results for the analyses of the cholesteryl ester, phospholipid, and free fatty acid fractions of the various tissues are not given in detail, as the effects of the different dietary treatments on the fatty acid compositions of these lipid fractions were similar to, but less pronounced than, those observed for the plasma and tissue triglycerides. The 18:2 content of the plasma phospholipids of the animals in the nonfasted state was ca. 46% and remained unaffected by dietary treatment. However, in the fasted state, the 18:2 content of the plasma phospholipids of the rabbits given the diet supplemented with 12:0 (group 2) was 46% as compared with a value of 35% found for the plasma phospholipids of the animals in group 1 (Table II).

In nutritional experiments designed to assess the effects of different dietary lipids upon the concentrations and fatty acid compositions of the various plasma lipid components, blood samples often are taken after an overnight fast. Although this procedure undoubtedly reduces between-animal variations, the extent to which such results are meaningful must be open to question because, in these experiments, the effect of dietary treatment upon plasma lipid composition observed in the fasted state differed markedly from that observed in the nonfasted state (Table II).

The results of the stereospecific analyses of the triglycerides of the adipose tissue and liver are listed in Table III. In the triglycerides of adipose tissue from all groups of rabbits, the highest concentrations of 16:0 and 18:0 were found in position 1, whereas 12:0, 14:0, and 16:1 were found predominantly in positions 1 and 2. The highest concentrations of 18:2 were found in positions 2 and 3. There was a more even distribution of 18:1 between the three positions; but, in position 3, there were higher concentrations of this acid than in position 2 which, in turn, contained greater concentrations than position 1. Similar distributions have been found in triglycerides of adipose tissue from many other species (1,2). Stereospecific

TABLE IV

Relative Proportions of Each Fatty Acid in Three Positions of *L*-glycerol Moiety of Triglycerides of Adipose Tissue and Liver from Five Groups of Rabbits, Each Containing Three Males, Fed Diets Containing Different Amounts of Fatty Acids[a] (Mean Values and Standard Deviations)

Tissue	Position of fatty acid on *L*-glycerol moiety		Fatty acid composition (%) 12:0	14:0	16:0	16:1	18:0	18:1	18:2
	1	Mean	47	39.0	59.8	38.2	78.6	28.6	22.0
		SD[b]		5.7	3.2	4.8	2.9	1.8	1.2
Adipose	2	Mean	31	46.4	26.2	47.2	6.0	33.8	37.0
		SD		12.1	2.9	4.9	2.1	0.8	1.2
	3	Mean	22	14.2	14.0	14.6	15.4	37.6	41.0
		SD		16.7	2.6	6.7	2.7	1.5	1.9
	1	Mean	43	45.6	57.2	48.6	51.0	24.6	18.2
		SD		12.8	9.7	13.7	8.9	2.6	4.1
Liver	2	Mean	18	34.6	18.6	42.4	12.6	31.8	46.0
		SD		10.9	5.1	9.4	5.9	4.6	3.6
	3	Mean	39	19.6	24.2	9.0	36.4	41.6	35.6
		SD		7.0	5.9	7.0	7.3	3.4	1.7

[a]Composition of fats fed to the five groups of rabbits are given in Table I.

[b]SD = standard deviation.

analysis of rabbit adipose tissue triglycerides has been reported by Brockerhoff, et al. (9); the diet and the breed of the animal were not recorded, and the total fatty acid composition of the triglycerides was very different from that found in this investigation. However, the distribution of fatty acids among the three positions was similar to that shown in Table III; the only difference was that Brockerhoff, et al., (9) found more 16:0 in positions 2 and 3 and correspondingly less in position 1.

Although the amounts of each fatty acid in the triglycerides of each group of rabbits varied considerably, they were divided among the three positions in remarkably constant proportions. The proportions (mean values and standard deviations) of each fatty acid in the three positions of the adipose tissue and liver triglycerides from the five groups of rabbits are listed in Table IV. As an example, although the concentration of 16:0 in the adipose tissue triglycerides varied between 10 and 23% of the total fatty acids in the 5 experimental treatments, 59.8 ± 3.2% of the total 16:0 was found in position 1, 26.2 ± 2.9% in position 2 and 14.0 ± 2.6% in position 3. Similarly, the relative proportions of 18:0, 18:1, and 18:2 in each position of the adipose tissue triglycerides did not vary greatly; and, although somewhat higher standard deviations were found with 14:0 and 16:1, these acids were among those present in the lowest proportions.

This is the first investigation in which fatty acid compositions of adipose tissue triglycerides have been varied systematically and the positional distributions of the fatty acids determined by stereospecific analysis procedures. As fatty acids were divided among the three positions in roughly constant proportions, although the absolute amount in each position varied considerably from group to group, the mechanism of triglyceride biosynthesis in rabbit adipose tissue would seem to be controlled largely by the availability of individual fatty acids. The α-glycerophosphate pathway of triglyceride biosynthesis (10) is believed to be the principal mechanism operating in adipose tissue. In rabbit adipose tissue, there would appear to be a single pool of fatty acids; and the acyltransferases responsible for esterification of each position accept and utilize fatty acids at constant rates relative to each other. In pig adipose tissue, on the other hand, there is evidence (3) that those fatty acids synthesized in the tissue form a separate pool and are incorporated into each position of the triglycerides in constant proportions but that fatty acids of dietary origin are not. Similar experiments with a much wider range of dietary fatty acids and other animal species must be performed, however, before definitive rules can be formulated.

The liver triglycerides were also highly asymmetric; and, although the overall fatty acid compositions of the triglycerides of each group of rabbits were different from those of the adipose tissue triglycerides, fatty acids were distributed among the three positions in a roughly similar manner. The only noticeable differences were a higher proportion of 18:2 in position 2 and more 16:0 and 18:0 in position 3 in the liver triglycerides than in the corresponding positions in the adipose tissue triglycerides. The proportionate distributions

TABLE V

Stereospecific Analysis of Triglycerides in Lipid Extracts from Plasma of Five Groups of Fasted and Nonfasted Rabbits, Each Containing Three Males, Fed Diets Containing Different Fatty Acids[a]

Nutritional status	Group	Position of fatty acid on *L*-glycerol moiety	12:0	14:0	16:0	16:1	18:0	18:1	18:2
		1	---	1.0	44.7	2.5	7.0	13.6	31.2
	1	2	---	1.8	11.5	0.5	1.0	18.9	66.3
		3	---	-0.1	2.6	0.3	0.4	21.2	75.6
		1	8.8	4.6	34.1	1.4	3.8	10.8	36.5
	2	2	3.7	2.9	20.6	1.2	1.0	19.8	50.8
		3	2.2	0.3	2.6	1.6	0.6	17.4	75.3
		1	---	39.3	26.4	1.6	1.2	4.6	26.9
Nonfasted	3	2	---	11.2	6.1	1.0	1.0	7.1	73.6
		3	---	5.9	5.6	0.1	0.5	8.4	79.5
		1	---	3.0	56.2	0.5	4.6	6.2	29.5
	4	2	---	1.9	18.0	1.0	1.6	9.2	68.3
		3	---	0.2	14.0	0.6	1.0	8.9	75.3
		1	---	0.9	33.0	1.1	18.4	15.9	30.7
	5	2	---	2.4	18.0	1.9	10.6	16.3	50.8
		3	---	1.2	2.1	0.6	1.6	17.6	76.9
		1	---	1.7	63.3	3.6	8.8	12.5	10.1
	1	2	---	2.1	11.6	4.0	4.4	52.8	25.1
		3	---	0.1	16.6	5.9	5.1	58.0	14.3
		1	0.9	1.9	50.1	1.9	3.7	21.2	20.3
	2	2	0.3	1.2	16.4	2.2	1.7	33.8	44.4
		3	0.9	1.1	13.0	0.7	1.5	32.3	50.5
		1	---	3.1	63.3	3.1	6.8	12.6	11.1
Fasted	3	2	---	1.9	8.0	2.1	4.3	52.0	31.7
		3	---	3.4	21.7	2.0	13.8	44.6	14.5
		1	---	0.4	66.9	7.4	3.5	16.9	4.9
	4	2	---	0.7	6.5	3.3	2.0	56.9	30.6
		3	---	0.7	12.7	4.6	4.7	66.9	10.4
		1	---	1.3	57.0	2.6	8.9	16.6	13.6
	5	2	---	1.2	19.0	1.0	2.9	31.4	44.5
		3	---	0.2	17.0	0.6	2.9	31.5	47.8

[a]Composition of fats fed to the five groups of rabbits are given in Table I.

were not as predictable as those in the adipose tissue triglycerides, however; and this was shown by the higher standard deviations obtained when the mean proportions were calculated (Table IV). Among the more obvious discrepancies, the proportion of the available 16:0 in position 1 of the liver triglycerides of those animals fed high levels of 18:0 was much higher than those fed any of the other diets. Rabbit liver triglycerides have not been analyzed by stereospecific analysis procedures before, but they resembled liver triglycerides of other species examined, including the rat (11), pig (8), sheep (12), man (13), and chicken (14). Liver triglycerides have a more complex biosynthetic origin than adipose tissue triglycerides as the diglyceride intermediates may arise not only by the α-glycerophosphate pathway but also by dephosphorylation of phosphoglycerides (15). As a result, it was not surprising that fatty acids were not distributed among the three positions according to any simple rule.

Plasma triglycerides from the nonfasted animals were similar to adipose tissue triglycerides in that fatty acids were distributed among the three positions in similar proportions, i.e. saturated fatty acids were in greatest concentration in position 1 and C_{18} unsaturated acids in positions 2 and 3 (Table V). The specific dietary saturated fatty acids tended to increase in concentration mainly in positions 1 and 2, however, and to a much smaller extent in position 3 so that the proportion of each fatty acid in each position in the 5 groups of rabbits apparently did not conform to any simple distribution rule. In addition, although there was ca. the same amount of 18:2 in the triglycerides of each experimental group, the distribution among the three positions varied noticeably. With groups 1, 3, and 4, 18:2 was found in positions 1, 2, and 3 in the approximate ratio 1:2.5:2.5, respectively; but, with groups 2 and 5 (those rabbits given diets containing 12:0 and 18:0, respectively), this ratio was ca. 1:2:3. It appears unlikely that any of these differences could have arisen as a result

of the small discrepancies in the levels of 18:2 in the diets.

The fatty acid compositions and fatty acid distributions (Table V) in the triglycerides of the plasma of the fasted animals were different from the results obtained from the animals in the nonfasted state, particularly with respect to distribution of 18:2. With groups 2 and 5 (given diets containing 12:0 and 18:0, respectively), the comparatively large concentration of 18:2 in the plasma triglycerides of the rabbits in the fasted state was distributed between positions 1, 2, and 3 in the approximate ratio 1:2.5:2.5, respectively; but in groups 1, 3, and 4, the corresponding ratio was ca. 1:2:1. The large amount of 18:1 in the plasma triglycerides from the rabbits in the fasted state was found mainly in positions 2 and 3.

Plasma triglycerides also have a rather complex biosynthetic origin, especially when the animal is in the nonfasted state as, in addition to triglycerides synthesized in and secreted from the liver, there is a significant contribution from chylomicrons entering the plasma from the lymphatic system. The latter contribution is negligible when the animals are in the fasted state. At the same time, triglycerides constantly are removed from the plasma and utilized by various organs. As a result of this complexity, the changes in the structure of plasma triglycerides with changes in the composition of the dietary fatty acids cannot readily be interpreted at present. The mechanisms by which dietary 12:0 and 18:0 increased the essential fatty acid (18:2) content of plasma triglycerides and their structure in both fasted and nonfasted animals are unknown by warrant further study.

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On the Levels of Alkyl and Alk-1-enyl Glycerolipids in Normal and Neoplastic Tissues: A Method of Quantification

KWEI LEE SU and **HARALD H.O. SCHMID**, The Hormel Institute, University of Minnesota, Austin, Minnesota 55912

ABSTRACT

A method is described for the quantification of the constituent *O*-alkyl and *O*-alk-1-enyl glycerols of neutral lipids or phospholipids. The method involves chemical degradation, the preparation of derivatives, and quantification by gas chromatography using internal standards. Alk-1-enyl moieties are converted to alkyl substituted dioxanes in the presence of 1,1-dimethoxyheptadecane as standard; alkyl glycerols are analyzed as isopropylidene derivatives using 1-*O*-heptadecyl glycerol as internal standard. The method is applied to the quantification of alkyl and alk-1-enyl glycerols derived from total lipids of rat heart, liver, testes, and brain and of various transplantable tumors, i.e. amelanotic melanoma, melanoma B16, sarcoma T241, and Novikoff hepatoma. The levels of alkyl glycerols range from 0.11-1.07% total lipids and those of alk-1-enyl glycerols from 0.49-5.03%. The data are compared to those obtained by other methods.

INTRODUCTION

O-Alkyl and *O*-alk-1-enyl glycerols occur in most mammalian and nonmammalian tissues as constituents of both neutral lipids and phospholipids (1). Methods for the quantification of either alk-1-enyl glycerols (2) or alkyl glycerols (3) are available. A reliable method for their simultaneous quantification is desirable because of their metabolic relationship (4) and because increased amounts of alkyl glycerols have been reported for certain tumors (5,6). Reduction of phospholipids (7) or neutral lipids (8) with lithium aluminum hydride yields alkyl and alk-1-enyl glycerols. They can be separated by thin layer chromatography (TLC) (9,10) and have been quantified (9) by densitometry (11) after charring. Such a method has been used for the quantification of the constituent alkyl and alk-1-enyl glycerols of neutral and polar lipids of various normal (9) and neoplastic (5,6) tissues. However, the values for alk-1-enyl glycerols were substantially lower than those obtained by other methods (12-15).

In this article, we report a procedure for the simultaneous quantitative analysis of alkyl and alk-1-enyl glycerols. 1,1-Dimethoxyheptadecane and 1-*O*-heptadecyl glycerol are used as internal standards; alk-1-enyl moieties of glycerol ethers are analyzed as alkyl substituted dioxanes (16) and alkyl glycerol ethers as isopropylidene derivatives (17). The method is applicable to the determination of the amounts of alkyl and alk-1-enyl glycerols derived from total neutral lipids and phospholipids or from individual lipid classes.

MATERIALS

All solvents were reagent grade and were distilled before use. Diethyl ether was dried over sodium. Vitride reagent (sodium bis [2-methoxyethoxy] aluminum hydride, 70% in benzene), 1,3-propanediol, and *p*-toluenesulfonic acid were purchased from Eastman Kodak Co., Rochester, N.Y., and were stored in a desiccator.

n-Hexadecane was obtained from Lachat Chemicals, Chicago, Ill. Methyl esters of fatty acids were obtained from The Hormel Institute Lipids Preparation Laboratory, Austin, Minn., and sphingomyelin (bovine) from Applied Science Laboratories, State College, Pa. Heptadecanol was prepared from methyl heptadecanoate by Vitride reduction and was used for the synthesis of 1-*O*-heptadecyl glycerol (18) and heptadecanal (19). Heptadecanal was converted to the dimethyl acetal (1,1-dimethoxyheptadecane) (20). (Both 1,1-dimethoxyheptadecane and 1-*O*-heptadecyl glycerol are now available from Nu-Chek Prep, Elysian, Minn.) 1-*O*-Alk-1′-enyl-2,3-dioleoyl glycerol was prepared semisynthetically from bovine heart plasmalogens as described (10).

Beef heart was obtained immediately after slaughter from George A. Hormel & Co., Austin, Minn. Male Sprague Dawley rats, both 18 day olds (20-25 g) and adults (300-350 g), C57BL mice (20 g), and Golden Syrian hamsters (80-100 g) were purchased from Sprague Dawley Co., Madison, Wis.

Hamsters bearing amelanotic melanoma were supplied by H. Smith, Glenwood Hills Hospital, Minneapolis, Minn., mice bearing melanoma B16 and sarcoma T241 by D.J. Hutchison, The Sloan-Kettering Institute for Cancer Research, New York, N.Y., and excised intramuscular Novikoff hepatomas by W. Steele and H.M. Jenkin, The Hormel Institute, Austin, Minn.

Tumors were maintained in their respective host animals through periodical subcutaneous transplantations. The tumors were harvested 21 days after transplantation, except for the Novikoff hepatomas which were 23 days old. Lipid extracts were prepared from fresh tissues according to Folch, et al. (21).

METHODS

Quantification of Alkyl and Alk-1-enyl Glycerols

As an example, the procedure is described for 100 mg total brain lipid from 18 day old rats.

Chemical degradation: The sample is dried and suspended in 10 ml anhydrous benzene in a 50 ml three-neck round bottom flask equipped with a water separation head, a dropping funnel and a condenser, both fitted with $CaCl_2$ drying tubes. 1,3-Propanediol (0.4 ml), 20 mg *p*-toluenesulfonic acid, 2 mg 1,1-dimethoxyheptadecane, and 0.2 mg 1-*O*-heptadecyl glycerol are added. The reaction mixture is stirred magnetically and refluxed for 5 hr. Water produced during the reaction is distilled off azeotropically, and the benzene is replenished through the dropping funnel. At the end of the reaction period, the mixture is cooled to room temperature and transferred to a dropping funnel. The lower phase, consisting mainly of excess 1,3-propanediol and *p*-toluenesulfonic acid, is discarded; and the upper phase is added dropwise at room temperature to 5 ml stirred Vitride reagent in a three-neck flask equipped with a condenser. The reaction is continued for 1 hr and then stopped by dropwise addition of 25% sulfuric acid to the reaction mixture maintained in an ice bath. A total of 75 ml of 25% sulfuric acid is required to obtain a clear solution. The resulting solution is transferred to a separatory funnel and extracted 3 times with 50 ml portions of diethyl ether. The combined ether extracts are washed once with water, 5% $NaHCO_3$, again with water until neutral, and dried over anhydrous sodium sulfate.

TLC: Reaction products are fractionated by TLC on Silica Gel H, 0.5 mm thick in the solvent system hexane-diethyl ether, 40:60 (v/v). Bands representing dioxanes (R_f=0.70) and alkyl glycerols (R_f=0.11) are scraped into glass columns, eluted with water-saturated diethyl ether, and dried over anhydrous sodium sulfate. Isopropylidene derivatives of alkyl glycerols are prepared by acid catalyzed reaction with acetone (17); they are purified by TLC using hexane-diethyl ether, 90:10 (v/v).

Gas liquid chromatography (GLC): All analyses are carried out with a gas chromatograph equipped with a hydrogen flame detector using ethylene glycol succinate at 200 C as stationary phase. Details are given in Figure 2. Wt percentage is determined by triangulation of peak areas, and the wt of the naturally occurring moieties (mainly C_{16}, C_{18}, and $C_{18:1}$) is compared to that of the internal C_{17} standard. To account for the difference in the mol wt of the standard 1,1-dimethoxyheptadecane (300.5) and the corresponding 1-*O*-heptadec-1′-enyl glycerol (328.5), a correction factor of 1.09 is used.

RESULTS AND DISCUSSION

The method reported here consists of a series of reactions and fractionations and utilizes synthetic internal standards for the quantification of both the constituent alkyl and alk-1-enyl glycerols of neutral lipids or phosphatides. First, the alk-1-enyl moieties are cleaved and converted to long chain alkyl substituted dioxanes as described by Venkata Rao, et al., (16) and the reaction mixture is subsequently subjected to hydrogenolysis with Vitride reagent. We have found Vitride to be as efficient for this reaction as the lithium aluminum hydride used previously, and it is advantageous because it is available in benzene solution, the reaction medium used for the cyclization.

As internal standards must be available in pure form and sufficiently stable for storage, we used 1,1-dimethoxyheptadecane as standard for the quantification of alk-1-enyl moieties. In contrast to free long chain aldehydes, their dimethyl acetals can be stored indefinitely under appropriate conditions (dry benzene, low temperature). Quantification of alk-1-enyl moieties by the method described depends upon virtually complete conversion of both the dimethyl acetal of heptadecanal used as internal standard and the naturally occurring alk-1-enyl glycerol ethers to long chain cyclic acetals. Therefore, both 1,1-dimethoxyheptadecane and a semisynthetic 1-*O*-alk-1′-enyl-2,3-dioleoyl glycerol were subjected to the acetalation reaction as described above.

Quantitative conversion of 1,1-dimethoxyheptadecane to 2-hexadecyl-1,3-dioxane was demonstrated by the absence of 1,1-dimethoxyheptadecane or heptadecanal after the cyclization reaction either on TLC or GLC. It further was confirmed by using *n*-hexadecane as an internal standard. The molar ratio of 1,1-dimethoxyheptadecane (mol wt 300.5) to *n*-hexadecane (mol wt 226.5) was 1.51 before the reaction, as determined by GLC, and that of 2-hexadecyl-1,3-dioxane (mol wt 312.5) to *n*-hexadecane was 1.50 after the reaction. The

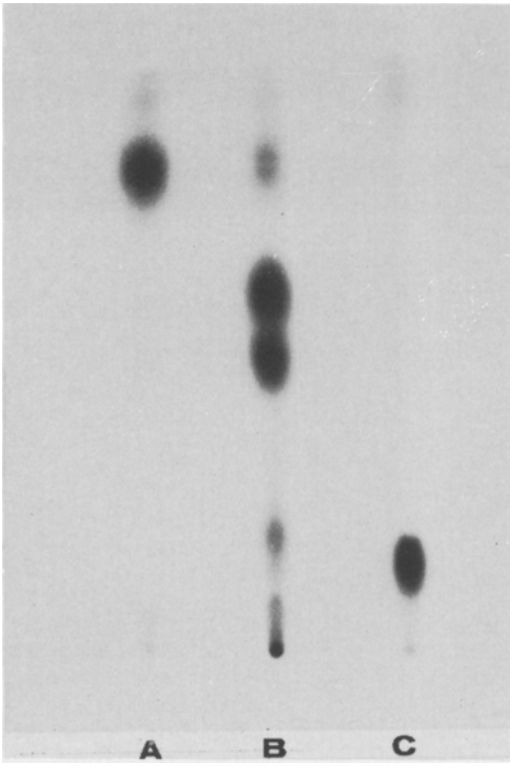

FIG. 1. Thin layer chromatogram of 2-hexadecyl-1,3-dioxane (a), reaction products of total lipids of adult rat brain, including internal standards (b), and 1-*O*-heptadecyl glycerol (c). Silica Gel H; hexane-diethyl ether, 40:60 (v/v). In the reaction products (b), the fraction slightly less polar than alkyl glycerols consists largely of an artifact derived from the reagent; it does not form isopropylidene derivatives and does not interfere with the quantification of alkyl glycerols by gas liquid chromatography.

experiment also proved that no decomposition of dioxanes had occurred on the EGSS-X column. Quantitative conversion of the alk-1-enyl moiety of a neutral plasmalogen to the cyclic acetal was demonstrated on a semisynthetic bovine 1-*O*-alk-1′-enyl-2,3-dioleoyl glycerol. The sample, 26 mg, was subjected to the same reaction sequence and the amount of alk-1-enyl glycerols was found to be 10.01 mg (calculated amount: 9.97 mg).

1-*O*-Heptadecyl glycerol has been used previously for the quantification of the constituent glycerol ethers of complex lipids (22,23).

Both dioxanes and glycerol ethers are separated easily from the major reaction products, such as alcohols (derived from acyl moieties), cholesterol, and sphingosine, by TLC as demonstrated in Figure 1.

Typical gas chromatographic fractionations of dioxanes and isopropylidene derivatives of glycerol ethers are shown in Figure 2.

It is well established (1) that the major long chain moieties of both alkyl and alk-1-enyl glycerols in mammalian tissues are 16:0, 18:0, and 18:1. Therefore, from the peak areas of 16:0, 17:0, 18:0, and 18:1, and amounts of internal standards added, one can calculate the quantities of these ether lipids present in total lipids or individual lipid classes. In general, the level of natural 17:0 alkyl or alk-1-enyl moieties is less than 2-3% of the total (1). Nevertheless, the presence of a natural 17:0 moiety would result in a lower value for total alkyl or alk-1-enyl ether content; by performing an analysis without addition of internal standards, one can determine the amount of 17:0 moiety and correct for it.

By using both TLC and GLC fractionations and internal standards for quantification, selective losses are prevented, and erroneously high levels of alkyl and alk-1-enyl glycerols due to interference of other lipids are eliminated. Free long chain aldehydes present in lipid extracts (24) would, in our method, be converted to dioxanes and, thus, add to the level of alk-1-enyl glycerols. However, the levels of free aldehydes in mammalian tissues are minimal; if necessary, they can be removed by TLC (24) prior to the analysis of alkyl and alk-1-enyl glycerols.

The amount of sample required for the method described here depends upon the levels of alkyl and alk-1-enyl glycerols present; usually 40 μg (ca. 0.1 μmole) of either alkyl or alk-1-enyl glycerols is sufficient for a complete analysis.

The method was used to quantify the constituent alkyl and alk-1-enyl glycerols in the total lipids of various normal rat organs and also in different types of neoplastic tissues. The results are shown in Table I.

The data listed in Table I show that the method of determining the levels of alkyl and alk-1-enyl glycerols in total lipids is well reproducible; repeated determinations were carried out using different amounts of sample, 50-150 mg, of the same lipid extracts. It is also evident from Table I that the levels of alkyl and alk-1-enyl glycerols in total lipids of normal and neoplastic tissues vary widely. Alkyl glycerols were lowest in the liver and highest in the Novikoff hepatoma; alk-1-enyl glycerols were lowest in the liver and highest in adult brain.

The data reported here are similar to those obtained for alk-1-enyl moieties by the methods of Rapport and Lerner (12) and Norton (13), but they do not agree with published data (5,9) obtained by the densitometric method.

When compared to the data reported here, those obtained by TLC-photodensitometry

TABLE I

Amounts of Alkyl and Alk-1-enyl Glycerols and Composition of Their Major Long Chain Moieties in Total Lipids of Normal and Neoplastic Tissues[a]

Tissue	Total lipid (% wet wt)	Alkyl glycerols (Percent total lipids)	Alkyl glycerols Composition (%) 16:0	18:0	18:1	Alk-1-enyl glycerols (Percent total lipids)	Alk-1-enyl glycerols Composition (%) 16:0	18:0	18:1	No. of determinations
Adult rat										
Heart	3.25	0.13 ± 0.05	38.21 ±0.25	11.73 ±0.79	50.06 ±0.74	3.25 ± 0.02	45.22 ±1.62	29.43 ±0.58	25.36 ±1.22	4
Liver	4.02	0.11 ± 0.03	43.24 ±0.15	16.50 ±0.06	40.27 ±0.09	0.49 ± 0.10	43.87 ±0.43	27.52 ±1.05	28.26 ±0.62	4
Testes	4.35	0.79 ± 0.03	77.70 ±1.11	18.29 ±0.83	4.01 ±0.48	1.29 ± 0.02	74.38 ±1.51	16.24 ±0.61	9.33 ±1.02	4
Brain	8.54	0.28 ± 0.01	52.92 ±0.23	29.34 ±3.16	17.74 ±1.93	5.03 ± 0.15	25.85 ±1.89	39.47 ±0.33	34.70 ±1.63	4
18 Day old rat										
Brain	6.93	0.37 ± 0.01	37.55 ±1.13	38.11 ±0.95	24.35 ±0.18	4.52 ± 0.24	36.80 ±2.27	45.96 ±0.68	17.25 ±1.58	2
Tumors										
Amelanotic melanoma	3.92	0.43 ± 0.02	35.53 ±0.93	13.96 ±0.88	53.46 ±1.47	1.96 ± 0.04	46.74 ±1.03	25.64 ±3.52	27.60 ±3.56	8
Melanoma B16	3.82	0.29 ± 0.02	41.00 ±2.96	21.28 ±0.73	37.73 ±2.32	3.11 ± 0.09	45.98 ±1.43	29.18 ±1.40	24.85 ±1.82	5
Sarcoma T241	3.01	0.59 ± 0.04	37.26 ±0.39	20.67 ±0.18	42.09 ±0.56	2.62 ± 0.02	43.87 ±0.43	27.52 ±1.05	28.62 ±0.62	4
Novikoff hepatoma	2.74	1.07 ± 0.01	41.93 ±1.24	30.22 ±1.41	27.85 ±0.67	2.26 ± 0.08	42.16 ±0.43	43.73 ±1.71	14.10 ±1.88	4

[a]Amounts are listed as wt percentages of alkyl and alk-1-enyl glycerols (average mol wt ~330). When lipid fractions are analyzed, wt percentages for the corresponding neutral lipids (alkyl and alk-1-enyl diacyl glycerols, mol wt ~860), ethanolamine phosphatides (mol wt ~730), and choline phosphatides (mol wt ~775) can be calculated by multiplying the data by 2.6, 2.2, and 2.3, respectively.

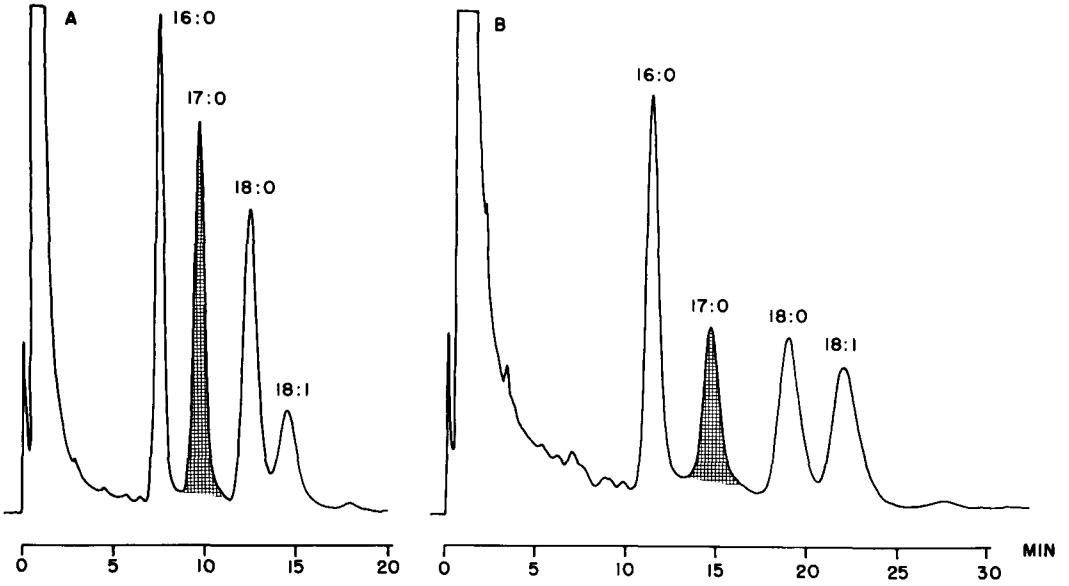

FIG. 2. Gas chromatograms of alkyl dioxanes (A) and isopropylidene derivatives of alkyl glycerols (B) derived from total lipids of Novikoff hepatoma, including internal standards (shaded areas). Victoreen 4000 instrument with hydrogen flame detector; aluminum column 180 x 0.4 cm inside diameter, 10% EGSS-X on Gas Chrom P, 100-120 mesh (Applied Science Laboratories); helium at 40 psi.

show 2-3 times higher amounts of alkyl glycerols and 3 times lower amounts of alk-1-enyl glycerols (9), calculated for total lipids of normal tissues. For tumor tissue, 4-5 times higher amounts of alkyl glycerols and 2-5 times lower amounts of alk-1-enyl glycerols were reported (5). Consequently, the alkyl to alk-1-enyl ratios were 5-10 times higher than those obtained from our data.

Perhaps one has to consider the fact that other dihydroxy compounds present in the lipid fraction or produced by $LiAlH_4$-reduction migrate with alkyl glycerols in TLC (25,26) and may, thus, lead to high alkyl glycerol values. Low values for alk-1-enyl glycerols may be explained by the possible formation of relatively volatile aldehydes caused by the sulfuric acid spray before charring. Therefore, we believe that synthetic alkyl glycerols should not be used as standards for the quantification of alk-1-enyl glycerols (9) by densitometry after charring on a thin layer plate.

ACKNOWLEDGMENTS

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SHORT COMMUNICATION

Slow Exchange of Erythrocyte and Plasma Phospholipids

ABSTRACT

^{32}P-Orthophosphate was injected intravenously into a pig, and the specific activities of serum phospholipids and phosphatidylcholine, serum and urine inorganic phosphorus, and erythrocyte phospholipids were followed for the next eight days. The specific activities of serum phospholipids, phosphatidylcholine, and inorganic phosphorus declined monoexponentially and were not significantly different from each other after the first day. These data imply that there are no large poorly labeled pools of inorganic phosphorus or phospholipids supplying serum phospholipid. Phospholipid metabolism is sufficiently rapid to obscure any immediate precursor product relationship. The specific activity of erthrocyte phospholipid rose slowly to a maximum on ca. the fourth day. The data were consistent with a precursor product relationship between serum phospholipids and part of the erythrocyte phospholipids.

INTRODUCTION

Several studies have demonstrated in vitro exchange of phospholipids between serum lipoproteins (1-7) and erythrocytes (8-10), most using ^{32}P-phospholipid as a tracer. The longer term in vivo changes in ^{32}P-phospholipid and inorganic phosphorus specific activity have not been reported. We have studied the changes in specific activity of serum and erythrocyte membrane phospholipids and serum and urine inorganic phosphorus over 8 days after ^{32}P administration.

METHODS

Lipids were extracted from serum and erythrocytes by a standard method (11), and phospholipids determined in duplicate (12). Phosphatidylcholine was determined after thin layer chromatography (13). Inorganic phosphorus in urine and serum was estimated in duplicate using an automated method (14). Nonprotein bound ^{32}P in serum was counted after trichloroacetic acid precipitation of protein (15).

Erythrocytes were washed 5 times in 0.15 M NaCl and the buffy coat removed with excess NaCl solution after each centrifugation. The haematocrit was measured in duplicate and the cells lysed by replacing the supernatant after centrifugation by an equal volume of distilled water, then freezing and thawing the suspen-

TABLE I

Specific Activity (SA) of Samples (μCi ^{32}P/g P)[a]

Time (days) after zero[b] serum	SA serum phospholipids (μCi/g P)	SA serum phosphatidylcholine (μCi/g P)	SA serum P_i (μCi/g P_i)	Time (days) after zero[c] urine	SA urine P_i (μCi/g P_i)
0			2805.0	0.25	147.4
0.125	15.1		207.7	0.89	52.7
0.79	30.9		52.8		
1.11	35.9	25.8	38.2	1.33	24.1
1.80	35.0		35.1	1.90	21.0
2.125	29.5	27.0	29.4	2.34	18.2
2.78	24.4		22.8	2.89	17.0
3.11	22.9	19.2	20.4	3.33	12.8
3.79	17.3	16.5	20.8	3.90	14.0
4.125	15.9		20.9	4.35	8.7
4.85	14.1	13.6	13.6	5.12	10.5
6.79	10.9	9.8	13.0	6.09	8.9
8.10	8.5	6.9	11.7	7.10	6.7

[a]Gaps in this table represent estimations not done because of loss of specimen or insufficient specimen.

[b]Zero time taken 15 min after $^{32}P_i$ injected into animal.

[c]Time taken T = mean of an exponential decay curve between T_1 and T_2; $T = T_1 + 0.32\ (T_2-T_1)$ ca.

sion.

^{32}P was counted in a liquid scintillation counter (Packard Tri-Carb) using an automatic external standard. Samples for counting were dissolved in toluene-PPO-POPOP with Triton-X100, after chloroform had been evaporated from the lipid extracts.

The experiments were performed on a healthy male 18 kg Large White pig aged 3 months. It was fed on a standard proprietary mash diet and kept in a metabolic cage, allowing quantitative collections of urine. Blood was obtained from the anterior vena cava by percutaneous puncture (16). Five mCi ^{32}P (as sodium orthophosphate, The Radiochemical Center, Amersham, England) were injected intravenously, and blood samples were taken over the next 8 days. Daily blood samples were taken for hemoglobin, hematocrit, leucocyte count and differential, platelet count, blood film, serum total protein, albumin, calcium, inorganic phosphorus, cholesterol, uric acid, creatinine, bilirubin, alkaline phosphatase, and aspartate aminotransferase, using standard laboratory techniques (14, 17). Standard statistical methods were used for data analysis (18).

RESULTS

In a preliminary experiment, 50 μCi ^{32}P (as sodium orthophosphate) were incubated with 25 ml serum at 37 C in a shaking waterbath and duplicate aliquots taken hourly. No significant incorporation of ^{32}P into phospholipid occurred over 5 hr (mean extraction 0.05%, range 0.008%-0.085%, not significantly different from chloroform-methanol extraction from water).

The specific activities of urine and serum inorganic phosphorus, total serum phospholipid, and serum phosphatidylcholine are shown in Table I. Results were corrected to allow for ^{32}P decay. The regressions of $\log_{10}$ specific activity upon time ($t > 1$ day) were calculated by the method of least squares. The biological half-lives ($T_{½}$) of urine and serum inorganic phosphorus, total serum phospholipids, and serum phosphatidylcholine were 3.3, 4.2, 3.2, and 3.3 days respectively (not significantly different from each other, $P < 0.05$). F-Distribution analysis (18) of the regression lines demonstrated a highly significant difference between urine P_i and the serum values for P_i, phospholipid, and phosphatidylcholine ($F_{6,29} = 6.9$, $P < 0.001$). However, any differences between the three regression lines for serum specific activities were not significant ($F_{4,21} = 1.8$, $P > 0.05$). The slightly lower values for urine specific activity may have been due to contamination of urine by food or faeces.

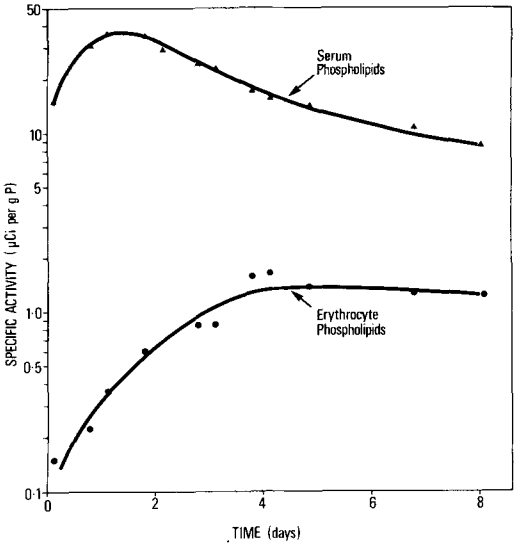

FIG. 1. Semilogarithmic plot of the specific activities of erythrocyte and serum phospholipids following injection of ^{32}P orthophosphate. Points represent means of duplicate estimations.

The specific activity of erythrocyte phospholipid did not reach a maximum until ca. the fourth day; the maximal value was an order of magnitude below that of plasma inorganic phosphate (Fig. 1). This indicates that ca. 90% erythrocyte phospholipid did not exchange with plasma phospholipid during the experiment and that the small portion of the erythrocyte phospholipid which exchanged with plasma phospholipid did so slowly.

No change occurred in the other haematological or biochemical variables measured.

DISCUSSION

Short term in vitro studies have shown that rapid phospholipid exchange between lipoprotein classes occurs (1-7), but information on the extent of exchange is sparse. One study (7) showed equilibration of a [Me-^{14}C] choline label after 4-5 hr incubation; this merely may have reflected rapid base exchange (19) rather than phospholipid exchange. Another suggested that [^{32}P] phospholipid equilibration between lipoprotein classes was incomplete after 20 hr incubation (1). If there was a significant non-exchangeable phospholipid component of lipoprotein (and it is well known that complete removal of phospholipid from apolipoprotein is difficult to achieve), one might expect there to be a difference in specific activity of phospholipid and inorganic serum phosphorus. There was no such difference, implying that there was no large poorly labeled pool of inorganic

phosphate or phospholipid feeding into the pool of serum phospholipid nor a large poorly labeled pool of serum phospholipid. These conclusions agree with those drawn from previous work. Zilversmit, et al., (20) in dogs, and Moser and Emerson (21) in man, derived precursor-product relationships between liver inorganic phosphate and glycerophosphate and liver phospholipids, and urine phosphate and plasma phospholipids, respectively. Although these experiments, in which the technique developed by Zilversmit, et al., (22) was used, suggest that no large poorly labeled pools of inorganic phosphate or phospholipid supply the pool of serum phospholipid, the complexities of phospholipid metabolism are such that calculation of turnover times is likely to be of limited validity.

The changes in specific activity of erythrocyte membrane phospholipid suggest a slow equilibration of part of the erythrocyte phospholipid pool with that of plasma. Other workers (reviewed by Shohet [23]) have demonstrated passive equilibration of part of the total erythrocyte phosphatidylcholine in serum. They also have shown that more rapid exchange of lysophosphatidylcholine occurs, with subsequent intracellular acylation to less exchangeable phosphatidylcholine or deacylation to exchangeable water-soluble glycerylphosphorylcholine (23). The changes we have observed support this concept that there is slow equilibration between serum phospholipids and part of the total erythrocyte phospholipids.

G.D. CALVERT
Department of Clinical Chemistry
Wolfson Research Laboratories
Queen Elizabeth Hospital
Queen Elizabeth Medical Center
Birmingham, B15 2TH, England
P.J. SCOTT
Department of Medicine
Auckland Hospital
Auckland 3, New Zealand

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LETTERS TO THE EDITOR

Concerning an Analysis of Mortality Rates

Sir: In a recent issue of *Lipids* (7:576, 1972), Dupont, Mathias, and Cabacungan present the following data on the mortality experience of rats in an experiment designed, in part, to determine whether there is an effect on mortality due to diets high in animal or vegetable fats.

Table I, which represents a modified form of the one given by Dupont, et al., may be interpreted as follows: 25 male rats on low fat (LF) diet enter the study at 3 months; 4 die between 3 and 12 months (16% mortality rate). Of the 21 survivors, 6 are sacrificed, leaving 15 animals to enter the 12-15 month period, and 2 die between 12-15 months. Of the 13 remaining animals, 6 more are sacrificed at 15 months. In the period 15-18 months, 2 of the remaining 7 animals die.

The use of percentages is misleading, and the authors' statement (Table I, footnote a) that the mean mortality over the experiment is 9.68% is incorrect. For example, suppose one observes 1 death in 2 animals, in 1 class, and 6 deaths in 8 animals in another. Then, over the 2 classes, one observes 7 deaths in 10 animals, or a 70% mortality. This differs from the average of the mortality rates for the 2 classes, i.e. $\frac{50\%+75\%}{2} = 62.5\%$.

The total mortality over the experiment is actually $\frac{27}{144} = 18.75\%$. However, because of the sacrificed animals, this figure may be an underestimate.

If the animals considered at 12-15 months were different animals from those in the 3-12 month period, a mean mortality rate could be calculated, e.g. in the case of male LF rats, this would be $\frac{4+2}{25+15} = \frac{6}{40} = 15\%$, not $\frac{16\%+13\%}{2} = 14.5\%$. However, because some of the animals are considered in both the 3-12 and 12-15 month period, this treatment of the data should be avoided. Also, the cumulative percentages given in the table do not provide relevant information. For example, for the male LF rats, $\frac{6}{25} = 24\%$, not 29% as stated, are observed to die by the end of 15 months.

In general, chi-square tests should be avoided when numbers as small as those in Table I are being considered. The χ^2 probability distribution is used as an approximation to the distribution of a quantity (test statistic), obtained when a test of significance is performed. Such an approximation may be poor when the data consist of small numbers.

A suitable analysis of the data involves computing exactly the probability (p) of obtaining, by chance, an observation as unfavorable or more unfavorable to the hypothesis being tested than the one observed. If this probability is small, this, then, suggests that the hypothesis is not supported by the data. (See,

TABLE I

Death from Apparent Chronic Respiratory Disease, Per Cent of Survivors and Number[a]

	Males			Females			Males[a]	Females[a]
Period	3-12 Months	12-15 Months	Cumulative	3-12 Months	12-15 Months	Cumulative	15-18 Months	15-18 Months
LF[b]	16%	13%	29%	0	0	0	28.6%	0
	4/25	2/15		0/23	0/17		2/7	0/11
BT[b]	12%	37.5%	49.5%	0	23%	23%	0	28.6%
	3/25	6/16		0/23	4/17		0/4	2/7
CO[b]	4%	5.5%	9.5%	4%	0	4%	9%	0
	1/24	1/18		1/24	0/18		1/11	0/12

[a]Chi square test of significant difference from the grand mean of 9.68% mortality showed males greater than females, all ages ($P<.05$); diet effect in males at both ages ($P<.01$) and females at 12-15 months ($P<.01$); and sex-diet interactions at both ages ($P<.01$).

[b]LF = Low fat (2% of calories as corn oil); BT = beef tallow (2% corn oil plus 40% beef fat), CO = corn oil (42% of calories as corn oil); see Table I.

[a]Additional data supplied by J. Dupont.

for example R.A. Fisher, *Statistical Methods for Research Workers,* Fourteenth Edition, Oliver and Boyd, 1970, p. 97.) Such an analysis indicates that at the 5% significance level, there is no diet effect in either 3-12 months old males ($p = 0.515$; cf. Dupont, et al.'s previous calculation of $p < .01$) or in 12-15 months old males ($p = .072$; cf. Dupont, et al.'s previous value of $p < .01$); the diet effect in 12-15 months old females is significant ($p = .018$; cf. Dupont, et al., $p < .01$); and there is a significant difference between male and female mortality in the age range of 3-12 months ($p = .034$) but not for the 12-15 months animals ($p = .141$) (cf. previous levels of $p < .05$ for both groups). Other analyses indicate that there is no diet-age group for which the male and female mortality experience is significantly different.

In general, it may be desirable to compare the mortality experience across the entire experiment. Such an analysis requires a slightly more sophisticated approach and may require additional assumptions.

Certain methods have been developed, e.g. M. Zelen, *Technometrics* 1:769 (1959); D.R. Cox, *J. Royal Stat. Soc.* (B) 34:187 (1972); and N. Mantel, *Cancer Chemotherapy Reports,* 50:163 (1966) which may be used in studies of mortality. In particular, Mantel's method would seem to apply to data of this type, since it allows for the study of the trend in mortality across the experiment and does not focus merely on a specific time period. Using this method, one obtains the following: (A) There is no significant difference in the mortality experience between rats raised on beef tallow (BT) and those raised on LF diets ($.20 < p < .10$) or between those raised on corn oil (CO) and those raised on LF diets ($p \sim .30$). (B) The mortality experience of animals raised on BT diets is significantly different from those raised on CO diets ($.001 < p < .01$). (C) The male and female mortality patterns are significantly different ($.01 < p < .02$).

It should be noted that Mantel's method uses a χ^2 distribution to obtain a significance level. Mantel, however, corrects his value for small numbers.

K. S. BROWN
Department of Statistics
University of Waterloo
Waterloo, Ontario, Canada

ACKNOWLEDGMENT

J. Dupont supplied additional information to Table I.

[Revised manuscript
received October 10, 1973]

Reply to K. S. Brown

Sir: The purpose of the use of statistical treatment of data is to obtain an objective evaluation of the validity of differences between experimental treatments. Dr. Brown has presented several objective means for analyzing our data, each of which gives different conclusions. One must, therefore, choose a statistical procedure subjectively. The data are nonparametric as published. Mantel's method would appear to give more precise analysis than the simple chi-square which we used. Use of survivor function would appear to be the most precise way to deal with the data which we have, but which were not published, since they were individual observations.

I wish to thank Dr. Brown for his informative comparison of statistical methodology and his communication with us about it.

JACQUELINE DUPONT
Food Science and Nutrition
Colorado State University
Fort Collins, Colorado 80521

[Received October 4, 1973]

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JACQUELINE DUPONT
Food Science and Nutrition
Colorado State University
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[Received October 4, 1973]

ERRATUM

Several errors occurred in the publication of "Leaf Wax of Oats" by A.P. Tulloch and L.L. Hoffman (*Lipids* 8:617 [1973]). The words "relative to" should be deleted from the fifth sentence of the fifth paragraph under the section "Experimental Procedures." In the first sentence under the subsection entitled "Separation of Wax," the word "liter" was deleted. Parentheses were deleted from the third sentence of the fifth paragraph of the "Results and Discussion" section. In the first sentence of the sixth paragraph of that section, the words "with 1" should be replaced by "-one." In the "References," ref. 10 should read: "Tulloch, A.P., Phytochemistry 12:2225 (1973). Portions of the article, with corrections, appear below.

Amounts of hydrocarbons were calculated from the response relative to *p*-dioctylbenzene, amounts of alcohols (as acetates), free acids (as methyl esters) and long chain esters were calculated relative to octadecyl octadecanoate.

Wax (11.3 g) was applied to Biosil A (Bio Rad, Richmond, Calif.), (200 g, activated at 100 C for 18 hr), and hydrocarbons (0.97 g) were eluted with hexane (1 liter).

MS examination gave peaks at m/e 409 and 394 (5-OH), 409 and 380 (6-OH), and 395 and 366 (7-OH) (these 2 very small).

Alkaline hydrolysis and separation into ketonic and acidic fractions and conversion of acids to methyl esters gave methyl palmitate and heptadecan-2-one showing that the parent dione was a 14,16-dione (also indicated by MS peaks at 281 $[COCH_2CO[CH_2]_{14}CH_3]$ and 239 $[CO[CH_2]_{14}CH_3]$).

Effect of Soluble and Membrane Proteins upon Diethyl Ether Extraction of Aqueous Phospholipid Dispersions

G. PARENTI-CASTELLI, E. BERTOLI, A.M. SECHI, M.G. SILVESTRINI, and G. LENAZ,
Instituto di Chimica Biologica, Università di Bologna, Bologna, Italy 40126

ABSTRACT

The effect of proteins upon diethyl ether extraction of phospholipids from aqueous dispersions has been investigated as a model for elucidating lipid-protein interactions in the more complex membrane systems. Mixed phospholipids having a net anionic charge or purified lecithin (a zwitterion) are extracted from water dispersions into ether only after addition of salts. Basic proteins (lysozyme and cytochrome *c*), by ionically binding phospholipids, allow extraction. Phospholipids are extracted together with the proteins in the form of neutralized lipid-protein complexes. On the other hand, lipid depleted mitochondria (a hydrophobic protein residue after acetone extraction of mitochondria), after reconstitution with phospholipids, do not allow phospholipid extraction unless salts or basic proteins also are added to the system. This observation indicates that, in reconstituted membranes, the phospholipids are largely in the bilayer form with the polar heads still charged and susceptible to ether extraction only after neutralization with salts or basic proteins.

INTRODUCTION

Organic solvents may be useful in the elucidation of the properties of biological membranes (1). The nature of the lipid-protein interactions in mitochondrial membranes has been investigated in our laboratory by using alcohols (2) and diethyl ether or pentane (3).

Phospholipids largely are extracted from aqueous suspensions of mitochondrial membranes by diethyl ether only when extrinsic water soluble proteins (4) have been removed previously from the membrane (3).

This observation suggests different types of bonds between lipids and proteins in the mitochondrial membranes; in particular, the extrinsic proteins appear to be lipid-bound in such a way that phospholipids are not available for partition into a nonpolar medium, while the intrinsic membrane proteins do not appear to prevent phospholipid extraction into the ether phase. These interesting effects may have profound implications upon membrane structure; on the other hand, we feel that there is insufficient knowledge of the effects of nonpolar solvents upon phospholipid extraction from aqueous systems and of the effect of proteins upon such extraction. For this reason, we thought it interesting to investigate ether extraction of phospholipid aqueous dispersions and of simple lipid-protein systems to obtain better knowledge of the effect of different types of lipid-protein interactions upon the extractability of phospholipids by ether.

MATERIALS AND METHODS

Membrane preparations: Beef heart mitochondria were prepared by a small scale procedure (5). Lipid depleted mitochondria (LDM) were prepared by extraction with cold 90% aqueous acetone containing ammonia, according to the procedure of Fleischer and Fleischer (6). LDM lost most phospholipids (90-95%) and only contain a residual amount (1-2 μg/mg protein) of tightly bound cardiolipin, as checked by thin layer chromatography (TLC) (7).

Phospholipids: Phospholipids used were either Asolectin (commercial soybean phospholipids supplied by Associated Concentrates, Long Island, N.Y.) or phospholipids derived from beef heart mitochondria, beef myelin, or rat liver (8) by extraction with chloroform-methanol according to Folch, et al., (9) followed by purification of the phospholipids on silicic acid (10). Dipalmitoyl lecithin was supplied by K and K Laboratories, Plainview, N.Y., and egg yolk lecithin by Koch-Light Laboratories, Colnbrook-Bucks, England. TLC (7) has shown the presence of traces of impurities in the egg lecithin preparation, mainly phosphatidyl ethanolamine. Lecithin was purified by DEAE cellulose chromatography according to Rouser, et al. (11).

All phospholipids were prepared as clear aqueous dispersions by sonic oscillation (6) using a Branson sonifier. Sonication was followed by centrifugation at 30,000 rpm for 40 min in the no. 40 rotor of the Spinco model L-65 ultracentrifuge, and only the unsedimented material was used throughout the experiments, to use vesicles as much as possible homogeneous in size.

Lipid-protein interactions: Recombination of phospholipids with LDM was accomplished

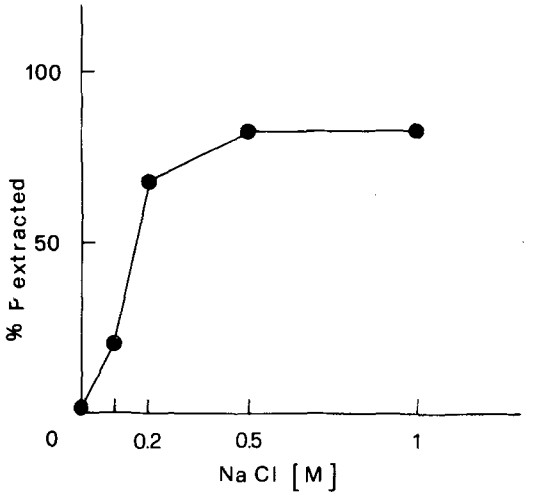

FIG. 1. Effect of NaCl upon phospholipid extraction from Asolectin aqueous dispersions into ether.

as previously described (12). The resulting reconstituted membranes resembled original membranes in lipid content and certain enzymatic activities. It must be remembered that, since LDM lost extrinsic proteins (13), the reconstituted particles represent membranes containing only intrinsic hydrophobic proteins.

Ionic binding of phospholipids with basic proteins (cytochrome *c*, Sigma, St. Louis, Mo., Type II-A; lysozyme, Biochemia, Milan, Italy) was accomplished by mixing the components as described below.

A ternary complex of mitochondrial protein-phospholipid-basic protein, where phospholipids are bound ionically to the basic protein and hydrophobically to the mitochondrial protein (14), was formed by incubating reconstituted mitochondria with the basic proteins in a

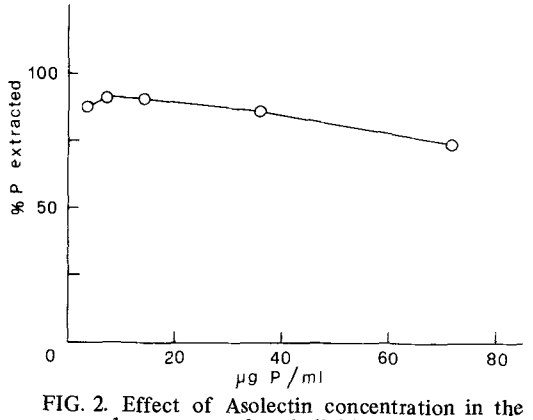

FIG. 2. Effect of Asolectin concentration in the aqueous phase upon phospholipid extraction into ether. The aqueous phase contained 0.15 M NaCl. No extraction occurs in absence of salt at all concentrations tested.

TABLE I

Diethyl Ether Extraction of Aqueous Phospholipid Dispersions[a]

Addition	Percent P extracted
—	0
NaCl 0.2 M	68
NaCl 1 M	83
NaSCN 0.2 M	47
NaSCN 1 M	98
HCl 10 mM	40
Urea 6 M	0
Urea 6 M + NaCl 0.1 M	26
NaCl 0.1 M	10

[a]Asolectin was ca. 130 μg/P (3.25 mg phospholipid).

medium as described by Kimelberg and Papahadjopoulos (15) for the interaction of basic proteins with phospholipid dispersions.

Diethyl ether extraction: The partition of phospholipids between an aqueous medium and diethyl ether was accomplished essentially as described in a previous paper on mitochondrial membranes (3). In a typical experiment, a phospholipid aqueous dispersion or membrane preparation containing 100-120 μg phospholipid phosphorus in a total volume of 10 ml was shaken for 10 min at room temperature (20 C) with 20 ml diethyl ether. The ether phase was separated, and the extraction was repeated twice. The combined ether phases were reduced to a final volume of 10 ml, and phosphorus was determined both in the ether and in the aqueous phase. Variations of the procedure described above, as well as additions to the aqueous phase, are described below. In some experiments, the aqueous phase was resolved further into soluble and sedimental fractions (40,000 rpm for 10 min in the Spinco); and phosphorus was assayed in both fractions.

Analytical procedures: Lipid phosphorus was assayed by the method of Marinetti (16). Protein was determined by a biuret method (17); when soluble proteins were to be determined, the absorption at 280 nm was taken as a relative measure of protein concentration.

RESULTS

Studies with Sonicated Soybean Phospholipids (Asolectin)

The phospholipid mixture routinely used throughout this investigation (Asolectin) has the following average composition according to Richardson, et al. (18): phosphatidyl choline, 31%; phosphatidyl ethanolamine, 26%; phosphatidic acid, 11%; cardiolipin, 4%; phospha-

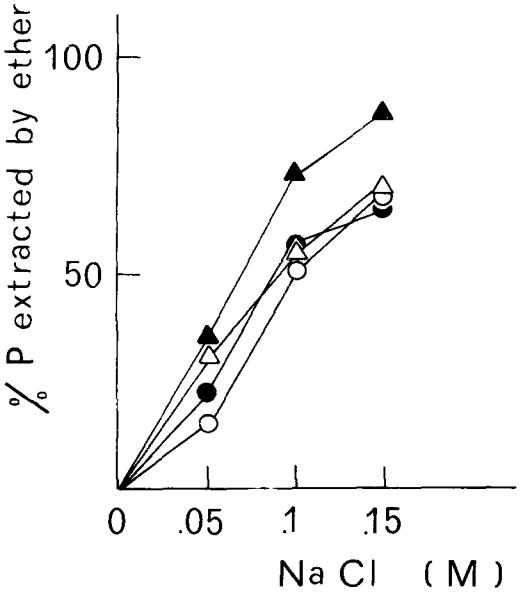

FIG. 3. Effect of NaCl upon ether extraction of aqueous dispersions of different phospholipid mixtures. Each phospholipid mixture contained ca. 120 μg lipid P in 10 ml aqueous phase. ○—○ = myelin phospholipids, ●—● = Asolectin, △—△ = rat liver phospholipids, and ▲—▲ = beef heart mitochondrial phospholipids.

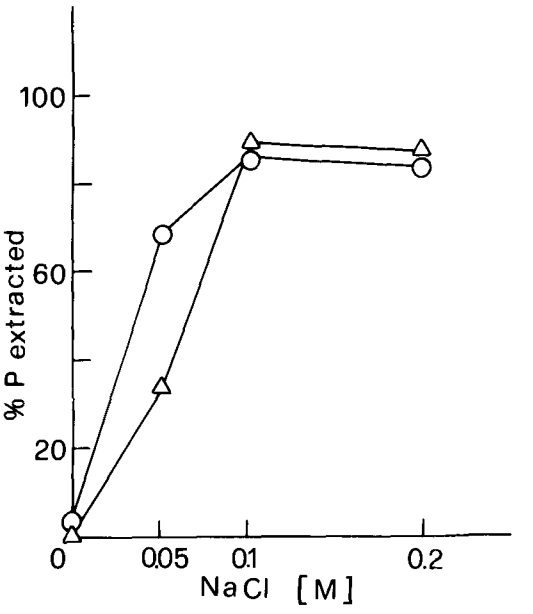

FIG. 4. Effect of NaCl upon ether extraction of purified egg lecithin and dipalmitoyl lecithin. ○—○ = egg lecithin purified by DEAE-cellulose chromatography and △—△ = synthetic dipalmitoyl lecithin.

tidyl inositol (components migrating with same R_f), 18%; and unidentified 9%.

The average surface charge of the phospholipid lamellae is anionic at pH near neutral and the bilayer vesicles formed by extensive sonication have a negative ζ potential (19).

Figure 1 shows the effect of NaCl concentration upon the extraction of phospholipids from the aqueous dispersion into the ether phase. Almost no extraction occurs if salt is omitted in the water phase; there is a progressive partition of phospholipids in ether by increasing the salt concentration. The nature of the salt does not appear to be specific (3), and the concentration necessary for extraction is low, pointing out that the effect must be electrostatic in nature (20,21). Table I summarizes the effects of different additions in the aqueous phase: dilute acid also allows phospholipid extraction in ether, while urea prevents extraction (3,22). It can be seen that agents which decrease the negative charges of the phospholipid vesicles are effective in transferring phospholipids into the organic phase. Figure 2 shows the effect of phospholipid concentration upon its extraction into the ether phase. No major changes in partition appear at different phospholipid concentrations at a constant NaCl concentration of 0.15 M.

Phospholipid Specificity

The specificity of phospholipids with regard to diethyl ether extraction has been investigated, with special attention to phospholipid mixtures having different fatty acid composition (8). No large reproducible differences were found between different phospholipid mixtures (Fig. 3). It must be pointed out that all of the mixtures investigated contain anionic phospholipids and form vesicles having net negative charges. No phospholipid is extracted significantly in the absence of salt.

We then investigated the ether extractability of phospholipids having no net surface charge. Lecithin is zwitterionic at a wide pH range; the pattern of the ether extraction of two lecithins having different fatty acid composition is shown in Figure 4. Again no phospholipid is extracted in ether at zero salt concentration. The lecithins studied are chromatographically pure and have no anionic phospholipid contamination after DEAE cellulose chromatography. A main physical difference exists between the two kinds of lecithins at the temperature of extraction; while egg lecithin is liquid-crystalline, dipalmitoyl lecithin is in a crystalline phase (23). Lipids in crystalline phase maintain a vesicular lamellar structure in aqueous media (24). Notwithstanding these differences, ether is effective in ca. the same way in extracting phospholipid from the aque-

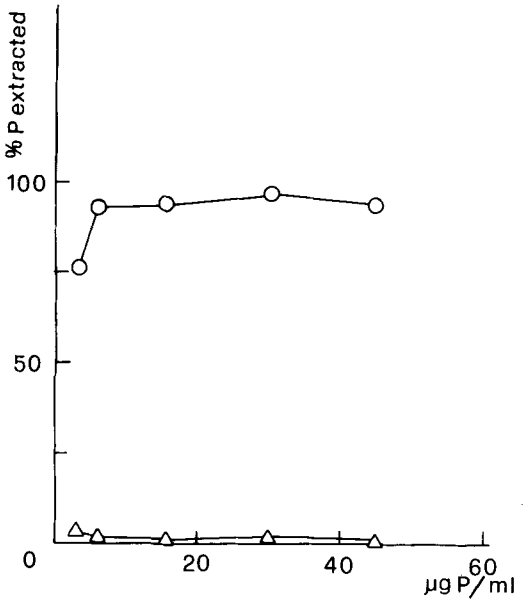

FIG. 5. Effect of lecithin concentration in the aqueous phase upon P extraction into ether. Purified egg lecithin in absence of salt (△——△) or in presence of 0.15 M NaCl (○——○).

ous phase.

Figure 5 shows the effect of purified egg lecithin concentration upon its extraction into the ether phase. In absence of salt, no lecithin is extracted into ether at any P concentrations tested; on the other hand, at 0.15 M NaCl, 80-95% lecithin is extracted from 3-45 μg P/ml aqueous phase.

Effect of Basic Proteins

Basic proteins bind anionic phospholipids by electrostatic interactions (20,21,25); it is known that the phospholipids form lamellar phases in water with the basic proteins disposed in the interlamellar spaces (21,26). Addition of the basic proteins, cytochrome *c* and lysozyme, to anionic phospholipid aqueous dispersions allows ether extraction of the phospholipids in absence of salt (Table II and Fig. 6). Protein is, however, extracted together with the lipids in the form of a nonpolar lipid-protein complex; protein extraction is prevented by salt, which breaks ionic bonds and allows extraction of the phospholipids alone, while the proteins remain in the aqueous phase. Similar results are known for the extraction of cytochrome *c* and anionic phospholipids into isooctane (20). Figure 7 shows that the phospholipid-lysozyme complex concentration has no effect upon P extraction into the ether phase.

TABLE II

Effect of Cytochrome *c* and Lysozyme upon Phospholipid Extraction by Diethyl Ether[a]

Addition to Asolectin	Percent P extracted
—	1
Cytochrome *c* (4 mg)	54
NaCl 1 M	70
Cytochrome *c* + NaCl 1 M	51
—	2
Lysozyme (3.5 mg)	76

[a]Asolectin P was ca. 130 μg (3.25 mg phospholipid).

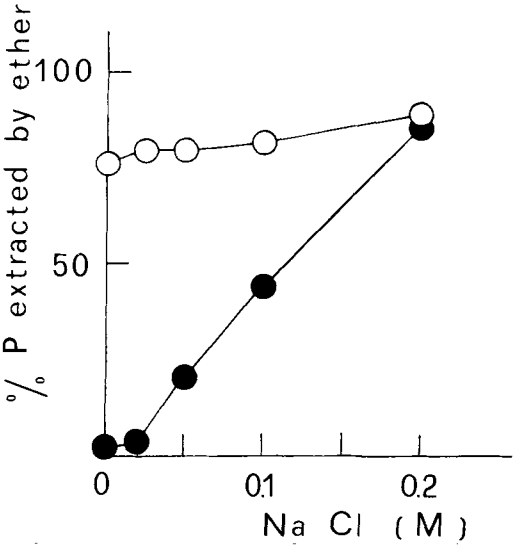

FIG. 6. Effect of lysozyme upon phospholipid extraction by diethyl ether. ●——● = Asolectin and ○——○ = Asolectin-lysozyme complex.

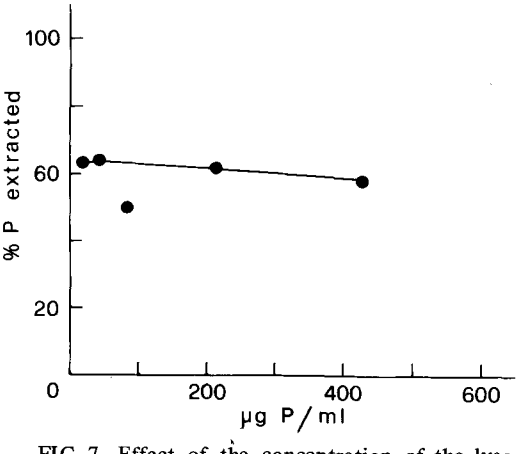

FIG. 7. Effect of the concentration of the lysozyme-Asolectin complex upon phospholipid extraction into ether. The data are represented as μg lipid P present in the complex.

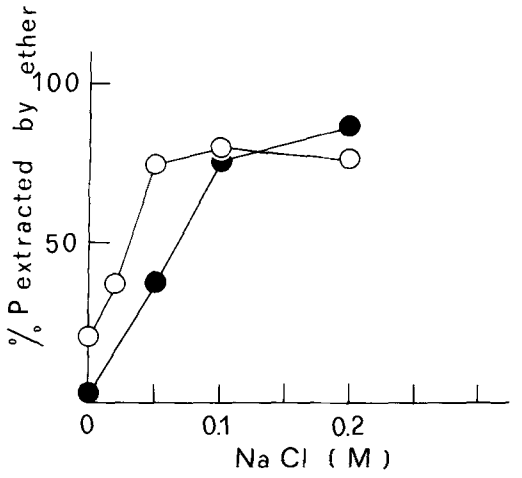

FIG. 8. Effect of lipid depleted mitochondria upon phospholipid extraction by diethyl ether. ●—● = Asolectin and ○—○ = Asolectin + lipid depleted mitochondria; reconstitution was accomplished as described previously (12) and the excess of unreacted phospholipids were washed. The complex contained 17 μg P/mg protein.

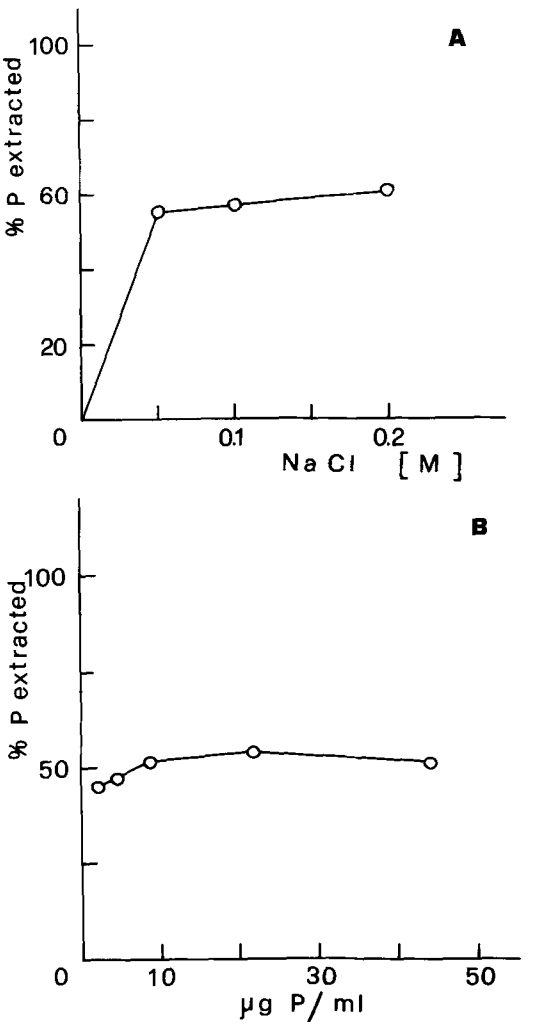

FIG. 9. Ether extraction of phospholipids from reconstituted mitochondria. This is a different preparation from the one of Figure 8. A—Effect of salt concentration. B—Effect of complex concentration, expressed in μg P/ml aqueous phase. The reconstituted mitochondria contained 14.6 μg P/mg protein.

Effect of LDM

LDM are membrane-like structures formed by the hydrophobic protein residue after aqueous alkaline acetone extraction. LDM bind phospholipids by nonionic interaction (12), presumably by means of hydrophobic bonds, giving rise to reconstituted membranes having part of the original properties of the mitochondrial membrane (27). We investigated ether extraction of mitochondrial membranes reconstituted from LDM and Asolectin. Figures 8 and 9A show the phospholipid extraction of such mitochondrial membranes as a function of salt concentration. No protein apparently is extracted into the ether phase. Addition of salt induces an increase of phospholipid extraction (in absence of salt) to high values, although variable for different experiments. Figure 9B shows the effect of membrane concentration upon phospholipid extraction by ether. Again, no major differences are apparent in the range explored.

Effect of a Mitochondrial Protein-Phospholipid-Basic Protein Ternary Complex

We have described elsewhere (14) the formation of a ternary complex of LDM with anionic phospholipids and basic proteins, such as lysozyme. The complex is formed by adding basic proteins to reconstituted membranes previously prepared by interacting LDM with phospholipids. Table III describes the formation and stoichiometry of the complex with lysozyme. Higher quantities of basic protein are bound to the phospholipids in the membrane than in the Asolectin vesicles. Correction of the binding for that in LDM allows a reasonable comparison, showing that the phospholipid negative charges are available for interaction with basic proteins in the reconstituted membranes.

Table IV shows that a significant aliquot of the phospholipids in the complex is extracted by ether bound to the basic protein when salt is not present, showing that they are in the same arrangement in Asolectin vesicles and in reconstituted membranes. Table V indicates that a substantial amount of phospholipid is extracted

TABLE III

Stoichiometry of Binding of Lysozyme to Phospholipid Vesicles and Reconstituted Mitochondria

Phospholipid vesicles		Reconstituted mitochondria[a]	
Total P in the assay (μmoles)	$\frac{\text{Lysozyme}}{\text{P}} \times 100$	Total P in the assay (μmoles)	$\frac{\text{Lysozyme}}{\text{P}} \times 100$
0.68	4.4	0.63	5.2
1.35	4.5	1.25	7.0
2.71	3.8	2.50	6.2
4.06	4.1	3.75	5.6
5.42	3.7	5.00	5.0

[a]Mitochondrial membranes reconstituted from lipid depleted mitochondria and Asolectin.

when lysozyme has interacted with natural membranes (submitochondrial electron transfer particles [ETP] [28]), showing again that, even in ETP, lysozyme is bound to the phospholipids.

DISCUSSION

Aqueous dispersions of mixed phospholipids are not extracted with diethyl ether if salt or dilute acid are not added in the water phase. This observation agrees with studies of Bruckdorfer, et al. (22). The most likely explanation of the salt effect, in our opinion, is the following: ether does not solubilize anionic lamellar phospholipids if the membrane potential has a high negative value. Neutral salts decrease the ζ potential of the lamellae to zero, facilitating a new distribution of phospholipid molecules in the ether phase in the form of inverted micelles with the anionic groups neutralized by metal cations. Evidence for the existence of a micellar structure of phospholipids in organic solvents was given by Lee, et al. (29). Unexpectedly, lecithin, which is zwitterionic at neutral pH, may not be extracted by ether in absence of salt. On the other hand, Misiorowski and Wells (30) have shown that lecithins bind metal cations in organic solvents. Little is, however, known about the conformation of phospholipid head groups in bilayers (31) and about the effect of salts on such conformation. Bruckdorfer, et al., (22) observed that lecithin can be extracted in the absence of salt if cholesterol is incorporated in the lecithin bilayer. Cholesterol apparently dilutes the charge density of the membrane and, thus, may allow extraction of the phospholipids. This interpretation also explains our observation that a pH decrease, which induces protonation of anionic groups, enhances phospholipid extraction. No major changes in extractability were found by varying the types of phospholipid mixtures nor the fatty acid composition of the phospholipids (purified egg lecithin, unsaturated, and dipalmitoyl lecithin, having two C16:0, are both extracted according to similar patterns). Moreover, no major changes were induced by varying the phospholipid concentrations in the aqueous phases.

TABLE IV

Effect of Lysozyme and Salt upon Diethyl Ether Extraction of Phospholipids from Asolectin Vesicles and Asolectin-Reconstituted Mitochondria

Membrane	Addition	Percent P extracted in ether
Asolectin	—	1.3
	NaCl 0.15 M	66.0
	Lysozyme[b]	47.4
RM[a]	—	4.6
	NaCl 0.15 M	51.0
	Lysozyme[b]	48.5

[a]Asolectin-reconstituted mitochondria, prepared according to ref. 12.

[b]0.4 μmoles of lysozyme were added to either 175 μg Asolectin phosphorus or to 130 μg RM phosphorus prior to ether extraction.

Cytochrome *c* and lysozyme are basic proteins which bind phospholipids by ionic interactions (20); their addition to phospholipid aqueous dispersions allows ether extraction in the absence of salt. Protein is, however, extracted together with the lipids, probably also in the form of inverted micelles; such an extraction of a proteolipid complex is prevented by salt, which breaks ionic bonds and allows extraction of lipids alone.

Gitler and Montal (32) have shown that cytochrome *c* or other proteins are extracted into decane complexed with phospholipids only in presence of divalent cations or at very low pH. In that case no, or very little, water is partitioned in the organic phase, while ether dissolves significant proportions of water and

may allow formation of inverted phases with water in contact with the buried neutralized polar groups.

When proteins are linked to phospholipids nonionically and presumably by hydrophobic bonds, as suggested by us for LDM, the pattern of ether extraction resembles that in absence of protein. No, or very little, protein is extracted in absence of salt; but, by increasing the salt concentration, increasing amounts of protein-free phospholipids were extracted into ether. This observation allows better interpretation of the previously found effects upon mitochondrial membranes. Membranes devoid of detectable associated proteins are susceptible to ether extraction of phospholipids, provided that salt is added to neutralize the phospholipid polar charges (3). In our system of reconstituted membranes the lipid-protein interaction must be such as to leave the polar head groups of phospholipid still charged and not susceptible to ether extraction unless salt is added for neutralization.

A final observation which can bear important consequences upon membrane structure is the formation and behavior of a mitochondrial protein-phospholipid-lysozyme ternary complex. Such a complex is formed by hydrophobic interaction of mitochondrial proteins (as LDM) and phospholipids, with subsequent interaction of the still free polar anionic groups of the phospholipids (see above) with the basic protein, lysozyme. The formation of such a complex represents a strong indication that the polar heads of the phospholipids are available on the surface of reconstituted membranes as they are in liposomes. Similar results were obtained with natural membranes (ETP) having their natural phospholipid complement. In all of these membranes, the bound lysozyme can be extracted into ether bound to membrane phospholipids, strongly suggesting that lysozyme has interacted mainly with the lipid rather than with the protein components of the membrane.

TABLE V

Effect of Lysozyme and Salt upon Diethyl Ether Extraction of Phospholipids from Submitochondrial Electron Transfer Particles

Membrane	Addition	Percent P extracted in ether
ETP[a]	—	10.6
	NaCl 0.15 M	53.6
	Lysozyme (0.25 μmoles)	40.0
	Lysozyme (0.25 μmoles) + NaCl 0.15 M	49.2

[a]For each extraction, a sample of ETP was used containing 2.0 mg protein and 41 μg lipid P.

ACKNOWLEDGMENTS

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Acyl Specificity in Glyceride Synthesis by Lactating Rat Mammary Gland[1]

HIROAKI TANIOKA[2], CHU YUAN LIN, STUART SMITH[3], and S. ABRAHAM,
Bruce Lyon Memorial Research Laboratory, Children's Hospital Medical Center, Oakland, California 94609

ABSTRACT

We have investigated the possibility that the nonrandom association of fatty acids in rat milk triglycerides results from specificity of the acyl transferases in the glycerolphosphate pathway. Subcellular fractionation of lactating rat mammary gland revealed that the microsomal fraction was the most active in acylation of 3-sn-[U-^{14}C] glycerolphosphate with various acyl-CoA's. The major products were diacylglycerolphosphate and diglyceride; no monoacylglycerolphosphate was detected. Maximum rate of acylation occurred at or below the critical micelle concentration for each acyl-CoA, indicating that only the monomeric substrate molecules were acceptable by the enzyme system. The observed acyl specificity, 16:0 > 18:0 ≐ 14:0 > 12:0 > 10:0 > 8:0 is consistent with the concept that, in general, milk triglycerides are synthesized by insertion of a short or medium chain fatty acid into a long chain diglyceride.

INTRODUCTION

The experimentally determined distribution of mol wt of milk fat triglycerides from many species deviate appreciably from that predicted by random association of fatty acids from a single pool (2, 3). It has been proposed that this nonrandom association of fatty acids is due to the positional specificity on the glycerol backbone of particular chain length fatty acids (4). The stereochemical analyses of milk fat triglycerides support this thesis, for long chain fatty acids predominate in positions 1 and 2 of the glycerol, whereas short chain fatty acids are found predominantly in position 3 (4, 5).

We have investigated the possibility that the nonrandom association of fatty acids in milk fat triglycerides may result from specificity of the acyl transferases involved in lipid synthesis in the lactating mammary gland.

We chose to use acyl-CoA derivative as substrates rather than fatty acids to avoid complications arising from specificity of the activating enzymes, although we recognize that more rapid rates of glyceride synthesis usually can be obtained with the free acids.

MATERIALS

Preparation of 3-sn-[U-^{14}C] glycerolphosphate: Glycerol kinase, purchased from Boehringer and Soehne, Mannheim, Germany, (adenosine 5′ triphosphate [ATP]:glycerol phosphotransferase, EC 2.7.1.30) was used to convert [U-^{14}C] glycerol (6) to 3-sn-[U-^{14}C] glycerolphosphate. The reaction mixture contained Tris buffer (pH 9.0), 600 μmoles; [U-^{14}C] glycerol (68 μCi/μmole), 15 μmoles; ATP, 150 μmoles; $MgCl_2$, 10 μmoles, and glycerol kinase, 30 international unit, in 4.0 ml. The mixture was incubated for 30 min at 30 C, cooled to 0 C, and diluted with 4 ml water. The 3-sn-[U-^{14}C] glycerolphosphate was purified by chromatography on a column (10 x 1 cm) of Dowex AG-1 formate (7). Glycerolphosphate was assayed spectrophotometrically (8).

Preparation of acyl-CoA derivatives: Acetyl-CoA, butyryl-CoA, and hexanoyl-CoA were prepared from the appropriate acid anhydrides (Eastman Kodak, Rochester, N.Y.) by an adaptation of the method of Simon and Shemin (9) and purified by chromatography on DEAE-cellulose (10). Longer chain acyl-CoA's were synthesized from their acyl chlorides (Hormel Institute, Austin, Minn.) and purified according to the procedure described by Seubert (11). Acyl-CoA concentrations were assayed by measuring, with 5,5-dithiobis (2-nitrobenzoate), sulfhydryl groups released following mild alkaline hydrolysis (12). The ratio of E232nm : E260nm for the acyl-CoA's was in the range of 0.56-0.60 : 1.0.

Other chemicals and cofactors were obtained from Sigma Chemical Co., St. Louis, Mo.; Calbiochem, La Jolla, Calif., and Boehringer and Soehne and were of the highest purity available.

EXPERIMENTAL PROCEDURES

Preparation of subcellular fractions: Lac-

[1]Presented at the 68th Annual Meeting of the American Dairy Science Association, Pullman, Wash., June 1973 (1).

[2]Research Fellow of the Bay Area Heart Research Committee; present address: Osaka University Dental School, Osaka, Japan.

[3]Established Investigator of the American Heart Association, to whom correspondence should be addressed.

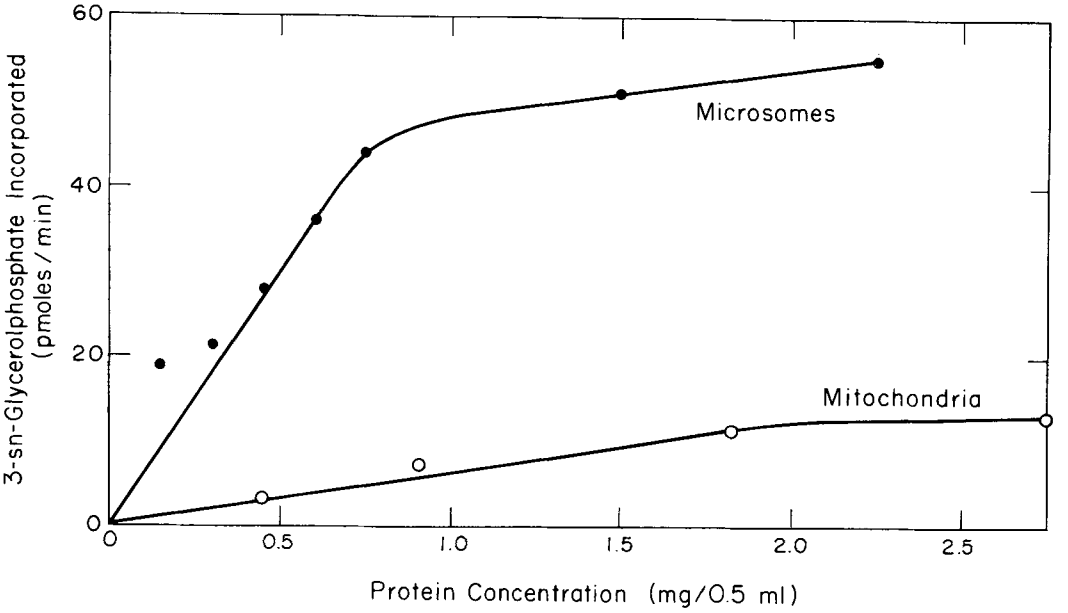

FIG. 1. Effect of protein concentration upon acylation of 3-sn-glycerolphosphate. Palmityl-CoA (2 μM) was used as acyl donor. See text for experimental details. The period of incubation was 5 min.

tating rats (10-18 days postpartum) of the Long-Evans strain, which were suckling at least 6 pups, were used for this study. Dams were supplied with Purina Rat Chow and water ad libitum and maintained in a regular cycle of 12 hr dark (5:00 p.m.-5:00 a.m.) and 12 hr light. Animals were killed between 8:00 a.m. and 10:00 a.m. by a blow on the head and the mammary glands were excised. The subsequent procedures were carried out at 0-5 C. The tissue was homogenized in 0.25 M sucrose, 3 volume/g tissue (13) and subcellular fractions prepared by differential centrifugation (14). Nuclei and cell debris were sedimented at 1,200 x g for 10 min; mitochondria at 2,500 x g for 20 min; a mixed mitochondrial, lysosomal, and microsomal fraction at 13,000 x g for 20 min; and microsomes at 100,000 x g for 60 min. The mixed fraction was discarded; and only the mitochondrial, microsomal, and cytosol fractions were used.

Assay system for acylation of 3-sn-glycerolphosphate: Reaction mixtures contained in a total volume of 0.5 ml were: potassium phosphate buffer pH 6.5 (100 mM), $MgCl_2$ (4mM), dithiothreitol (2 mM); 3-sn-[U-^{14}C]glycerolphosphate (0.5 mM, 10 μCi/μmole), acyl-CoA (usually not greater than the critical micelle concentration), and protein. The mixtures were incubated for 1-5 min at 30 C. Short time intervals (1 min) were used in studies on the effect of acyl-CoA concentration upon reaction rate to ensure that at low substrate concentrations, the initial velocity, was measured. The reactions were terminated by the addition of methanol and lipids extracted from the incubation mixture as described by de Jiménez and Cleland (15); radioactivity was determined by liquid scintillation spectrometry.

Protein was determined by the method of Gornall, et al. (16), using defatted human serum albumin as standard.

RESULTS

Protein Concentration

Initial experiments using microsomal and mitochondrial fractions established ranges of protein concentration suitable for the assay system (Fig. 1). We found very little acyl transferase activity in the cytosol, the specific activity of the microsomal fraction was 10 times that of the mitochondrial fraction.

Products

Two radioactive lipids corresponding to phosphatidic acid and diglyceride were found to be synthesized by the microsomal fraction (Fig. 2). The rate of accumulation of label in phosphatidate was more rapid initially, but then declined, while the labeling of diglyceride increased steadily, indicative of a precursor product relationship.

To determine whether radioactivity which cochromatographed with phosphatidic acid in the specified solvent system was associated with

TABLE I

Acylation of 3-sn-[U^{14}C] Glycerolphosphate with [9, 10 ^{3}H] Palmityl-CoA by Microsomes from Lactating Rat Mammary Gland[a]

R_f	Identification	Substrate incorporated (pmoles)		Palmityl-CoA / glycerolphosphate ratio
		Palmityl-CoA	Glycerolphosphate	
0.2-0.3	Phosphatidic acid	73.7	32.7	2.2
0.8-0.9	Neutral glycerides	122	19.4	6.3

[a]Reaction mixtures, which contained 77 μg microsomal protein and 2 μM [9,10^{3}H] palmityl-CoA (65 μCi/μmole) were incubated for 3 min, as described in the text. The radioactive lipids were extracted and fractionated by thin layer chromatography on silica gel using the solvent system chloroform-methanol-methylamine (25%), 130-60-18 (v/v/v/). Authentic phosphatidic acid and diglyceride markers were used. Similar values for the ratio of palmityl-CoA incorporated to those shown in the $\frac{\text{palmityl-CoA}}{\text{glycerolphosphate}}$ table were obtained when incubations were carried out for 5 min.

diacylglycerolphosphate or whether some labeled monoacyl glycerolphosphate (lysophosphatidate) might be present, we performed the experiment described below.

Reaction mixtures which contained both 3-sn-[U-^{14}C] glycerolphosphate and [9, 10^{3}H] palmityl-CoA were incubated and the radioactive lipids fractionated by thin layer chromatography (TLC) (Table I). Radioactivity was confined to two areas of the chromatogram, (R_f's 0.2-0.3 and 0.8-0.9) corresponding to phosphatidic acid and neutral glyceride, respectively. The ratio of palmityl-CoA: glycerolphosphate incorporated into the material with R_f 0.2-0.3, was 2.2:1, consistent with the identification of the compound as diacylglycerolphosphate. The ratio of palmityl-CoA:glycerolphosphate incorporated into the R_f 0.8-0.9 material was 6.3:1. It seems likely that some [9, 10^{3}H] palmityl-CoA had been transferred to an unlabeled acceptor, possibly endogenous diglyceride. Since this material was not resolved into mono-, di-, or triglycerides, it is possible that some of the ^{3}H-label was present as triglyceride. We should emphasize, however, that this experiment demonstrated that most of the 3-sn-[U-^{14}C] glycerolphosphate was incorporated into phosphatidate, rather than into neutral glyceride (Table I).

3-sn-Glycerolphosphate Requirement

Palmityl-CoA was used as acyl donor and microsomes as the source of enzyme to study the dependence of the reaction rate upon 3-sn-glycerolphosphate concentration (Fig. 3). The Km for 3-sn-glycerolphosphate, derived from the Lineweaver-Burk plot, was 0.11 mM.

Acyl-CoA Requirement

The dependence of the reaction rate upon

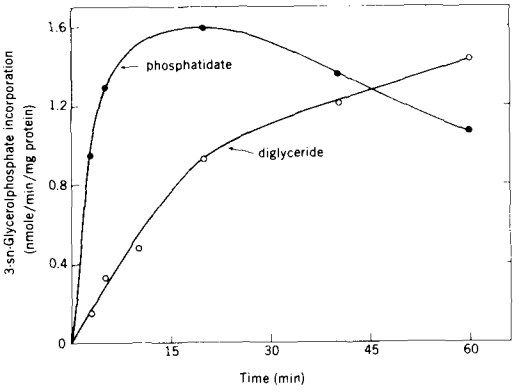

FIG. 2. Time sequence for appearance of products of the acylation of 3-sn-glycerolphosphate. Reaction mixtures included 76 μg microsomal protein and 2 μM palmityl-CoA. The radioactive lipids were fractionated by thin layer chromatography on silica gel using the solvent system hexane-diethylether-acetic acid, 170-30-3 (v/v/v); authentic phosphatidic acid, mono-, di-, and triglycerides were used as markers.

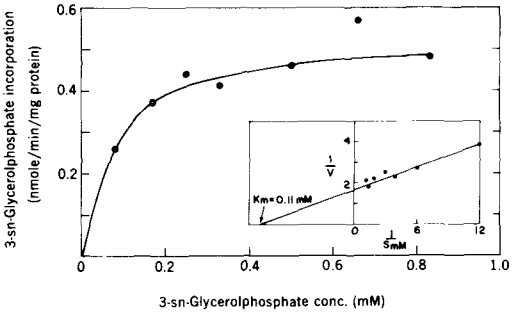

FIG. 3. 3-sn-Glycerolphosphate requirement for acylation with palmityl-CoA by microsomes from lactating rat mammary gland. Reaction mixtures contained 60 μg microsomal protein and 2 μM palmityl-CoA and were incubated for 5 min.

TABLE II

Chain Length Specificity for Acylation of 3-sn-[U-^{14}C] Glycerolphosphate by Subcellular Fractions of Lactating Mammary Gland[a]

Acyl chain length	nmoles glycerolphosphate acylated/mg protein/min		
	Microsomes	Mitochondria	Cytosol
C_2-C_8	0.02	0	0
C_{10}	0.1	0	0
C_{12}	0.2	0.01	0
C_{14}	1.0	0.01	0.003
C_{16}	1.8	0.13	0.008
C_{18}	1.1	0.01	0.001

[a]Incubations included microsomes (80 μg protein) mitochondria (128 μg), or cytosol (128 μg). The subcellular fractions were prepared from different animals. Acyl-CoA concentration was 2 μM, and the incubations were for 5 min in the case of mitochondrial and cytosol preparations. With the microsomal preparation, acyl-CoA concentration was 3 μM, and the incubations were for 1 min.

acyl-CoA concentration was studied in detail with several acyl-CoA derivatives (Fig. 4). There was considerable variation in the activity of different batches of microsomes. Using Palmityl-CoA as substrate with 8 microsomal preparations, the mean activity was 0.89 nmoles glycerolphosphate acylated/min/mg protein and the standard error 0.31. The experiments with each acyl donor were performed with separate microsomal preparations, and, in view of the variation between batches of microsomes, we expressed the reaction rates as percentages of the maximum velocity obtained with each acyl-CoA. A comparison of the actual rates obtained with various acyl-CoA's using the same microsomal preparation is presented in another section. Although there were some small differences in the velocity/concentration profiles from one microsomal preparation to another, in general, the Vmax was observed at concentrations of acyl-CoA at or below the critical micelle concentrations. Thus, the Vmax with decanoyl-CoA, myristyl-CoA, and palmityl-CoA were observed at acyl-CoA concentrations of ca. 9μM, 5μM, and 3μM, respectively; the critical micelle concentrations for myristyl-CoA and palmityl-CoA are 4 μM (17) and 3-4 μM (18), respectively. Decanoyl-CoA apparently does not form micelles below 60 μM, according to the pinacyanol adsorption technique (17), an observation we have confirmed in our laboratory. We were unable to make reliable measurements of the Km's for acyl-CoA's since the V against S plots frequently were of a sigmoidal character, and the Lineweaver-Burk plots were consequently nonlinear. Some factors which might influence the V against S profiles are dealt with below.

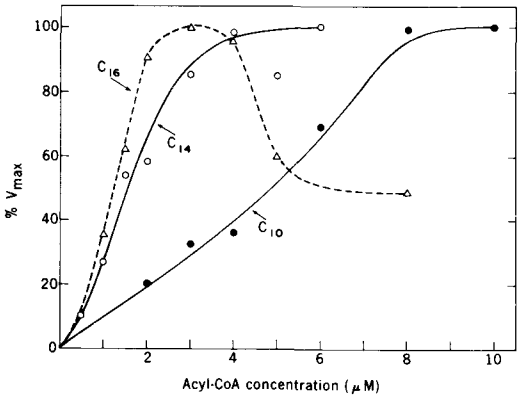

FIG. 4. Acyl-CoA requirement for acylation of 3-sn-glycerolphosphate with microsomes from lactating rat mammary gland. Experiments with each acyl-CoA derivative were carried out with microsomal preparations obtained from different mammary glands; for the experiment with decanoyl-CoA (C_{10}), reaction mixtures contained 116 μg microsomal protein; for myristyl-CoA (C_{14}), 70 μg microsomal protein; and for palmityl-CoA (C_{16}), 64 μg microsomal protein. Reaction mixtures were incubated for 1 min.

Acyl-CoA Specificity

The effectiveness of a number of acyl-CoA derivatives to function as acyl donors was compared at a single acyl-CoA concentration using mitochondrial, microsomal, and cytosol fractions. Reaction rates were highest with palmityl-CoA using each of the subcellular fractions (Table II). The activities of the mitochondrial and cytosol fractions were lower than that of the microsomal fraction.

When the reaction rates were compared at Vmax for each particular acyl-CoA, again palmityl-CoA was the most effective acyl donor.

DISCUSSION

Triglycerides are synthesized by the lactating

rat mammary gland via the glycerolphosphate pathway; the monoglyceride pathway plays an insignificant role (19, 20). The results of our in vitro study indicate clearly that acylation of 3-sn-glycerolphosphate in the gland takes place predominantly in the microsomal fraction of the cell. These findings are consistent with the in vivo observations of Stein and Stein (21). These workers injected radioactive palmitate and oleate intravenously into lactating mice and found by electron microscope radioautography that most of the radioactivity in the mammary gland was seen first over the rough endoplasmic reticulum and subsequently over intra and extracellular lipid droplets. Since the labeled lipid in the mammary gland was found in the esterified form, Stein and Stein concluded that the fatty acids were esterified in the rough endoplasmic reticulum and then released as lipid droplets into the lumen. We, as well as Dils and Clark (19), found some glycerolphosphate acylating activity associated with the mitochondrial fraction of the lactating rat mammary gland. That is consistent with the observation of Stein and Stein who observed a few silver grains deposited over the mitochondria in their radioautographic studies. Whereas it seems certain that the glyceride synthesizing activity associated with the endoplasmic reticulum is involved in the production of milk fat glycerides, we cannot say whether the small amount of activity associated with the mitochondria is so involved or whether its function is to produce lipids for internal use.

In interpreting kinetic data obtained with acyl-CoA derivatives, careful consideration must be given to the fact that these compounds form micelles and that the critical micelle concentration is dependent upon the length of the acyl chain (18, 22). A detailed analysis of the kinetics of interaction of enzymes with lipid substrates has been made by Gatt, et al. (23). In our experiments, Vmax was observed at ca. the critical micelle concentration for each acyl-CoA. This would indicate that only the monomeric acyl-CoA molecules are acceptable as substrates for the acyl transferase enzymes. Thus, we expected to observe classical Michaelis-Menton kinetics, only when all of the added substrate was in the monomeric form. As mentioned earlier, however, the Lineweaver-Burk plots were frequently nonlinear. Some factors which might influence V against S profiles include binding of acyl-CoA's to microsomal protein, hydrolysis of acyl-CoA's by a thiolase, and allosteric effects upon the acyl transferases. We did, in fact, investigate the binding of palmityl-CoA to microsomal protein at various concentrations of the acyl-CoA. When we corrected the apparent concentration of palmityl-CoA in the acyl transferase assay for the amount of palmityl-CoA bound to the microsomal protein, although the resulting V against S plots showed less pronounced sigmoidal characteristics, we felt that the Lineweaver-Burk plots were still not acceptable for estimation of the Km values. We did not investigate the possibility that the addition of albumin might abolish the sigmoidal nature of the Lineweaver-Burk plots (23).

Both the 1 and 2 positions of 3-sn-glycerolphosphate were acylated under the conditions used in our experiments. The diacylglycerolphosphate has been shown to be the major product (15, 24, 25) with particulate preparations from several mammalian tissues, although recently Okuma, et al., (25) and Yamashita, et al. (26), have successfully resolved the rat liver microsomal system into a glycerolphosphate acyl transferase and a 1-acylglycerolphosphate acyl transferase.

Our studies on the chain length specificity of the acyl transferases involved in phosphatidate synthesis by microsomes of lactating rat mammary gland demonstrate a preference for the long chain thioesters. Similar results have been obtained with microsomes of lactating cow mammary gland (27). In this respect, the lactating rat mammary gland microsomal system resembles that of rat liver (28). Daae (28), using carnitine esters as acyl donors in the presence of an acylcarnitine-CoA, acyl transferase enzyme, found the following relationship of chain length to rate of acylation of glycerolphosphate:

$$C_8 < C_{10} < C_{12} < C_{14} < C_{16} > C_{18}$$

Whereas decanoic acid and palmitic acid constitute up to 20 and 30 moles %, respectively, of rat milk triglycerides (29), the specificity of the acyl transferases involved in synthesis of the diglycerides from these two acids differs enormously. The lactating rat mammary gland synthesizes considerably more decanoate (34 moles %) than palmitate (12 moles %); but palmitate is almost certainly also supplied to the gland by the action in the capillary endothelium of lipoprotein lipase on circulating chylomicrons (30). We feel, therefore, that decanoate and palmitate are probably available in the gland in comparable amounts. If the acyl transferases involved in synthesis of the phosphatidate strongly favor palmitate, how can we account for the proportion of palmitate to decanoate that is found in rat milk triglycerides? It is possible that the acyl transferase involved in the conversion of the diglycerides to

triglycerides has a different specificity, one which is more favorable toward the medium chain acids. Such a situation also would be compatible with the observed nonrandom association of fatty acids in rat milk triglycerides. We currently are studying the chain length specificity of the diglyceride, acyl-CoA acyl transferase to test this hypothesis further.

ACKNOWLEDGMENT

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Bis-(monoacylglyceryl) Phosphate and Acyl Phosphatidylglycerol Isolated from Human Livers of Lipidosis Induced by 4,4'-Diethylaminoethoxyhexesterol

KAZUO KASAMA, KATSUMI YOSHIDA, SUSUMU TAKEDA, and **SHOZO AKEDA,** Department of Public Health and Pathology, Mie University School of Medicine, Tsu, 514 Japan, and **KAZUO KAWAI,** Shizuoka Central Prefectural Hospital, Shizuoka, 420 Japan

ABSTRACT

Bis-(monoacylglyceryl) phosphate and acyl phosphatidylglycerol were isolated from the liver of two patients with lipidosis induced by 4,4'-diethylaminoethoxyhexesterol. Identification was based upon the results of alkaline hydrolysis, acetolysis, IR spectrometry, and upon the determination of molar ratio of phosphorus-glycerol-ester. The contents of the bis-(monoacylglyceryl) phosphate were 10 and 16% total phospholipid phosphorus in them. The bis-(monoacylglyceryl) phosphate contained mainly docosahexaenoic (42%), oleic (29%), and linoleic acid (14%) and had the hemolytic activity of ca. one-eighth lysolecithin from egg yolk. Acidic lipids from the liver also were found to contain a lipid which is less polar than bis-(monoacylglyceryl) phosphate. The results of lipid analysis showed that the lipid possessed the structure of an acyl phosphatidylglycerol, and its content was ca. 2% total phospholipid phosphorus. Accumulation of 4,4'-diethylaminoethoxyhexesterol and its derivatives was found in clinical cases by thin layer chromatography and IR spectrometry. This fact suggested that human liver has an ability to metabolize the drug.

INTRODUCTION

It is known that foam cell syndrome (1-3) and phospholipidosis of the liver cell (4) were induced by the administration of 4,4'-diethylaminoethoxyhexesterol (5). Yamamoto, et al., (5) has shown the increases of bis-(monoacylglyceryl) phosphate, phosphatidylinositol, and desmosterol by this drug in clincial cases. This disease also was reproduced in rats (6). This article describes the isolation and characterization of bis-(monoacylglyceryl) phosphate and acyl phosphatidylglycerol from the liver of two patients with lipidosis induced by 4,4'-diethylaminoethoxyhexesterol.

MATERIALS AND METHODS

The liver samples used in this study were obtained from two autopsy cases at the Shizuoka Central Prefectural Hospital (case 1, age 36, male; case 2, age 69, female). The total amount of the drug administrated was 55.8 g in the last 16 months in case 1 and 25.4 g in the last 18 months in case 2. Electron microscopy section of the liver tissue obtained by biopsy showed it to be filled with multiple inclusion bodies with concentrically membranous structures in both cases. The autopsy was carried out within a few hr after death of the patients, and the samples of the livers were stored at -70 C until analyzed.

Authentic Compounds

Phospholipid standards were prepared by silicic acid column chromatography, thin layer chromatography (TLC), and enzymatic hydrolysis from egg yolk, beef heart, rat liver, and spinach leaf. Gas liquid chromatographic (GLC) standards were purchased from Applied Sciences Laboratory, State College, Pa.

Extraction of Lipids

Lipids were extracted from liver tissues with chloroform-methanol (C/M) 2:1 and washed free of nonlipid contaminants with 0.1 M potassium chloride (7).

Isolation of Bis-(monoacylglyceryl) Phosphate and Acyl Phosphatidylglycerol

The silicic acid (Mallinkrodt) was washed with methanol 3 or 4 times to remove fine particles and activated by heating at 110 C for 16 hr. The total lipids (equivalent to 20 mg lipid P) were dissolved in a small amount of chloroform and applied to a column (3.0 cm diameter) of silicic acid (25 g) treated as above. Elution was carried out with 400 ml chloroform, 200 ml C/M 9:1, 250 ml C/M 4:1, 300 ml C/M 3:2, and 350 ml methanol. Fractions were checked by TLC using C/M/28% aqueous ammonia (65:25:4 by volume) or C/M/water (65:25:4 by volume) as the developing solvent. The bis-(monoacylglyceryl) phosphate was isolated from C/M 9:1 and 4:1 fractions by preparative TLC using C/M/28% aqueous ammonia (65:25:4 by volume). The acyl phosphatidylglycerol also was isolated from C/M 9:1 fraction by TLC described as above and puri-

TABLE I

Lipid Compsoition of Liver from Lipidosis Induced by 4,4′-Diethylaminoethoxyhexesterol

Lipid class	Case 1	Case 2	Normal liver[a]
	Percentage on wet wt of tissue		
Triglycerides	1.45	0.74	0.47
Cholesterol esters	0.36	0.20	0.19
Cholesterol	1.11	0.91	0.17
Fatty acids	2.75	0.75	—
Phospholipids	4.67	5.62	2.95
	Percentage on total phospholipid phosphorus		
Phosphatidylethanolamine	15.3	16.7	28.7
Phosphatidylcholine	35.8	34.9	48.5
Phosphatidylinositol	10.1	10.2	6.4
Phosphatidylserine	4.0	3.9	3.8
Caldiolipin	3.0	3.6	4.2
Sphingomyelin	5.5	4.2	5.8
Bis- (monoacylglyceryl) phosphate	10.3	16.3	0.6
Acyl phosphatidylglycerol	2.1	1.7	—
Lysophosphatidylcholine	4.4	3.2	0.4
Lysophosphatidylethanolamine	6.5	2.5	—
Origin	1.4	1.8	—

[a]Values were taken from the work of Yamamoto, et al. (5).

fied by DEAE-cellulose column chromatography (8).

Chemical Analyses

The neutral lipids (20-25 mg) were separated by preparative TLC on Silica Gel G (Merck) plate using hexane/diethyl ether/acetic acid (80:20:1 by volume). Individual neutral lipid classes were eluted with chloroform from the plate and quantitated on the basis of dry wt. The acidic lipids and nonacidic lipids were separated from total lipids by DEAE-cellulose column (8).

The phospholipids were determined according to the method of Rouser, et al. (8). Total lipids or acidic lipids (10 μg as lipid P) were chromatographed on Silica Gel H plate using the following solvent system: the first dimension, C/M/28% aqueous ammonia/water (120:80:10:5 by volume) or C/M/28% aqueous ammonia (65:25:4 by volume); the second dimension, C/acetone/M/acetic acid/water (100:40:30:20:12 by volume) or C/M/acetic acid/water (65:25:7:3 by volume).

Mild alkaline hydrolysis of phospholipids was carried out according to the method of Dawson (9). The water-soluble hydrolysis products were chromatographed on Toyo no. 50 paper with phenol/water/ethanol/acetic acid (80:20:12:10, w/v/v/v) and isopropanol/water/28% aqueous ammonia (7:2:1 by volume) solvent system.

Acid hydrolysis of the phospholipids was carried out by heating in 90% acetic acid for 90 min at 100 C (10). The acid hydrolysis products were separated by preparative TLC on Silica Gel H plate in a solvent of C/acetone/M/acetic acid/water (100:40:30:20:12 by volume).

Acetolysis was carried out by heating at 145 C for 4 hr with 2 ml acetic acid/acetic anhydride (3:2 by volume) according to the method of Renkonen (11). Acetolysis products were chromatographed on Silica Gel G plate in a solvent of hexane/diethyl ether (60:40 by volume).

Methyl esters of fatty acids were prepared by heating the phospholipids at 100 C for 60 min in sealed tubes with 3 ml 5% dry hydrogen chloride in methanol. The methyl esters of fatty acids obtained were analyzed by GLC using a Shimazu GC-4A gas chromatograph. The following conditions were employed: column, 10% diethylene glycol succinate on Shimalite 60-80 mesh (2 m x 3 mm); column temperature, 195 C; carrier gas, nitrogen; flow rate of carrier gas, 40 ml.

Phosphorus assays (12, 13) and determination of ester (14) and glycerol (15) were carried out by the established procedures.

TLC of the 4,4′-diethylaminoethoxyhexesterol and its derivatives was carried out on Silica Gel G plate in a solvent of diethyl ether/M/28% aqueous ammonia (95:3:2 by volume) and C/M/water (65:25:4 by volume). The position of the drug and its derivatives was visualized by spraying the plate with 50% H_2SO_4 followed by Dragendorff reagent or charring after spraying with 50% H_2SO_4.

IR analysis was made on 1% KBr discs of the

sample with a Shimazu IR spectrophotometer IR-27. The absorption intensities of major bands were obtained by the base line method (16), and relative intensities were computed using the absorbance of the C-H absorption band at 1470 cm^{-1} as reference.

Hemolysis was determined for human red cell by the method of Matsumoto (17).

RESULTS

General

The results of lipid analysis are in Table I. The total lipids from the liver were present in high content of 10.3 and 8.3% on wet wt of the tissue in case 1 and 2, respectively. Accumulation of the lipids was found in all lipid classes and especially in free cholesterol which increased more than five times in comparison with normal liver (5, 18). The free fatty acids which have not been demonstrated in normal liver also were found to be predominant in these cases. However, the problem on presence of considerable amount of fatty acids and lysophospholipids described later remained unsettled as to whether they were contained originally or produced by hydrolysis, because the samples were stored 12 months until analysis. Lipidosis by this drug is characterized by increases in free cholesterol and phospholipids and appears to differ from experimental fatty liver induced by carbon tetrachloride, ethionine, etc.

TLC of phospholipids of case 2 is shown in Figure 1. The large spot which was found in the position between neutral lipids and phosphatidylethanolamine was found to be the bis-(monoacylglyceryl) phosphate. As shown in Table I, the results of phospholipid analysis indicated the abnormal accumulation of bis-(monoacylglyceryl) phosphate and acyl phosphatidylglycerol, the increases of phosphatidylinositol and lysophospholipids, and the relative decreases of phosphatidylethanolamine and phosphatidylcholine. These findings generally agree with a previous work by Yamamoto, et al. (5), but they do not report the presence of acyl phosphatidylglycerol.

Characterization of Bis-(monoacylglyceryl) Phosphate

The results of silicic acid column chromatography of liver lipids showed that the bis-(monoacylglyceryl) phosphate was eluted in C/M 9:1 and 4:1 fractions. The C/M 9:1 fraction contained bis-(monoacylglyceryl) phosphate, cardiolipin, acyl phosphatidylglycerol, and unknown lipid; and C/M 4:1 fraction contained bis-(monoacylglycryl) phosphate and phosphatidylethanolamine. The pure bis-(monoacylglyceryl) phosphate was isolated by the preparative TLC as described in "Methods." The recovery of bis-(monoacylglyceryl) phosphate was found to be more than 95% on the basis of results of two dimensional TLC. As shown in Figure 2, the bis-(monoacylglyceryl) phosphate migrated below cardiolipin by the solvent of C/M/water (65:25:4 by volume) and sufficiently above cardiolipin by C/M/28% aqueous ammonia (65:25:4 by volume). This lipid also was found to be in the acidic lipids from DEAE-cellulose column (Fig. 2, lane G).

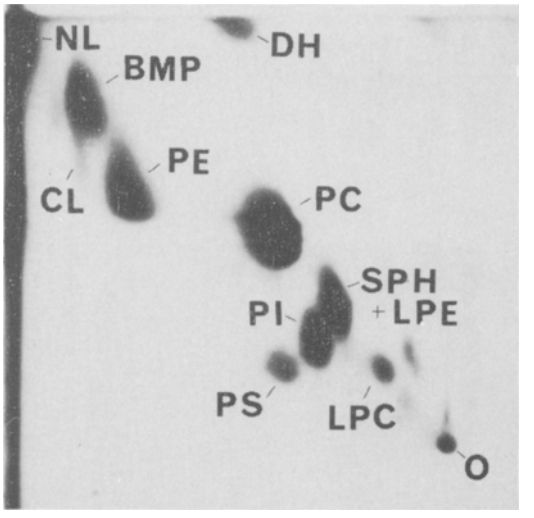

FIG. 1. Thin layer chromatogram of the liver lipids from lipidosis induced by 4,4'-diethylaminoethoxyhexesterol. Adsorbent, Silica Gel H. Solvent system, vertical direction (first development)–chloroform/methanol/28% aqueous ammonia/water (120:80:10:5 by volume); horizontal direction (second development)–chloroform/acetone/methanol/acetic acid/water (100:40:30:20:12 by volume). NL = neutral lipids, BMP = bis-(monoacylglyeryl) phosphate, CL = cardiolipin, PE = phosphatidylethanolamine, PC = phosphatidylcholine, SPH = sphingomyelin, LPE = lysophosphatidylethanolamine, PI = phosphatidylinositol, PS = phosphatidylserine, LPC = lysophosphatidylcholine, DH = 4,4'-diethylaminoethoxyhexesterol, and O = origin.

The purified lipid had a phosphorus content of 3.53% (theoretical value, 4.0% as bis-(monoacylglyceryl) phosphate having only stearic acid) and had a molar ratio of phosphorus-glycerol-fatty acid esters of 1:1.92:2.06. Figure 3 shows a paper chromatogram of water-soluble phosphate esters obtained by Dawson mild alkaline hydrolysis of bis-(monoacylglyceryl) phosphate and several authentic phospholipids. Mild alkaline hydrolysis gave 1 water-soluble phosphate ester with the chromatographic properties of glycerylphosphorylglycerol, and 92% of original lipid phosphorus was converted to the water-soluble phosphate ester. On the other

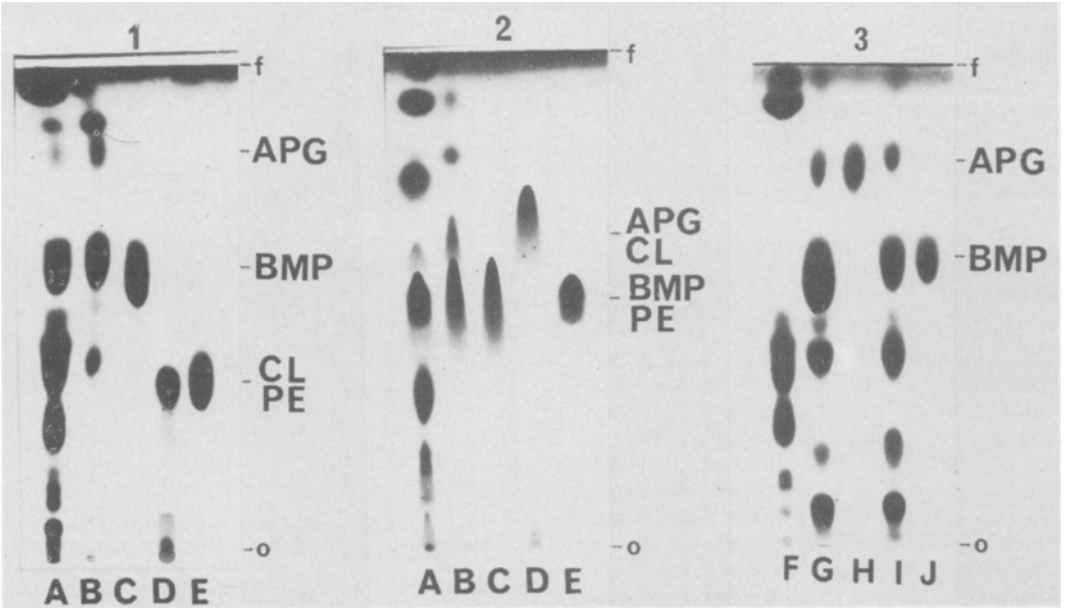

FIG. 2. Thin layer chromatogram of the lipids from silicic acid column and DEAE-cellulose column. Solvent system of 1 and 3, chloroform/methanol/28% aqueous ammonia (65:25:4 by volume); solvent system of 2, chloroform/methanol/water (65:25:4 by volume). Adsorbent, Silica Gel G. A = total lipids, B = chloroform/methanol 9:1 fraction from silicic acid column, C = bis-(monoacylglyceryl) phosphate, D = cardiolipin, E = phosphatidylethanolamine, F = nonacidic lipids from DEAE-cellulose column, G = acidic lipids from DEAE-cellulose column, H = acyl phosphatidylglycerol (APG), I = acidic lipids from rat liver treated with 4,4'-diethylaminoethoxyhexesterol, and J = bis-(monoacylglyceryl) phosphate. See Figure 1 for definitions of abbreviations.

hand, the chloroform/isobutanol-soluble products were found to consist of two components of fatty acids and their ethyl esters on TLC. Acetolysis of the bis-(monoacylglyceryl) phosphate produced only one glycerylacetate which was identified as a monoacylglyceryldiacetate (Fig. 4). The hydrolysis products by 90% acetic acid of bis-(monoacylglyceryl) phosphate were found to contain mainly the monoacylglycerol and unknown lipid which were recovered from TLC plate in ca. equal proportion (Fig. 5). The isolated unknown lipid had a molar ratio of

TABLE II

Fatty Acid Composition of Bis- (monoacylglyceryl) Phosphate and Acyl Phosphatidylglycerol

	Bis- (monoacylglyceryl) phosphate		Acyl phosphatidylglycerol
Fatty acid[a]	Case 1	Case 2	Case 1
16:0	3.0	2.5	17.0
16:1	1.0	1.5	1.9
18:0	2.3	1.5	16.4
18:1	27.2	30.5	24.3
18:2	12.8	14.5	11.9
18:3	0.7	0.4	0.2
20:3	1.9	0.5	0.6
20:4	3.5	2.1	2.3
20:u[b]	3.1	1.6	0.8
22:5	1.1	0.4	0.9
22:6	41.5	43.2	21.7
Other	2.0	1.3	1.9

[a]First number = chain length and second number = number of double bonds.
[b]Unidentified unsaturated acid.

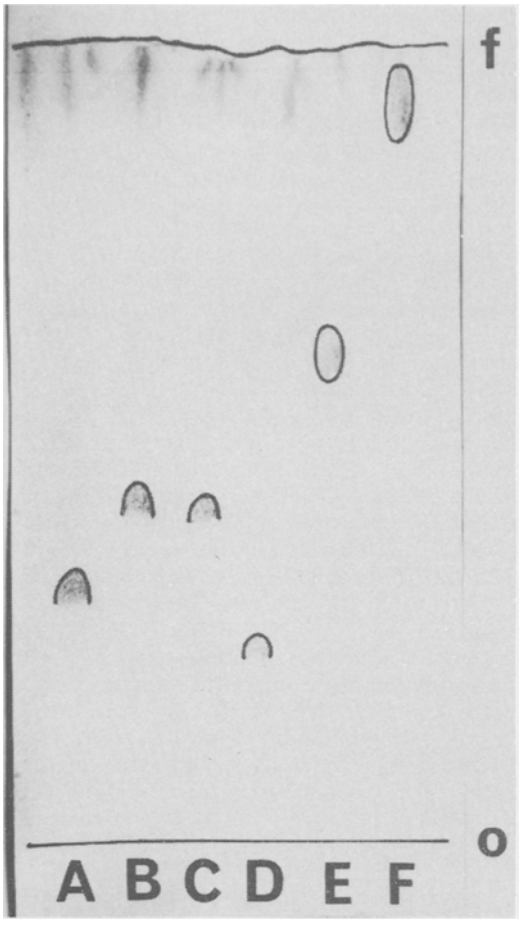

FIG. 3. Paper chromatogram of the water-soluble phosphate from mild alkaline hydrolysis of bis-(monoacylglyceryl) phosphate and authentic phospholipids. Solvent, phenol/water/ethanol/acetic acid (40:10:6:5, w/v/v/v). Water-soluble hydrolysate from (A) phosphatidic acid, (B) bis-(monoacylglyceryl) phosphate, (C) phosphatidylglycerol, (D) cardiolipin, (E) phosphatidylethanolamine, and (F) phosphatidylcholine.

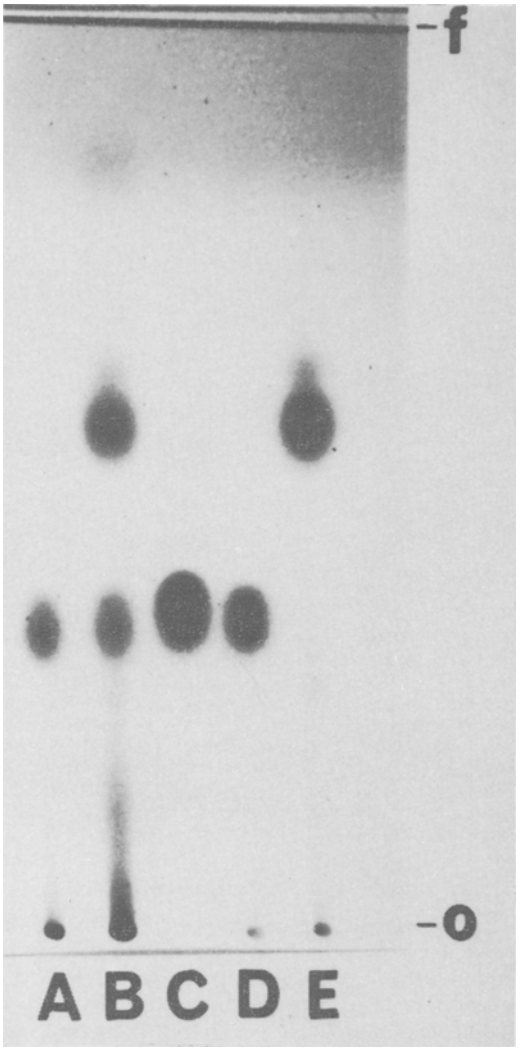

FIG. 4. Thin layer chromatogram of the acetolysis products of bis-(monoacylglyeryl) phosphate, acyl phosphatidylglyercol, and authentic lipids. Adsorbent, Silica Gel G. Solvent system, hexane/diethyl ether (60:40 by volume). Acetolysis products of (A) bis-(monoacylglyeryl) phosphate, (B) acyl phosphatidylglycerol, (C) 1-monopalmitin, (D) 2-monopalmitin, and (E) lecithin.

phosphorus-fatty acid esters of 1:1.10, and the phosphate ester after the mild alkaline hydrolysis of the lipid agreed with glyceryl phosphate on chromatographic properties. It was suggested that acetic acid hydrolysis of bis-(monoacylglyceryl) phosphate produced a monoacylglycerol and monoacylglycerylphosphate. The IR spectrum of the bis-(monoacylglyceryl) phosphate was compared with that of phosphatidylglycerol from spinach leaf (Fig. 6). Both spectra were similar in the presence and intensities of major absorption bands and showed characteristic absorption bands of phospholipids reported by Abbramson, et al., (19) and Chapman (20). From the above evidence, it was concluded that the lipid is bis-(monoacylglyceryl) phosphate. The fatty acids from the bis-(monoacylglyceryl) phosphate were similar to each other in case 1 and 2, and the sum of unsaturated acids was over 95% of total fatty acids. The main fatty acids were 22:6 (42%), 18:1 (29%), and 18:2 (14%) (Table II). Hemolytic activity of bis-(monoacylglyceryl) phosphate was compared with that of lysolecithin from egg yolk (Table III). The results showed that this lipid had a low hemolytic activity of ca. one-eighth of that of lysolecithin.

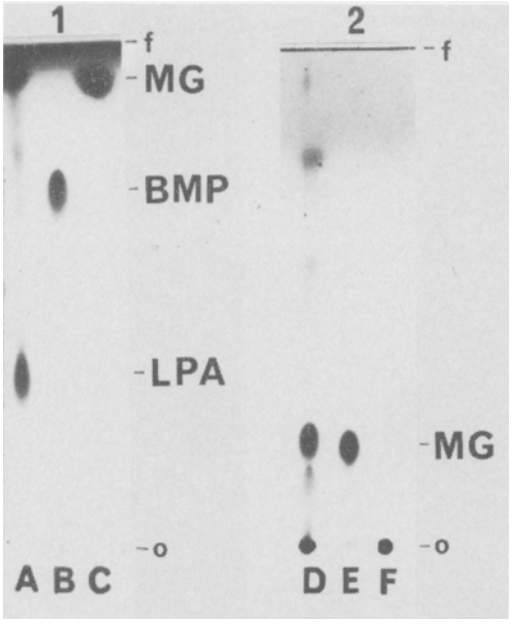

FIG. 5. Thin layer chromatogram of 90% acetic acid hydrolysate of bis-(monoacylglyceryl) phosphate. Adsorbent, Silica Gel H (1) and Silica Gel G (2). Solvent system of 1, chloroform/acetone/methanol/acetic acid/water (100:40:30:20:12 by volume); solvent system of 2, hexane/diethyl ether (20:80 by volume). A and D, 90% acetic acid hydrolysate of bis-(monoacylglyceryl) phosphate (BMP); B and F, bis-(monoacylglyceryl) phosphate; and C and E, 1-monopalmitin (MG). LPA = lysophosphatidic acid.

Characterization of Acyl Phosphatidylglycerol

As shown in Fig. 2, lane G, the acidic lipids from DEAE-cellulose column contained a lipid which is less polar than bis-(monoacylglyceryl) phosphate. The lipid was separated completely from bis-(monoacylglyceryl) phosphate and other lipids by preparative TLC and DEAE-cellulose column chromatography as described in "Methods" (Fig. 2, lane H). The purified lipid contained the phosphorus of 3.02% (theoretical value, 2.96% as acyl phosphatidylglycerol having only stearic acid) and had a molar ratio of 1:1.94:3.08 of phosphorus-glycerol-fatty acid esters. The water-soluble phosphate ester after alkaline hydrolysis was found to give only one component with chromatographic properties of glycerylphosphorylglycerol. Acetolysis of the phospholipid gave two major acetates in ca. equal proportions. As shown in Fig. 4, the acetates obtained were a diacylglycerylmonoacetate and monoacylglyceryldiacetate. In IR spectrum of acyl phosphatidylglycerol, the major absorption bands and those intensities were similar to those of bis-(monoacylglyceryl) phosphate and phosphatidylglycerol, but the absorption intensity of the hydroxyl group near 3300 cm^{-1} of acyl phosphatidylglycerol was a low intensity ca. less than half of those of bis-(monoacylglyceryl) phosphate and phosphatidylglycerol (Fig. 6). On the basis of these results, it was concluded that the lipid possessed the structure of an acyl phosphatidylglycerol. The main fatty acids were 18:1 (24%), 22:6 (22%), 16:0 (17%), 18:0 (16%), and 18:2 (12%) (Table II). The ratio of unsaturated to saturated acids in this lipid is 1:1.91 and suggests a loss of saturated fatty acids at 1 primary hydroxyl group in the structure. Acidic lipids from rat liver treated by this drug (3 weeks, 50 mg/kg body wt/day) also was found to give a spot which had an R_f value identical with a acyl phosphatidylglycerol on TLC (Fig. 2, lane I).

Accumulation of Drug and Its Derivatives

Figure 7 shows a TLC of the drug and its derivatives. The drug and its derivatives could be separated completely from the phospholipids on TLC when the development was made in diethyl ether/M/28% aqueous ammonia. The spots of these compounds appeared more strongly than phosphatidylcholine by Daragendorff reagent. Spot 5 from the liver had an R_f value identical with 4,4′-diethylaminoethoxyhexesterol in two solvent systems used, and its IR spectrum agreed completely with that of the drug. The IR spectrum of the spot 4 also was

TABLE III

Hemolysis of Human Red Cell by Bis- (monoacylglyceryl) Phosphate[a]

Amount of lysophospholipids added to cell suspension (μg/ml)	800	400	200	100	50	25	12.5	6.25	3	1.5
Bis- (monoacylglyceryl) phosphate	+	+	+	+	–	–	–	–	–	–
Lysolecithin from egg yolk				+	+	+	+	–	–	–

[a]Assay of hemolysis was as follow: a mixture of 1 ml 0.5% human red cells and 0.5 ml lysophospholipid solution was incubated at 37 C for 30 min. After cooling, the degree of hemolysis was read.

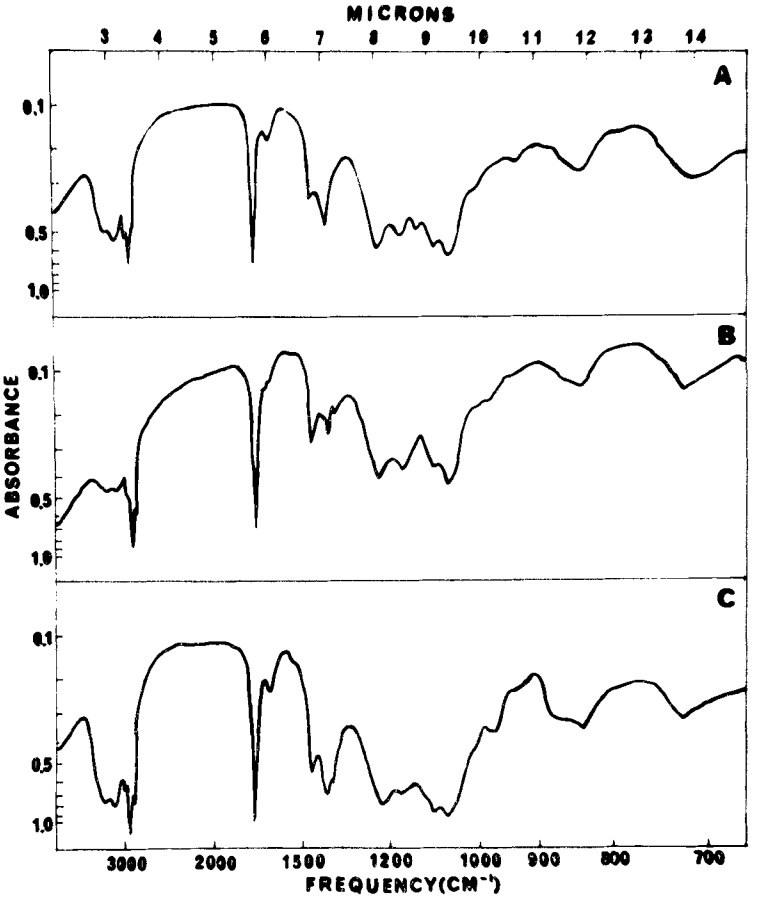

FIG. 6. IR absorption spectra of (A) bis-(monoacylglyceryl) phosphate, (B) acyl phosphatidylglycerol, and (C) phosphatidylglycerol.

similar to that of the drug except for the following: the absorption intensity at 1031 cm^{-1} and the absorption bands at the region of 854.7 and 800 cm^{-1}. This spectrum did not show the absorption band of the hydroxyl group near 3300 cm^{-1} which Matsuzawa, et al., (21) found in the metabolite of the drug in rat liver. The results of TLC of phospholipids from the rat liver treated showed the presence of three drug derivatives (spots 1, 3, and 6 in lane D).

DISCUSSION

Accumulation of bis-(monoacylglyceryl) phosphate in disease was first found by Rouser, et al., (22) in livers of patients with Niemann-Pick disease and with other unidentified diseases. This lipid also was shown to accumulate remarkably in the liver of patients with foamy cell syndrom indicated by 4,4′-diethylaminoethoxyhexesterol (5). Recently, Werrett and Huterer (23) indicated that bis-(monoacylglyceryl) phosphate is enriched in the lyosome fraction of rat liver and suggested that the nonspecific increase of this lipid found in several lipid storage diseases reflects an increase in the number of lysosomes.

Acyl phosphatidylglycerol has been identified in the lipids of rabbit lung (24), mycoplasma (25), and *Salmonella thyphimurium* (26), but the accumulation of this lipid in disease has not been reported. This study showed that the bis-(monoacylglyceryl) phosphate and acyl phosphatidylglycerol are abnormally accumulated in human livers from lipidosis induced by 4,4′-diethylaminoethoxyhexesterol.

In these lipids, the position of the free hydroxyl group and the stereochemical configuration of two glycerols remain to be determined. The results of the fatty acid analysis suggest that free hydroxyl groups would be all primary in the bis-(monoacylglyceryl) phos-

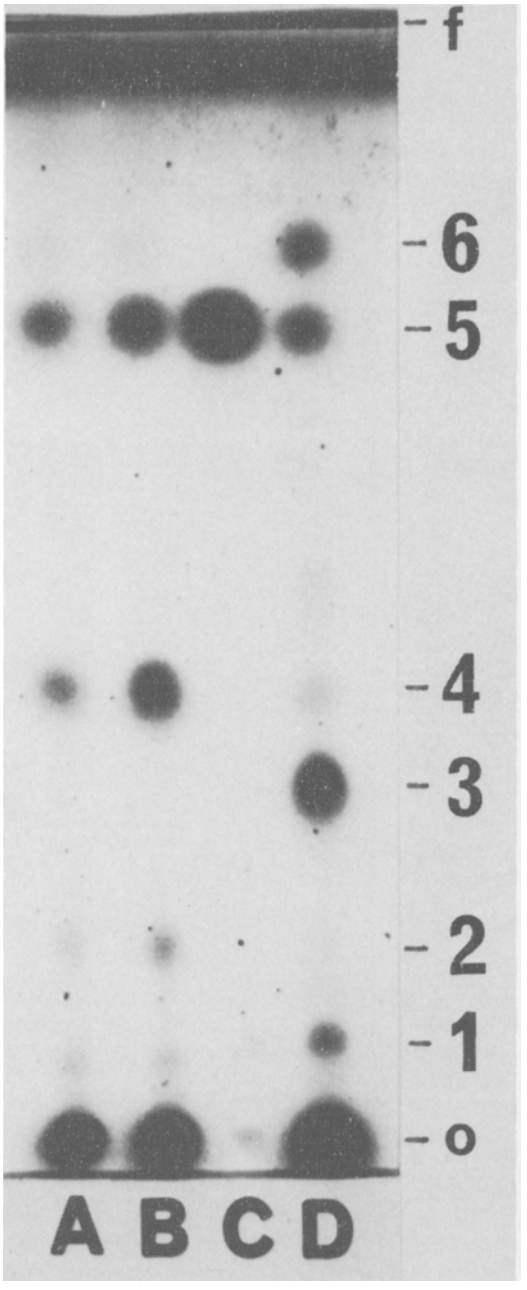

FIG. 7. Thin layer chromatogram of 4,4′-diethylaminoethoxyhexesterol and its derivatives. Adsorbent, Silica Gel G. Solvent system, diethyl ether/methanol/28% aqueous ammonia (95:3:2 by volume). Spots visualized by charring after spraying with 50% H_2SO_4. A = phospholipid fraction of case 1, B = phospholipid fraction of case 2, C = 4,4′-diethylaminoethoxyhexesterol, and D = phospholipid fraction of rat liver treated with the drug.

phate and in the acyl phosphatidylglycerol as indicated by Body and Gray (24) and Seng, et al., (27). Accordingly, the acyl phosphatidylglycerol obtained might differ from that of *Salmonella thyphimurium* in the position of hydroxyl group and the biosynthetic derivation.

The origin of bis-(monoacylglyceryl) phosphate and acyl phosphatidylglycerol is not yet known, but the biosynthetic derivation from phosphatidyldiacylglycerol, phosphatidylglycerol, and diphosphatidylglycerol has been suggested in recent publications (23, 24). From the structural similarity and the contents of both lipids, it seems likely that the acyl phosphatidylglycerol serves as a direct precursor for the bis-(monoacylglyceryl) phosphate. Also, the fatty acid composition of the lipids isolated is considerably different from that of cardiolipin of mamalian tissues (28, 29). The difference of fatty acid composition between the lipids isolated and cardiolipin suggests that the former is not the biocleavage product of the latter.

Accumulation of 4,4′-diethylaminoethoxyhexesterol and its metabolite was recently reported by Matsuzawa, et al., (21) in rat liver after the administration of this drug, while the metabolite of the drug was not found in clinical cases. Our data indicated that the drug derivatives which are different from those of rat accumulate in these clinical cases. It seems that the human liver also has the ability to metabolize the drug, but this is somewhat different from that of rat liver.

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Occurrence of *cis*-5,*cis*-9-Hexacosadienoic and *cis*-5,*cis*-9,*cis*-19-Hexacosatrienoic Acids in the Marine Sponge *Microciona prolifera*

ELAINE JEFFERTS,[1] **REGINALD W. MORALES,** and **CARTER LITCHFIELD,**
Department of Biochemistry, Nelson Biological Laboratories, Rutgers University, New Brunswick, New Jersey 08903

ABSTRACT

Fatty acid analysis of the total lipids from the marine sponge *Microciona prolifera* by gas liquid chromatography on an EGSS-X column revealed two major peaks with equivalent chain length values of 27.08 and 27.74. Each of these components was isolated as a separate band by thin layer chromatography on $AgNO_3$-silicic acid. Characterization of the two unknowns by IR spectroscopy, NMR, hydrogenation, and gas liquid chromatography revealed that the unknown acids were *n*-26:2 and *n*-26:3 containing only nonmethylene interrupted *cis*-double bonds. Reductive ozonolysis identified the 26:2 as *cis*-5,*cis*-9-hexacosadienoic acid and the 26:3 as *cis*-5,*cis*-9,*cis*-19-hexacosatrienoic acid. Analysis of the fatty acid composition of *Microciona* total lipids showed 14% 26:2 and 31% 26:3. The neutral lipids, phosphatidylethanolamine, and phosphatidylserine all contained >41% C_{26} acids; but only 4% C_{26} was present in the phosphatidylcholine.

INTRODUCTION

In the course of an investigation of lipid metabolism in the marine sponge *Microciona prolifera*, we observed an unusual distribution of fatty acids, indicating that large amounts of two unsaturated C_{26} acids might be present. This article describes the isolation and characterization of these two C_{26} acids which, as far as we are aware, have not been reported previously.

EXPERIMENTAL PROCEDURES

Materials

Microciona prolifera sponge colonies obtained by dredging at a depth of 10 m in Raritan Bay, N.J., in September 1971 were used for initial qualitative studies on fatty acid composition. Additional samples of the same sponge were collected at a depth of 2 m in Barnegat Bay near Waretown, N.J., in March 1972; all quantitative analyses reported are on this material. A sample of *Microciona* sp. sponge from Florida coastal waters was purchased in March 1972 from the Gulf Specimen Co., Panacea, Fla., and used only for comparative analyses on 26:2 and 26:3 content.

The following standards were purchased from reliable sources and used in the identification of fatty acids and the products of reductive ozonolysis by gas liquid chromatography (GLC): methyl esters of 14:0, 16:0, 18:0, 18:1ω9, 18:1ω12, 18:2ω6, 20:0, 20:1ω15, 22:0, 22:1ω9, 24:0, 24:1ω9, and 28:0; a mixture of C_{10} to C_{20} α-olefinic hydrocarbons; 1,5-hexadiene; and 1,11-dodecadiene. Methyl esters of cod liver oil and of *Tropaeolum speciosum* seed fat (containing 26:1ω9 [1]) were prepared by KOH-catalyzed methanolysis (2). Qualitative mixtures of suitable neutral lipid and phospholipid standards for thin layer chromatography (TLC) were purchased from Supelco Inc., Bellefonte, Pa.

Methods

The sponges were washed in seawater, blotted dry, and all visible algae and debris were carefully removed. The total lipids (0.96% blotted wt) were extracted from the tissue with chloroform-methanol 2:1 according to the method of Bligh and Dyer (3).

The total lipids were refluxed for 1.5 hr with 0.05 *N* KOH in methanol. Water then was added, and the unsaponifiable material was extracted with petroleum ether. After acidification with HCl, the free fatty acids were extracted with petroleum ether. Methyl esters then were prepared by H_2SO_4-catalyzed methanolysis as described by Johnston (4). Methyl esters were purified by preparative TLC on 1.0 mm thick silicic acid impregnated with rhodamine 6 G by developing in petroleum ether-diethyl ether-acetic acid 93:7:1. The methyl ester band, located under UV light by comparison with known standards, was scraped off the plate; and the methyl esters were recovered using diethyl ether (5).

[1]Present address: Rockefeller University, New York, N.Y. 10021.

The GLC analyses of methyl esters were run on 1.82 m x 2.4 mm inside diameter stainless steel columns packed with 10% EGSS-X or 10% EGSS-Y on 100-120 mesh Gas Chrom P (Applied Science Laboratories, State College, Pa.) at 207 or 220 C. A Bendix 2600 gas chromatograph equipped with an Infotronics CRS-104 integrator was used. Peaks were identified by cochromatography with known compounds and by graphical correlation of equivalent chain length (ECL) values (6,7).

Methyl ester hydrogenations normally were run with PtO_2 catalyst in methanol according to the method of Litchfield (8). However, hydrogenation with hydrazine to determine the double bond locations in 26:3 was carried out with the method of Privett and Nickell (9).

Methyl esters were separated according to degree of unsaturation by preparative TLC on 1.0 mm thick $AgNO_3$-impregnated silicic acid (10) using two developments with petroleum ether-diethyl ether 80:20 or 95:5. Bands were located after spraying with 2′,7′-dichlorofluorescein by observation under UV light and were recovered by extraction with diethyl ether as described above.

IR analyses were carried out using a Perkin-Elmer 700 spectrophotometer. Samples were dissolved in CCl_4, and the 4000-650 cm^{-1} spectrum was automatically recorded. The 60 MHz NMR spectra were run on a Varian T-60 instrument using 20-35 mg samples dissolved in CCl_4 with $Si(CH_3)_4$ as a reference marker.

Reductive ozonolysis of 1-5 mg methyl ester samples was carried out in CH_2Cl_2 at -70 C using the procedure of Stein and Nicolaides (11). Aldehydic products were identified by GLC as described above except that isothermal column temperatures between 95 and 180 C were used.

Total *Microciona* lipids were separated into lipid classes on 0.25 mm thick Q-1 precoated TLC plates (Quantum Industries, Fairfield, N.J.). Plates were predeveloped, first in diethyl ether and then in methanol-acetone-water 51:46:3, and then activated. Sample was applied with a streaking device, and the plates were developed once in chloroform-methanol-conc. NH_4OH 62:33:5. Bands were located by charring with conc. H_2SO_4 and identified by cochromatography with known standards and by their color reactions with ninhydrin, Dragendorff, and α-naphthol spray reagents. For preparative TLC, the position of the bands was determined by exposing a narrow vertical strip to iodine vapor, while the rest of the adsorbent was kept covered. Each band was scraped from the unexposed area of the plate; the lipids were recovered with $CHCl_3$-CH_3OH 2:1 and then converted to methyl esters by the method of Brockerhoff (2). Blank runs with methyl octacosanoate as an internal GLC standard showed no significant amount of impurities present.

RESULTS

Analysis of the purified methyl esters of *Microciona prolifera* lipids by GLC on a new EGSS-X column at 207 C revealed two major peaks eluting after 22:6ω3 and having ECL values of 27.08 and 27.74 (these values decreased as much as 0.5 units as the column aged). Separation of the purified methyl esters by preparative argentation TLC produced five major bands, which were recovered and analyzed by GLC. The ECL 27.08 and ECL 27.74 components were recovered in ~95% purity from the third and fourth bands (counting from the top down) respectively.

Identification of 26:2

A portion of the ECL 27.08 ester was hydrogenated fully producing a product having an ECL value of 26.00 on an EGSS-X column. Hence, an unsaturated n-C_{26} structure was indicated. The IR spectrum of the unsaturated ester was typical for a fatty acid methyl ester containing double bonds and showed no band at 960-970 cm^{-1}. Thus, all double bonds present were of *cis*-configuration (12). The NMR spectrum also indicated an unsaturated fatty acid methyl ester. The ratio of the -CH=CH- signal at 5.3 δ to the CH_3O- signal at 3.6 δ was 3.9 to 3.0, indicating that the compound was a diene. However, there was no =C-CH_2-C= signal at 2.7 δ, from which it was concluded that no 1,4-diene structure was present (13).

Reductive ozonolysis of the 26:2 methyl ester produced three major GLC peaks. Cochromatography of the reaction products with authentic standards identified these peaks as a C_5 aldehyde ester, a C_4 dialdehyde, and a C_{17} aldehyde. Hence, the unknown compound is *cis*-5,*cis*-9-hexacosadienoic acid.

Identification of 26:3

The ECL 27.74 ester was identified in the same manner as the 26:2. Complete hydrogenation produced n-26:0 as characterized by GLC. The IR spectrum was again typical of an unsaturated methyl ester having only *cis*-double bonds. The NMR spectrum was very similar to that of the 26:2, except that the ratio of the -CH=CH- signal to the CH_3O- signal was 6.0 to 3.0, indicating a trienoic compound. Once again there was no =C-CH_2-C= signal at 2.7 δ showing the absence of any 1,4-diene structures.

TABLE I

26:2 and 26:3 Content of *Microciona prolifera* Lipids[a]

	Fatty acids (wt %)	
	26:2	26:3
Total lipids	14	31
Neutral lipids (mainly sterol esters)	35	23
Phosphatidylethanolamine	12	29
Phosphatidylcholine	1	3
Phosphatidylserine	22	48

[a]Specimen collected in Barnegat Bay, N.J., March, 1972.

Ozonolysis of the 26:3 methyl ester produced four fragments which were identified by cochromatography on GLC as a C_5 aldehyde ester, C_4 and C_{10} dialdehydes, and a C_7 aldehyde. Either a 5,9,19- or a 5,15,19-hexacosatrienoate could have produced these results. To identify the position of the middle double bond, the 26:3 ester was hydrogenated partially with hydrazine, a reaction which does not cause any migration of unsaturation along the hydrocarbon chain (9). The 26:1 was isolated from the reaction products by argentation TLC and subjected to reductive ozonolysis. Two of the major fragments characterized by GLC were a C_9 aldehyde-ester and a C_{17} aldehyde, indicating that the unknown compound is *cis*-5,*cis*-9,*cis*-19-hexacosatrienoic acid.

C_{26} Content of *Microciona prolifera* Lipids

The purified methyl esters derived from *Microciona prolifera* total lipids contained 14 wt % 26:2 and 31% 26:3.

Characterization of intact *Microciona* lipids by TLC indicated five major lipid classes were present: sterol esters, free sterols, phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine. Other minor bands were observed but not identified. To determine how the unusual 26:2 and 26:3 acids are distributed among the major lipid classes, *Microciona* lipids were separated by preparative TLC using a $CHCl_3$-CH_3OH-conc. NH_4OH 62:33:5 solvent system. The methyl esters derived from each major lipid class were subjected to quantitative GLC assay. The results (Table I) show that high levels of 26:2 and 26:3 are found in the neutral lipids (sterol esters), the phosphatidylethanolamine, and the phosphatidylserine; but only minor amounts are present in the phosphatidylcholine.

The unusual nature of these two C_{26} acids prompted us to compare their content in *Microciona* samples taken from New Jersey and Florida coastal waters. The New Jersey sample contained 14% 26:2 and 31% 26:3 in the total fatty acids, while the Florida material showed 42% 26:2 and 10% 26:3.

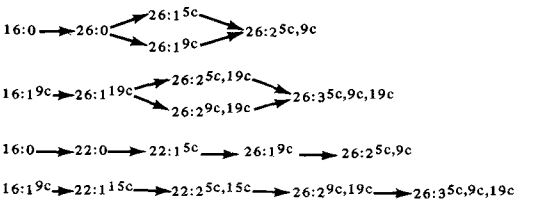
DISCUSSION

To our knowledge, *cis*-5,*cis*-9-hexacosadienoic and *cis*-5,*cis*-9,*cis*-19-hexacosatrienoic acids have not been previously reported in living organisms. They differ from the usual fatty acids in two respects: their extremely long chain length and their nonmethylene interrupted polyunsaturation.

Other marine sponges are known to contain large amounts of C_{26} and C_{28} acids. Bergmann and Swift (14) characterized 9-hexacosenoic and 17,20-hexacosadienoic acids in *Spheciospongia vesparia*, identified an octacosatrienoic acid in *Suberites compacta*, and presented evidence for high levels (>50 wt %) of C_{24}, C_{26}, and C_{28} fatty acid chains in both species. The present report, considered with Bergmann and Swift's work, suggests that unsaturated C_{26} and C_{28} fatty acids might well be characteristic of sponges. However, recent analyses in our laboratory (R.W. Morales, unpublished) have found at least one species in which none are present.

Except for the sponges, C_{26} polyunsaturated fatty acids rarely are found in significant amounts in nature. One exception is the 1.0% hexacosadienoic acid in muskrat scent glands reported by Erickson and Hix (15). Traces of C_{26} polyunsaturates often are reported in fish oils, such as tuna (16), herring (17), and cod liver (18) oils; but levels are so low that they probably lack any metabolic importance.

The biosynthesis of these unusual nonmethylene interrupted 26:2 and 26:3 acids is obviously an interesting matter for speculation. Since they have not been reported as components of any planktonic species which could conceivably be ingested by *Microciona*, we assume that they are synthesized by the sponge itself. The similar positioning of the *cis*-5 and *cis*-9 double bonds in the 26:2 and 26:3 molecules suggests a parallel origin from palmitic and palmitoleic acids by elongation and desaturation. Possible pathways might be



There is precedent for the occurrence of both Δ5 and Δ9 desaturase activity in the same biosynthetic pathway. Davidoff and Korn (19) have isolated $16{:}2^{5c,9c}$, $17{:}2^{5c,9c}$, and $18{:}2^{5c,9c}$ from the slime mold *Dictyostelium discoideum*. Desaturation at the C_{22} stage is suggested by the presence of small amounts of unsaturated C_{22} acids in our gas chromatograms which could not be identified by the usual graphical (6,7) and Ag^+ TLC (10) approaches.

Since *Microciona* lipids mainly are composed of membrane components, rather than energy storage molecules, such as wax esters or triglycerides, the presence of such high levels of 26:2 and 26:3 must have considerable influence on membrane structure. The total C_{26} content of the New Jersey *Microciona* sample (45%) did not differ greatly from that of the Florida sample (52%). However, there was a wide variation in the levels of the two acids: 14% vs. 42% for 26:2 and 31% vs. 10% for 26:3, with the New Jersey sample being the more unsaturated. This suggests an interchangeability between the two C_{26} acids, perhaps to maintain constant membrane flexibility with variations in environmental temperature as in *Escherichia coli* (20).

In the well known Danielli-Davson bilayer model (21) for membranes, the hydrophobic tails of the fatty acids are pointed toward each other in the interior of the bilayer and reach ca. halfway across the membrane's width. This forms an interior hydrophobic face along which the membrane can be fractured (22,23).

The questions that arise from having fatty acids with chains 26-carbons in length are intriguing. The presence of these very long fatty acids may well determine some of the chemical and physical parameters of the membrane, such as width, molecular mobility, phase transitions, or interactions with membrane protein (24). Though bending caused by the *cis*-unsaturation could represent solutions for some of the questions stemming from chain length, the very bulk of these chains would lead to problems involving packing and interactions with other hydrocarbon chains. Perhaps the C_{26} chains might fold around adjacent sterol molecules in the membrane, as has been proposed for shorter chain polyunsaturated fatty acids (25).

The asymmetric composition of membranes also has been noted (24,26). According to Bretscher's theory, phosphatidyl choline is located predominantly at the outer surface of the erythrocyte membrane. Our observation that the C_{26} acids of *Microciona* are mostly absent from the phosphatidyl choline fraction suggests that these acids may be oriented in some definite fashion to fulfill a specific function in sponge metabolism. Possibly the C_{26}-rich lipids are located mainly on certain membrane surfaces, or perhaps they are clustered about specific protein components in the membrane. This is obviously a fascinating problem for future research.

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Incorporation and Metabolism of Fatty Acids by Cultured Dissociated Cells from Rat Cerebrum

EPHRAIM YAVIN and **JOHN H. MENKES**, Division of Pediatric Neurology,
University of California at Los Angeles and Brentwood Veterans' Hospital, Los Angeles, California

ABSTRACT

Dissociated brain cells in culture incorporate a variety of saturated and unsaturated fatty acids into their cellular lipids. Of the various fatty acids studied, uptake of radioactivity was greatest for stearic acid and decreased progressively with decreasing chain length. Incorporation of radioactivity from linoleic and linolenic acids was more extensive than from oleic acid. Cellular phospholipids and triacylglycerols were labeled preferentially from all fatty acid precursors, with the relative amount of label in phospholipids being greatest when cells were incubated with linolenic acid. Fatty acids underwent desaturation and chain elongation. Changes in the labeling pattern of phospholipid fatty acids in the course of incubation demonstrated precursor-product relationships for laurate (12:0), myristate (14:0), palmitate (16:0), and stearate (18:0) and for linolenate (18:3), eicosapentaenoate (20:5), docosapentaenoate (22:5), and docosahexaenoate (22:6). The appearance of label in 22:5 and 22:6 paralleled the entrance of label into the ethanolamine phosphoglyceride fraction. Conversion of linoleate (18:2 ω6) to arachidonate (20:4 ω6) could be demonstrated but did not proceed via 18:3 ω6.

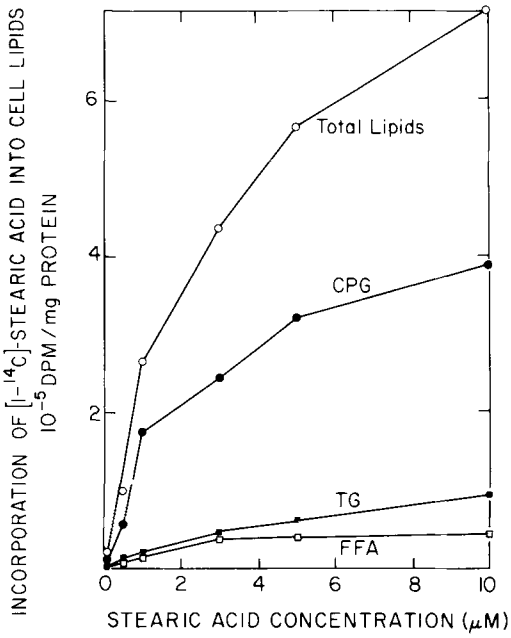

FIG. 1. Uptake of [1-^{14}C] stearic acid into total cell lipids and major lipid fractions. Brain cells cultivated for 14 days in vitro were incubated for 3 hr with [1-^{14}C] stearic acid in concentrations varying from 0.1-10 μM. The molar ratio free fatty acid/albumin (1:2) was maintained constant in each set of experiments. Lipids were extracted and fractionated and total counts measured as described in the text. Each value represents the mean of 3-4 flasks and is expressed as 10^{-5} dpm 1-^{14} C stearic acid incorporated/mg protein. CPG = choline phosphoglyceride, TG = triacylglycerol, and FFA = free fatty acid.

INTRODUCTION

Recent reports from a number of laboratories, including our own (1-4), have shown that cells from the immature nervous system can be maintained in culture following dissociation by either enzymatic or mechanical measures. When placed in a chemically defined medium, these cells utilize exogenous fatty acids and glycerol in the synthesis of cellular triacylglycerols (TG) and phospholipids (5).

In this article we wish to present data on the uptake and metabolism of a variety of saturated and unsaturated fatty acids and demonstrate that in culture dissociated brain cells are able to desaturate and elongate fatty acids.

MATERIALS AND METHODS

Preparation and maintenance of cultures: A description of the preparation of cultures of dissociated cells from cerebral hemispheres of newborn or 14-17 day rat embryos and their morphology already has been presented (3). Cells were grown in 60 mm Petri dishes with Eagles Minimal Essential Medium (Grand Island Biochemical Co., Grand Island, N.Y.) fortified with 20% heat inactivated fetal calf serum (Rehatuin Reheis Chemical Co., Chicago, Ill.), 6 g/l glucose, and antibiotics. The cells harvested from 1 Petri dish contained 1.0-2.0 mg. protein.

Substrates: The following fatty acids were obtained from Amersham-Searle, Des Plaines, Ill.: [1-^{14}C]-lauric acid (32 mCi/mmole), [1-^{14}C]-myristic acid (45 mCi/mmole), [1-^{14}C]-palmitic acid (57.7 mCi/mmole),

[1-^{14}C]-linoleic acid (57 mCi/mmole), and [1-^{14}C]-linolenic acid (52.4 mCi/mmole). [1-^{14}C]-Stearic acid (55.5 mCi/mmole) and [1-^{14}C] oleic acid (59.6 mCi/mmole) were purchased from ICN, Irvine, Calif., and from New England Nuclear Corp., Boston, Mass., respectively. The purity of fatty acids was vertified by both thin layer and gas liquid chromatography (TLC, GLC). Albumin complexes of fatty acids were prepared from fatty acid-free albumin, as previously described (5), and were stable for several months when stored at -20 C.

Metabolic studies: All metabolic studies were carried out after cultures had reached confluency. This usually occurred between 8-14 days in vitro (3). At that time, the nutrient medium was withdrawn and the cells washed with experimental medium M-199 (Grand Island Biochemical Co.) to remove residual amounts of serum. The experimental medium, consisting of 2-3 ml M-199 augmented with 6 g/l glucose and containing the radioactive substrate as its albumin complex, was added. No antibiotics were used during the metabolic studies; the cultures were kept free of contaminating microorganisms. Cells were incubated at 37 C in an atmosphere of 95% air, 5% CO_2 for the periods of time specified in the body of the text.

Extraction and fractionation of cellular lipids: Most of the procedures used have been described elsewhere (3, 5). In essence the cellular lipids were extracted with chloroform-methanol (1:2, v/v). To the organic extract, chloroform and water were added to give a final ratio of chloroform-methanol-water (8:4:3), and the ensuing aqueous phase was removed. The washed lipid extract was fractionated on silicic acid columns (2 g/mg lipid) using a modification of the method of Sun and Horrocks (6). The less polar groups, including neutral lipids, free fatty acids, and phosphatidic acid, were eluted with chloroform (50 ml) and chloroform-methanol (96:4, v/v; 50 ml). Phospholipids, including glycolipids, were removed with methanol-water (97:3, v/v; 60 ml). Lipid fractions were fractioned further using TLC, as previously described (5). To separate diacylglycerols (DG) from free fatty acids and monoacylglycerols (MG), the neutral lipid fraction was placed on TLC plates and developed in one direction successively with dichloroethane and a mixture of petroleum ether (boiling range [br] 30-60 C)-diethyl ether-formic acid (50:50:1.5, v/v/v).

Choline phosphoglycerides (CPG), and ethanolamine phosphoglycerides (EPG) were eluted from the silica column with methanol and

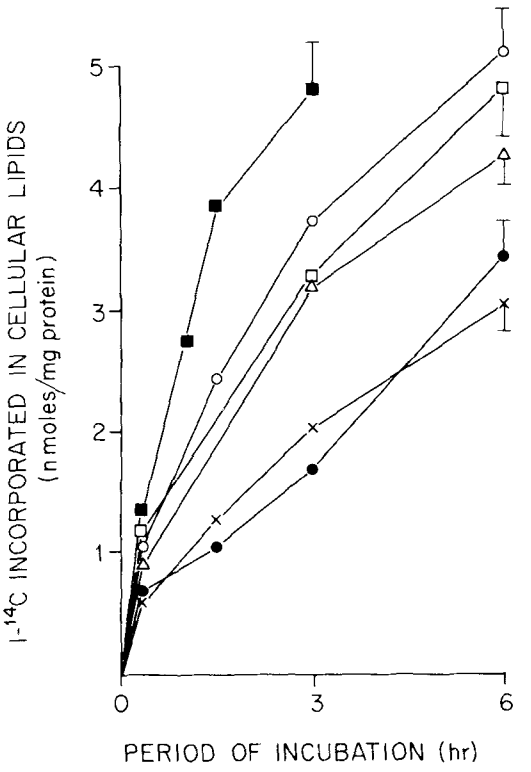

FIG. 2. Incorporation of radioactivity from various fatty acid precursors into total cell lipids. Brain cells cultivated for 13 days in vitro in 60 mm Petri dishes were incubated with 3 ml medium containing 7 μM radioactive substrate as described in the text. Total lipids were extracted, and aliquots were counted for radioactivity. The experiment was performed in triplicate. Values at 6 hr are presented as mean and standard error of mean of 3-4 Petri dishes. x—x = lauric acid, •—• = myristic acid, △—△ = palmitic acid, ○—○ = stearic acid, □—□ = linoleic acid, and ■—■ = linolenic acid.

separated by preparative TLC, employing Silica Gel G (Merck, Darmstadt, Germany) using authentic CPG and EPG (Supelco, Bellefonte, Pa.) as markers. Plates were developed at 5 C in the solvent system chloroform-methanol-acetic acid-water (25:15:3:2, v/v/v/v) as described by Skipsky, et al. (7). After the plates had been developed they were dried under nitrogen. The tissue phospholipids were covered with a glass plate while the markers were exposed briefly to iodine vapors. The areas corresponding to CPG and EPG were scraped off, and their radioactivity was counted (5). As judged by rechromatography in the above TLC system and in chloroform-methanol-7 N ammonia (65:35:5 v/v/v), the EPG and the CPG isolated by this method were chromatographically pure and free of radioactive contaminants.

GLC: The fatty acid constituents of the

TABLE I

Distribution of Radioactivity in Lipids after Incubation with Fatty Acids of Various Chain Lengths[a]

Lipid fraction	Fatty acid precursor						
	12:0	14:0	16:0	18:0	18:1	18:2	18:3[b]
Neutral lipids	30.0	30.7	19.0	17.5	22.2	12.4	13.4
Phosphatidic acid[c]	2.8	1.4	1.5	1.8	1.2	0.9	0.5
Diglyceride	5.3	3.9	2.8	3.2	3.8	2.7	3.0
Triglyceride	12.5	16.8	9.2	8.0	10.5	5.1	7.8
Cholesterol ester	2.1	4.2	0.9	1.6	2.2	2.1	1.4
Free fatty acid	6.1	2.3	3.9	2.5	3.5	1.3	0.4
Phospholipids	70.0	69.3	81.0	82.5	77.8	87.5	86.6
Serine phosphoglyceride[d]	3.4	3.7	4.5	7.5	8.2	6.1	10.5
Choline-phosphoglyceride	56.9	55.4	62.8	55.5	51.1	63.1	53.6
Ethanolamine phosphoglyceride	4.6	4.2	11.7	12.4	13.6	14.5	21.7
Total lipids[e]	273	308	547	635	440	612	502

[a]Cells were incubated for 6 hr under the same conditions as described for Figure 2. Lipid classes were separated by silicic acid columns and resolved by thin layer chromatography as described in the text. Values for neutral lipids and phospholipids are given as percentage of total radioactivity incorporated into the lipids.

[b]Incubation carried for 3 hr.

[c]Also contains monoglyceride.

[d]Also contains inositol phosphoglyceride.

[e]Values expressed as disintegration/min x 10^{-3}/mg protein.

various lipids purified by means of TLC were converted into their corresponding methyl esters by dissolving them in 2 ml 0.5 N HCl in methanol and subjecting them to transesterification in sealed ampoules under nitrogen at 80 C for 4 hr. The methyl esters were extracted with pentane and analyzed on a Barber-Colman model 5001 gas chromatograph equipped with a Nuclear-Chicago model 5190 radioactivity monitoring system. The stationary phase of the column was 15% DEGS on Chromosorb WAW (Supelco, Bellefonte, Pa.). The column was operated at 200 C under 25 lb argon pressure. Peak areas were quantified by triangulation.

RESULTS AND DISCUSSION

After an incubation time of 3 hr, the incorporation of [1-^{14}C] stearic acid into total cellular lipids ranged from 20-50% of the amount added to the medium. Since M-199 contained no free stearic acid, as verified by GLC, this radioactivity represents net incorporation of free fatty acid. Below concentrations of 1μM, the uptake of fatty acid by the cells after 3 hr incubation was ca. proportional to its concentration in the medium, while at higher substrate concentrations the fraction of radioactivity incorporated into cellular lipids or remaining as cellular free fatty acid decreased (Fig. 1).

This phenomenon suggests the presence of carrier mechanisms for the uptake of fatty acids from the incubating medium. In this respect, brain cells differ from fibroblast cultures which fail to show saturation of fatty acid uptake up to concentrations of 200 μM oleic acid (8). At 3 hr incubation, a large part of the radioactivity derived from [1-^{14}C] stearic acid was recovered in CPG (Fig. 1). Increasing the concentration of stearic acid in the incubating medium failed to alter the percentage distribution between CPG and TG. In this respect, as well, brain cells differ from fibroblast cultures. In the latter, an increase in the concentration of fatty acids in the medium causes proportionately more radioactivity to enter the TG fraction (8).

Incorporation into cellular lipids was greatest for stearic acid and decreased progressively with decreasing chain length. Total recovery of radioactivity from cellular lipids, proteins, and from the medium ranged from 80% for stearic acid to 45% for lauric acid (12:0). It is likely that the remaining amount of labeled fatty acid underwent oxidation. Incorporation of 18-carbon fatty acids was more extensive than that of the lower homologs, i.e. 12-14-, and 16-carbon fatty acids (Fig. 2). In all instances, incorporation of labeled fatty acids appeared to follow a biphasic curve, with a rapid initial uptake, probably a function of cellular binding and esterification to form TG and DG, followed by slower uptake which decreased progressively in the course of incuba-

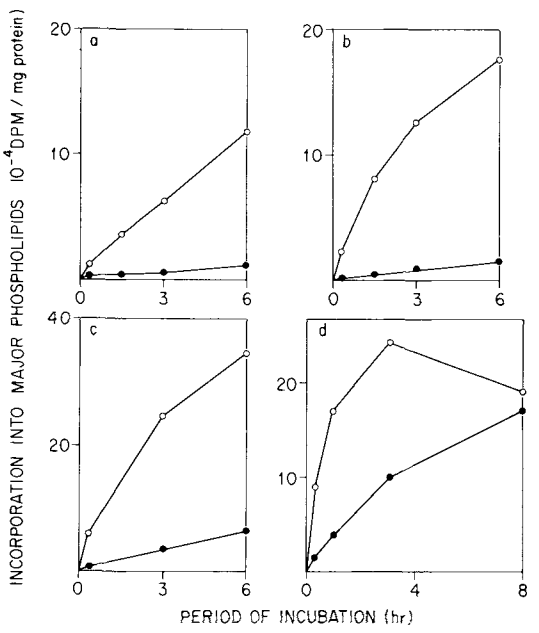

FIG. 3. Incorporation of radioactivity from various fatty acids into major phospholipids. Experimental conditions were the same as described for Figure 2. Phospholipids were separated by thin layer chromatography and counted as described in the text. Figures A-D represent incorporation of fatty acids into choline phosphoglyceride (open circles) and ethanolamine phosphoglycerides (closed circles). Values are given as disintegration/min incorporated/mg protein. 3a Lauric acid. 3b Myristic acid. 3c Palmitic. 3d Linolenic acid.

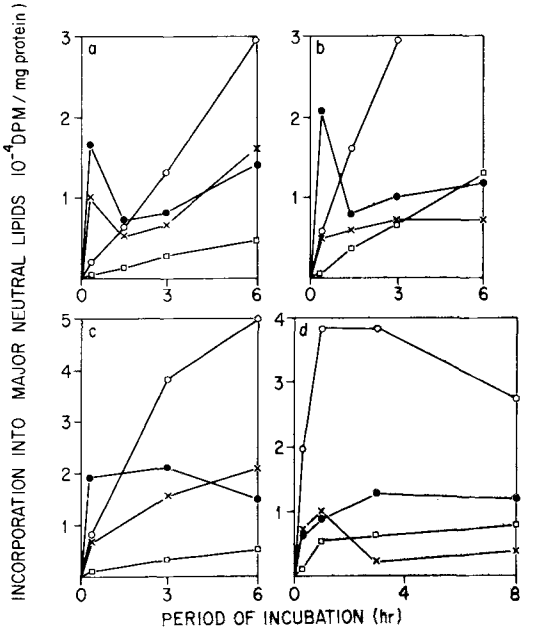

FIG. 4. Incorporation of radioactivity from various fatty acids into major neutral lipids. Experimental conditions were the same as described for Figure 2. Neutral lipids were separated by thin layer chromatography and counted as described in the text. 4a Lauric acid. 4b Myristic acid. 4c Palmitic acid. 4d Linolenic acid. Values are given as disintegration/min incorporated/mg protein ○—○ = triacylglycerols, ●—● = diacylglycerols, □—□ = cholesterol esters, x—x = free fatty acid.

tion (Fig. 2). Since 30-50% labeled fatty acids had entered the cellular lipids from the incubating medium by the end of 6 hr, the latter phenomenon appeared to be the result of substrate depletion, rather than saturation of cellular binding sites for fatty acids (9).

As previously demonstrated for stearic acid (5), other fatty acids also were incorporated preferentially into phospholipids and TG. When the distribution of label between the various phospholipid species were examined after 6 hr incubation, we found that, with increasing chain length and increasing unsaturation of the fatty acid precursors, the proportion of radioactivity in EPG to that entering CPG increased progressively (Table I). In all instances, CPG had more radioactivity than EPG. By contrast with the experience of Sun and Horrocks who studied in vivo incorporation of labeled palmitic acid into subcellular fractions of brain and found a significant amount of radioactivity in alkenyl groups (10), we were unable to find that saturated fatty acids entered alkenyl groups to any significant extent.

During the first 4 hr of incubation, label from [1-^{14}C] linolenic acid entered CPG preferentially. Thereafter, there was a progressive reduction in the proportion of radioactivity in CPG, while incorporation of labeled linolenic acid into EPG continued to increase (Fig. 3d). When incorporation was expressed in terms of dpm/nmoles phosphorus, the maximum ratio of CPG to EPG was observed after 60 min of incubation. Thereafter, it decreased progressively to a ratio of 0.29 by 24 hr. This observation contrasted with the relatively constant ratio of radioactive CPG and EPG when cells were incubated with labeled lauric (Fig. 3a), myristic (Fig. 3b), and palmitic acids (Fig. 3c).

In the neutral lipid fraction, incorporation of label was generally in the order of TG > DG > MG + phosphatidic acid (Table I). On studying the time course for fatty acid incorporation into the neutral lipid fraction, the amount of radioactivity in the DG fraction was found to be greater than in TG after 30 min incubation when 12:0, 14:0, and 16:0 were used as precursors (Fig. 4a, b, c). With further incubation, the amount of radioactivity in TG increased progressively, while that in DG fell or remained relatively constant. When cells were incubated with linolenic acid (18:3) (Fig.

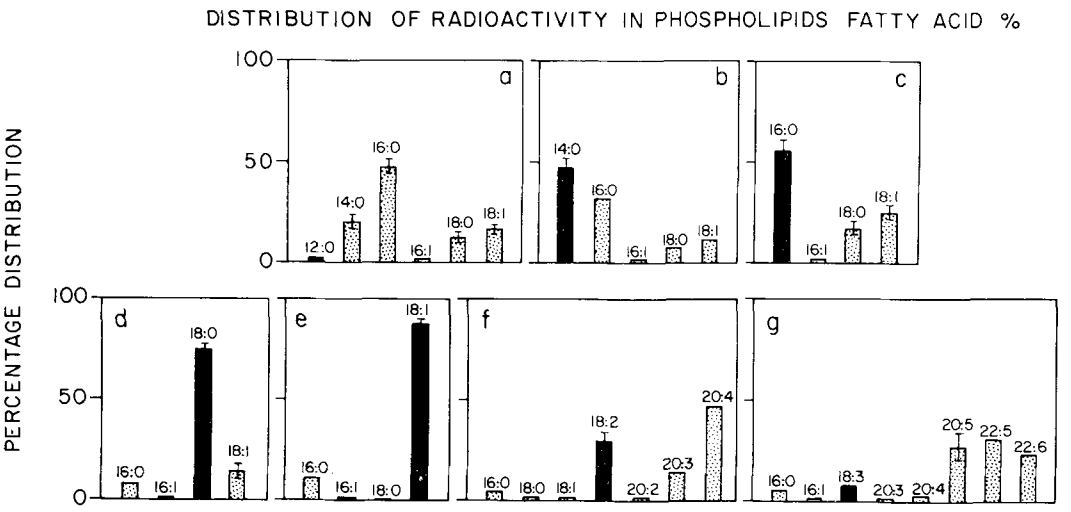

FIG. 5. Distribution of radioactivity in fatty acid esters of the phospholipids after prolonged incubation. Experimental conditions were the same as described for Figure 2, except that incubation was carried out for 24 hr. The phospholipids accounted for more than 80% of total radioactivity incorporated into the cell lipids. Values are given as percentage of radioactivity injected into the gas liquid chromatography columns and are expressed as mean and standard error of mean for two or three experiments. 5a Lauric acid 5b Myristic acid 5c Palmitic acid. 5d Stearic acid. 5e Oleic acid. 5f Linoleic acid. 5g Linolenic acid. The closed bars represent the initial precursors.

4d), radioactivity rapidly entered the neutral lipid fraction; and, at the first time point studied, TG was already more extensively labeled than DG. Labeling of the MG and phosphatidic acid fraction also proceeded rapidly, but as yet we are unable to compare the initial rates for fatty acid entry into phosphatidic acid, MG, and DG.

Since the quantity of DG and TG undergoes little alteration with incubations of 24 hrs or less (4), the time course for fatty acid esterification is consistent with an initial incorporation into DG and subsequent transfer to TG and phospholipids.

In the course of incorporation into cellular lipids, labeled fatty acids underwent chain elongation and desaturation (Fig. 5). The proportion of 1-^{14}C labeled saturated fatty acids

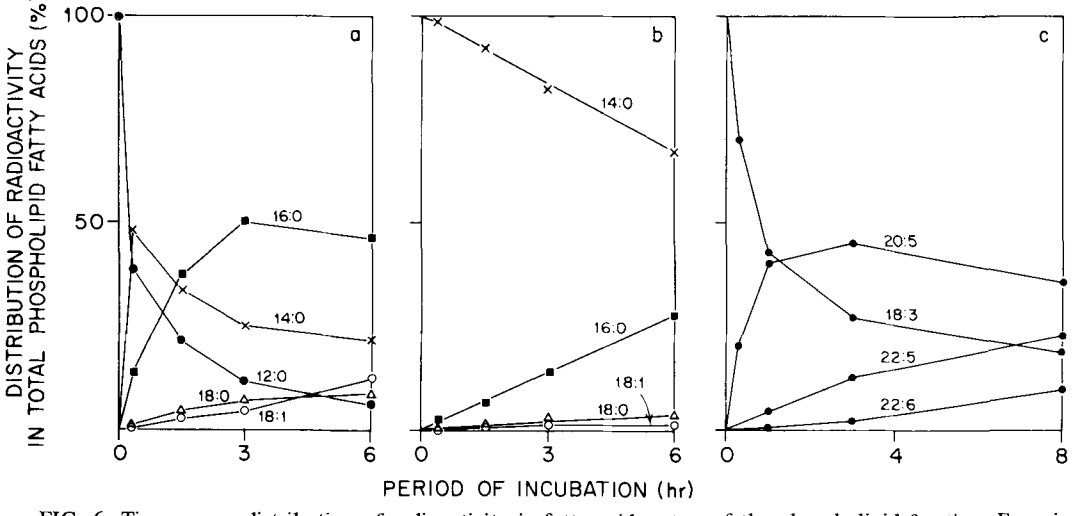

FIG. 6. Time course distribution of radioactivity in fatty acids esters of the phospholipid fraction. Experimental conditions were the same as described for Figures 2, 4, and 5. Values are expressed as percentage of the radioactivity injected into the gas liquid chromatography column. 6a Lauric acid. 6b Myristic acid. 6c Linolenic acid.

having undergone chain elongation at the end of 24 hr incubation was greatest for 12:0, and diminished in the order of 12:0 > 14:0 > 16:0 > 18:0. Since uptake of labeled fatty acids into cellular lipids was of the reverse order (Fig. 2), this finding suggests that, with increasing chain length, there is a progressively reduced accessibility of fatty acids to the cellular chain elongation system. Oleic acid (18:1), although taken up into cellular lipids in significant amounts, remained entirely unchanged. With all fatty acid precursors studied, only small amounts of radioactivity were found in fatty acids of shorter chain lengths, indicating β-oxidation and recondensation of two carbon fragments to be relatively inactive.

Formation of labeled phospholipid oleic acid from 12:0, 14:0, and 16:0 precursors appeared to depend upon the amount of labeled stearic acid derived from them, as the ratio of labeled stearic/oleic acid in cellular phospholipids was constant in all 3 instances.

There was extensive conversion of linoleic acid to arachidonic acid (20:4 ω6) and of linolenic acid to eicosapentaenoic (20:5 ω3) and docosahexaenoic acids (22:6 ω3). In this respect, cell cultures of fetal rat brain behave analogously to neonatal and adult rat brain in vivo (11-14). Although we have not yet fully examined the structure of fatty acids derived from linoleic and linolenic acids, on the basis of GLC retention times in comparison to known standards, 20:4 derived from linoleic acid belongs to the ω6 series, while 20:4, 20:5, 22:5, and 22:6 derived from linolenic acid belong to the ω3 series.

When labeling patterns of phospholipid fatty acids derived from linolenic acid are examined at various points in the course of incubation, the curves obtained are consistent with the expected precursor-product relationship between 18:3, 20:5, 22:5, and 22:6 (Fig. 6c).

Similar kinetic relationships also were found for 12:0, 14:0, 16:0, and 18:0 (Fig. 6a). Labeling of 22:5 and 22:6 occurs slowly and is still increasing after 8 hr incubation. This process occurs in parellel with labeling of the EPG fraction (Fig. 3d), and there is little doubt that polyunsaturated fatty acids have to be formed before these are incorporated into EPG. When labeled linoleic acid (18:2 ω6) was incubated with brain cell cultures, 18:3 ω6 remained essentially unlabeled, suggesting that under the conditions used in these experiments conversion of 18:2 ω6 to 20:4 ω6 did not proceed via 18:3 ω6. We also were unable to demonstrate labeling of 18:4 ω3 when labeled linolenic acid (18:3 ω3) was added to cultures.

In conclusion, we have found that dissociated brain cells in culture are able to incorporate and esterify a variety of fatty acids. In the course of incorporation, saturated fatty acids undergo chain elongation and desaturation, while polyunsaturated fatty acids are metabolized extensively to the other polyunsaturated fatty acids which are major components of the cultured brain cells.

These studies again serve to show the versatility of brain cells in culture in terms of utilization for metabolic studies and for an examination of the biochemical features of developing nervous tissue.

ACKNOWLEDGMENTS

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Elution Characteristics of Fatty Acid Methyl Esters on Capillary Columns

GARY J. NELSON[1], Bio-Medical Division, Lawrence Livermore Laboratory, University of California, Livermore, California 94550

ABSTRACT

The elution characteristics of fatty acid methyl esters on support-coated and open tubular capillary columns were investigated using reference standards and natural mixtures of fatty acid methyl esters. Over an extended range of fatty acid methyl esters chain lengths (C-11-C-26), the plot of the log of the adjusted retention time, t'_R, vs. number of carbon atoms in the fatty acid chain was not linear, as has been previously assumed by many investigators. With support-coated open tubular columns and with diethylene glycol succinate as the stationary phase, the relationship between the log of the retention time and carbon number was best approximated by a second-order equation: $\log (t'_R)_x = \alpha + \beta(CN_x) + \gamma(CN_x)^2$ where α, β, and γ are the virial coefficients of the equation. In addition, for the longer fatty acid methyl esters, the plots all tended to converge. Hence, for data from capillary columns, especially over a wide range of carbon numbers, all tentative indications based upon linear log plots and parallel lines for different homologous series of fatty acid isomers should be viewed with caution. A method is presented for identifying peaks from capillary columns; it uses quadratic equations and three reference fatty acid methyl ester standards for each homologous series being studied.

INTRODUCTION

The separation and subsequent identification of fatty acids was the first application of gas liquid chromatography (GLC) (1). Since then there has been a steady development in both the theory and practice of the GLC analysis of fatty acids, but accurate identification of all the components in a natural sample, especially when using high resolution capillary columns, is still not a simple task (2-4). Retention time, t_R, is the most convenient parameter on which to base a tentative identification; and, if suitable reference materials exist, comparing t_R is often a useful procedure to confirm identities. Unfortunately, adequate reference materials often are not available; hence, for tentative identification of many of the components in his sample, the non-GLC specialist usually relies upon data from other reputable laboratories and on various graphical and mathematical relationships between structure and elution times (2-7).

It is obvious that actual retention times alone are almost useless for comparison between different laboratories, because even minor variations in operating conditions affect the retention of a component on a GLC column (8, 9). The relative retention time, if normalized to a standard fatty acid methyl ester (FAME), such as methyl stearate, is somewhat better for this purpose; but, with the commonly used polyester stationary phases, column conditions rarely can be matched adequately among different laboratories to provide useful comparisons (2, 10).

In practice, most investigators use a combination of techniques that involve the idea of the carbon number or equivalent chain length developed by Woodford and Van Gent (11) and Miwa, et al. (12). This idea is based upon the linear relationship between the number of carbons in the fatty acid chain of a normal saturated methyl ester and the log of its retention time (1, 13). Initially both Woodford and Van Gent and Miwa et al., developed their method graphically, but a simple equation can be used equally well (2).

From variations of this idea, methods were developed rapidly by which the retention time of any member of a homologous series of FAME could be predicted if any two members were identified (2, 4, 14, 15). Ackman (16-18) and others (3, 19, 20) also extended this concept by the use of separation factors, in which the addition of one or more functional groups, e.g. a double bond, to a molecule had a definite and predictable effect upon its elution time.

The advent of capillary columns, both open tubular (OTC) and support-coated open tubular (SCOT) types (21), provided better resolution and allowed more components to be separated in a single run. Several reports (21-25) indicate that the principles pertaining to packed columns for the identification of FAME can be applied directly to capillary columns. This may be true if only a narrow range of carbon

[1]Present address: New York State Institute for Research in Mental Retardation, Staten Island, New York 10314

numbers is being studied. However, experience in this laboratory with capillary-column analyses of FAME, particularly on SCOT columns, casts doubt upon the validity of the linear relationship between the log of the retention time and the carbon chain length of FAME and consequently upon linear log plots and for separation factors methods of predicting retention times.

The observations described here show that with capillary columns utilizing polyester and some other stationary phases, FAME of both saturated and unsaturated homologs do not obey a linear relationship between chain length and the log of their retention time, but rather follow a higher order curve, either exponential or polynomial. The SCOT columns studied in detail in this work all exhibited positive deviations from linearity; however, OTC, examined only briefly here, were found to have both negative and positive deviations from linearity depending upon the nature of the stationary phase. And it may be that similar elution characteristics are exhibited in the GLC of FAME on packed columns but are masked by the inferior resolution, broader peaks, and higher concentration of stationary phase, commonly found in packed columns.

EXPERIMENTAL PROCEDURES

All chromatographic separations were performed with a Perkin-Elmer model 900 gas chromatograph equipped with a hydrogen flame ionization detector. The injection port was maintained at 275 C and the outlet manifold (approximate detector temperature) at 300 C. The carrier gas was helium. The detector was operated at 17 psig hydrogen and 31 psig compressed air.

All capillary columns were made of stainless steel and were purchased from the Perkin-Elmer Corp., Norwalk, Conn. The following columns were used. Open tubular columns: diethylene glycol succinate (DEGS) and butanediol succinate (BDS), 150 ft x 0.01 in. inside diameter, Apiezon L, 100 ft x 0.01 in. inside diameter. (Metric dimensions: 45.7 m x 0.025 cm inside diameter for DEGS and BDS; 30.5 m x 0.025 cm inside diameter for Apeizon L.) Support-coated open tubular columns: diethylene glycol succinate and butanediol succinate, 150 ft x 0.02 in. inside diameter, Apeizon L, 100 ft x 0.02 in. inside diameter. (Metric dimensions: 45.7 m x 0.05 cm inside diameter for Apiezon L.)

Samples of FAME were obtained either from the Lipids Preparation Laboratory, Hormel Institute, Austin, Minn., or Nu-Chek-Prep, Elysium, Minn. FAME from experimental samples were prepared by transmethylation in methanolic HCl as described elsewhere (26). All FAME were injected into the chromatograph as dilute solutions in hexane using a Beckman injection syringe; the injection volume was ca. 1 μl hexane. The number 1 splitter supplied by Perkin-Elmer was used for all injections on both SCOT and OTC columns.

All retention times were determined by measuring distances recorded on a Honeywell Model 16 electronic strip-chart recorder, 5.0 mV full scale, operated at a chart speed of 1/2 in./min. The retention time for any one peak was determined, if it was symmetrical in shape, by the distance between the peak for an unretained substance (air peak), which was essentially identical to the front of the hexane deflexion, and the peak maximum. In a few cases where peaks were asymmetric, the retention time was measured from the air peak to the midpoint of the base width as determined by triangulation; however, it should be mentioned that, if more than one peak in a run was skewed, that run was not used for data calculations, and in the SCOT column runs all peaks were symmetric. As expected, the distance from the injection point to the peak maximum gave results that failed to fit any convenient relationship between carbon-chain length and retention time; the error in the fit could be reduced greatly by subtracting the distance between the injection point and the air peak, t_M. In packed columns, t_M is usually short, so that such errors are not noticeable except at very short retention times. In either case, the air peak was the best point from which to measure retention time for both packed and capillary columns. This value usually is referred to in the GLC literature as the adjusted retention time and designated t'_R.

Arithmetic calculations involving logs, computations of various coefficients, and determination of carbon numbers were performed on a Hewlett-Packard model 9100A desktop calculator. The resulting curves were plotted automatically using the associated Hewlett-Packard model 9125 A calculator-plotter.

RESULTS

The elution times of a homologous series of FAME separated on a capillary GLC column do not yield a linear plot when log t'_R is plotted against the numbers of carbon atoms in the fatty acid chain. For SCOT columns when a linear relationship was used, the longer (C-20-C-24) and shorter (C-10-C-14) chain saturated FAME consistently fell above the line if

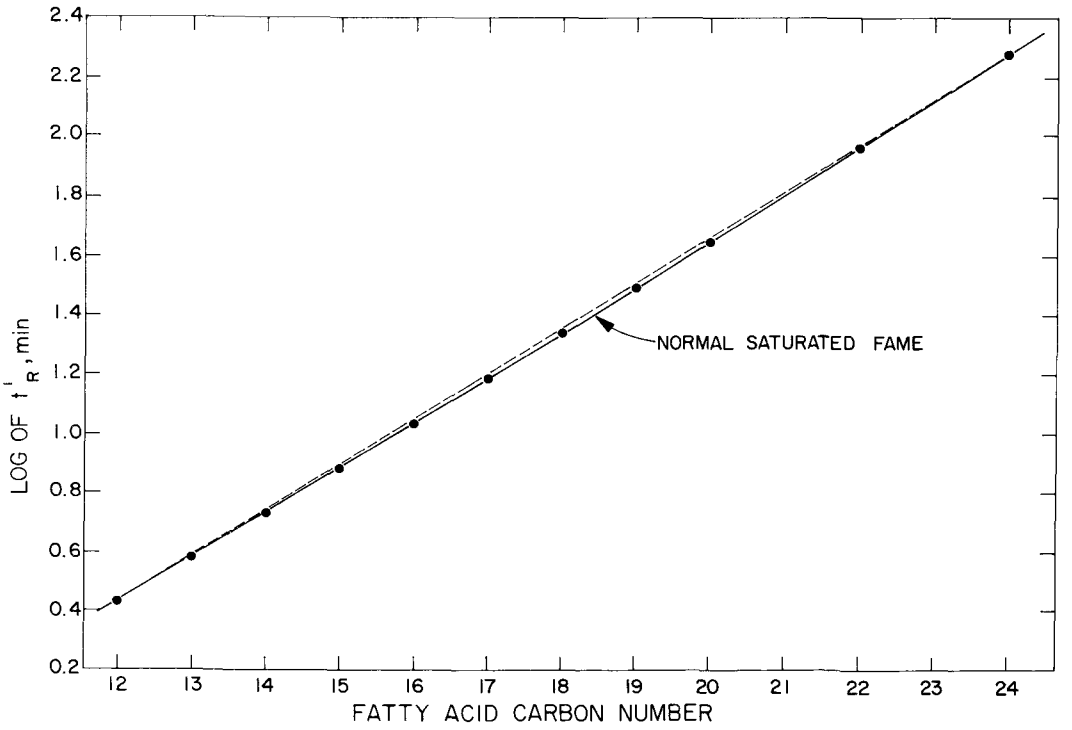

FIG. 1. The relationship between log t'_R and number of carbon atoms in the fatty acid for normal chain saturated FAME between 12-24 carbons. The points are the experimental data; the dotted line is a linear fit using log t'_R of the 12 and 24 carbon FAME to determine the slope; the solid line is the quadratic fit of data using the log of the retention times of the 16, 20, and 24 carbon FAME to determine the parabola. The deviation from the linear fit is evident. The column was a DEGS-SCOT column, 150 ft x 0.02 in. inside diameter, operated at 175 C at an inlet pressure of 18 psi.

the log t'_R of palmitate and stearate were used to determine the slop of the curve. Conversely, if the log t'_R of a short and long chain FAME, such as myristate and behenate, were chosen as the reference points, the intermediate chain length FAME were below the line (Fig. 1). These observations, which were made with a SCOT-DEGS column at 180 C and 18 psi helium at the inlet, suggested strongly that the true relationship between log t'_R and chain length followed a higher order curve than previously believed. The fact that the deviations from linearity were positive for both long and short chain FAME suggested a quadratic relationship.

To solve a second order curve exactly, the t'_R of three reference FAME must be used instead of the two adjusted retention times used for a linear fit. Then, three simultaneous equations can be written in the following form:

$$\log (t'_R)_1 = \alpha + \beta (CN_1) + \gamma (CN_1)^2 \quad [1]$$

$$\log (t'_R)_2 = \alpha + \beta (CN_2) + \gamma (CN_2)^2 \quad [2]$$

$$\log (t'_R)_3 = \alpha + \beta (CN_3) + \gamma (CN_3)^2 \quad [3]$$

in which $(t'_R)_1$, $(t'_R)_2$, and $(t'_R)_3$ are the respective adjusted retention times of the FAME with carbon numbers CN_1, CN_2, and CN_3; and α, β, and γ are virial coefficients of the set of equations (1), (2), and (3).

The values of the virial coefficients can be determined by solving a 3 x 3 determinant. The general expression for the carbon number then is given by the solution of a quadratic equation of the form:

$$CN_x = \frac{-\beta \pm \sqrt{\beta^2 - 4\gamma [\alpha - \log (t_R)_x]}}{2\alpha} \quad [4]$$

where CN_x is the carbon number of any member of homologous series and $(t'_R)_x$ is its retention time. While the solution of a 3 x 3 determinant is mathematically straightforward, it is extremely tedious if done manually. However, most modern desktop, programable calculators or any larger digital computer can perform these calculations simply and rapidly.

Table I gives examples of carbon numbers calculated for a linear, a frontal tangent, and a

TABLE I

Comparison of Carbon Numbers for Normal Chain Saturated Fatty Acid Methyl Esters (FAME) Calculated by Linear and Quadratic Methods on Data Obtained from SCOT-DEGS Column[a]

FAME	t'_R, min	Calculated carbon number		
		Linear fit[b]	Linear fit using frontal Tangent[b]	Quadratic fit[c]
11:0	1.94	11.119	10.903	11.034
12:0	2.71	12.078	11.948	12.020
13:0	3.83	13.070	13.000	13.034
14:0	5.35	14.028	13.993	14.009
15:0	7.53	15.009	14.994	15.001
16:0	10.64	16.000	16.000	16.000
17:0	15.05	16.995	16.994	16.997
18:0	21.37	18.000	18.000	18.000
19:0	30.28	18.999	18.995	18.992
20:0	43.10	20.012	19.993	19.993
21:0	60.22	20.971	20.992	20.994
22:0	87.98	22.058	22.013	22.000
23:0	125.65	23.081	23.019	22.995
24:0	182.45	24.150	24.071	24.032
25:0	258.37	25.148	25.052	24.995
26:0	378.62	26.244	26.129	26.047

[a]Column conditions: 175 C, 18 psi He; dimensions, 150 ft x 0.02 in. inside diameter.
[b]16:0 and 18:0 FAME retention time used as reference points.
[c]16:0, 18:0, and 22:0 FAME retention time used as reference points.

quadratic fit for the normal chain, saturated FAME containing 11-26 carbons separated on a SCOT-DEGS column. The frontal tangent fit will be discussed below. The quadratic fit is as good or better than the other fits for all the points. The deviation from linearity is shown graphically in Figure 1; the reference points for the linear fit were the t'_R of FAME containing 12 and 24 carbons. For this particular column, the deviation, though small, is consistant. Also, Figure 1 demonstrates that for any relatively small group of FAME, not varying more than 6 or 8 carbons in chain length, the linear fit is a good approximation.

Another way of illustrating this point is to look at the actual values of the virial coefficients α, β, and γ in a typical GLC run. In the run tabulated in Table I, $\alpha = 1.2897$, $\beta = 0.1389$, and $\gamma = 0.000369$. The coefficient, γ of the squared term in equations 1-3 is very small and, if ignored, reduces these equations to the linear form. Hence, the coefficient α is a measure of the deviation of the fit from linearity. It is not surprising, considering the magnitude of α, that this curvature has been overlooked previously on both packed and capillary columns.

Figure 2 illustrates a second aspect of the performance of a SCOT-DEGS column in separating mixtures of saturated and unsaturated FAME. As Ackman (17) pointed out some years ago, using data from packed columns, the adjusted retention times for either a saturated or unsaturated FAME series can be plotted on semilog paper to yield a straight line; but the line for the saturated series is not necessarily parallel to that obtained from an unsaturated series. He suggested, however, that the various homologous series of unsaturated FAME would be parallel to each other. With packed columns, the slight convergence of the unsaturated series probably would be unnoticed; and they would, indeed, appear truly parallel.

Ackman in his recent review concerned with capillary column separation of FAME (4) has modified this statement and concluded that while the lines for certain families, such as the $4\omega3$ and $3\omega6$, may be parallel, they may not be exactly parallel to those for the $2\omega6$ and $3\omega3$ series. On capillary columns, none of the homologous series are parallel, because there is no linear relationship between the log of adjusted retention time and carbon chain length. As shown in Figure 2, however, there is some convergence of all the curves at the longer chain lengths. In partial agreement with Ackman's observation, the saturated series tends to converge with the monoethylenic unsaturated series more rapidly than the members of the unsaturated series converge with themselves.

The data in Figure 2 are from runs for which reference FAME could be obtained for at least

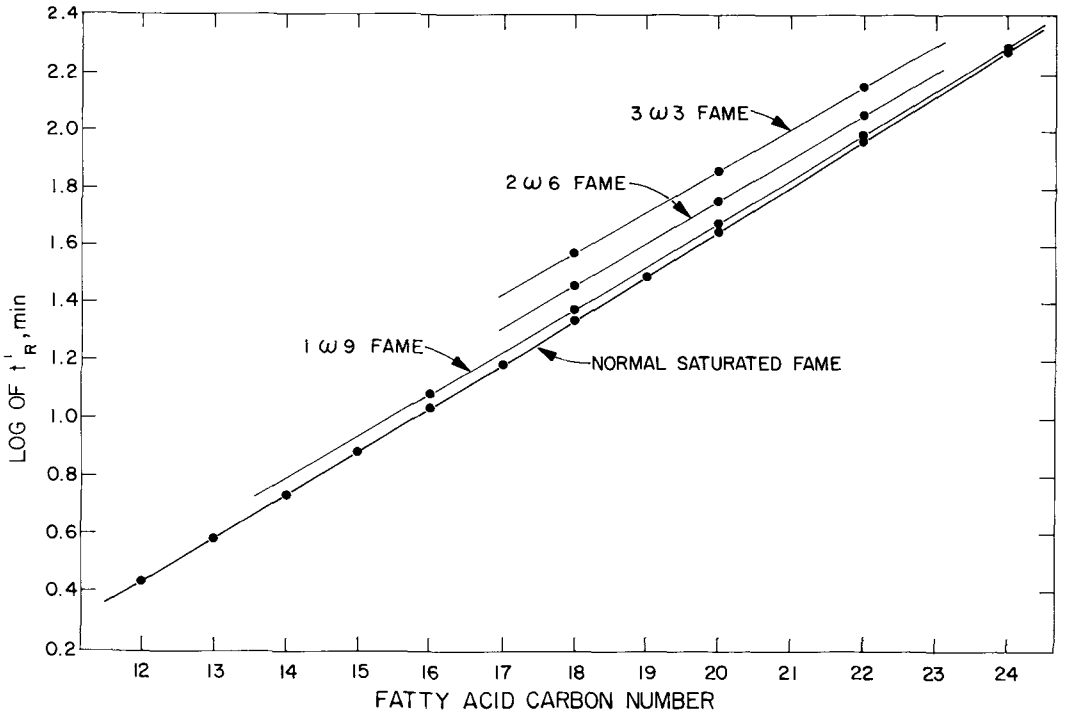

FIG. 2. Quadratic fit of log t'_R vs. carbon number for several homologous series of normal-chain FAME, showing the convergence between the saturated and monoethylenic FAME curves is shown. Convergence between the various unsaturated homologous series is less marked but also can be demonstrated; it would be more obvious if additional references compounds were available. Column and conditions were as given in the caption to Figure 1.

three members of each series. In the 2ω6 and 3ω3 series, only 3 members were available as reference points, but good quadratic fits could be obtained in each case.

The nonparallelism and convergence of the quadratic fits for homologous series of unsaturated FAME is well illustrated with the various monoethylenic series of positional isomers. These compounds are present in most biological samples (27) and, indeed, are often the major unsaturated fatty acids present. Ackman and Castell (28) studied the elution of monoethylenic FAME from polar and nonpolar OTC columns in some detail. These compounds can be separated on capillary columns, both SCOT and OTC, particularly if the double bonds are past the midpoint of the chain toward the methyl end of the molecule. In samples containing FAME of chain lengths from 14-24 carbons, the assumption of a linear fit would result in considerable confusion and perhaps misassignment of the identity of many of the isomers, because of both the nonlinearity and convergence of the curves for the homologous series, even if the 1ω9 series is used as the reference line as suggested by Ackman (17).

Figure 3 shows the convergence of values for the homologous series, The 18:1ω9 and the 19:1ω7 are well resolved on this column, which has ca. 25,000 HETP at the time of the run, but the 24:1ω9 and 24:1ω7 are poorly resolved. Figure 3 also shows that with the quadratic fit, most of the positional isomers of the monoethylenic fatty acid methyl esters can be resolved and identified unambiguously.

With this type of analysis, once enough reference compounds are identified in each homologous series, each FAME in a sample run can be identified by an arbitrary value which is equivalent to the number of carbons in the fatty acid molecule or carbon chain length number. (Table II provides examples of carbon chain length numbers.) The carbon chain length number should always be a whole number or at least a close approximation to a whole number, rather than the arbitrary number assigned in the systems of Haken (3) or Jamieson and Reid (29). For example, in Table II, 21:2ω6 will have a carbon chain length number of 21.004 based upon calculation from 18:2ω6 as 18:000, 20:2ω6 as 20.000, and 24:2ω6 as 24.000.

If the virial coefficients of the equation for the 2ω6 series are determined, then for each

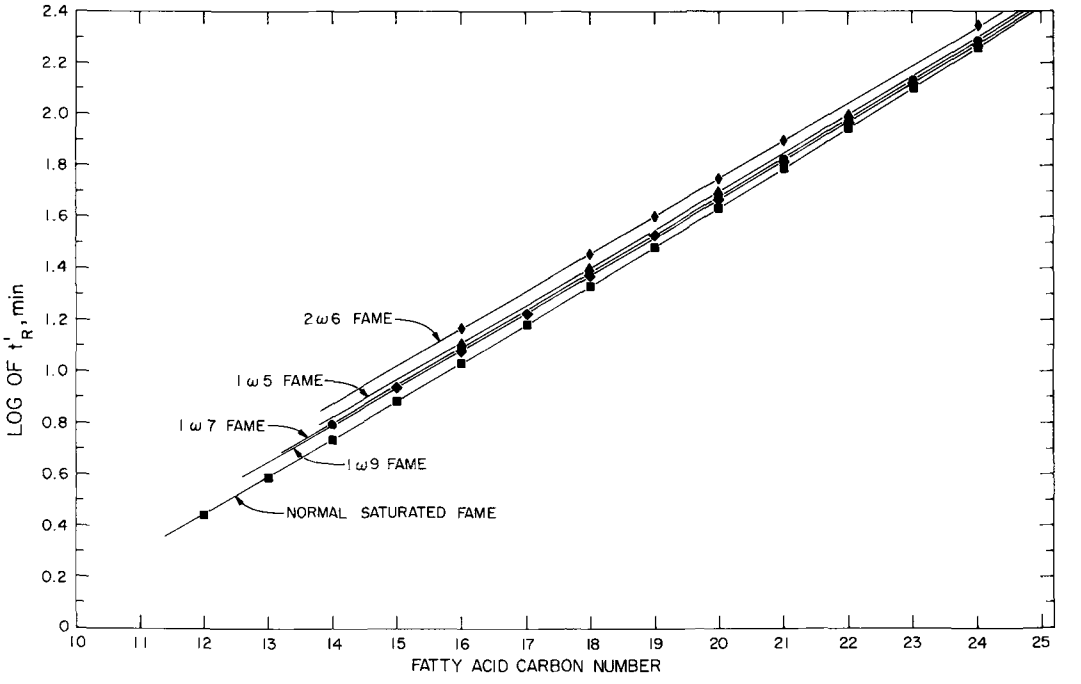

FIG. 3. Plot of carbon number vs. log t'_R for various homologous series of FAME from a sample of dolphin red blood cells. The solid lines are quadratic fits of the experimental data. Only series with three or more members identified are plotted. Convergence of all series, including the monoethylenic positional isomers, can be noted. The sample was run on a DEGS-SCOT column operated as described in the caption of Figure 1.

member of the series with a whole number, i.e. 16, 18, 20, and 22, a retention time can be predicted by means of the equation:

$$(t'_R)_X = e^{2.303[\alpha + \beta (CN_X) + \gamma (CN_X)^2]} \cdot \quad [5]$$

Thus, instead of trying to fit unknown peaks to various curves, one can predict exactly where each member of a series will be in each run. With equation (5), observed peaks can be matched or eliminated on the basis of their carbon chain length number and retention times with much less ambiguity than previously.

DISCUSSION

The nonlinear relationship between retention time and chain length of FAME is not unexpected, since elution characteristics depend, to a large extent, upon the solubilities of the molecules in the stationary phase (30). The convergence of the curves at longer chain lengths also could be predicted, because the physical properties of FAME become quite similar for the long chain compounds, regardless of the degree of unsaturation or branching in the molecule (31). Thus, such compounds should have very similar, though perhaps not identical, chromatographic behavior.

However, the effects described here should not be confused with the changes in t'_R caused by overloading the column as mentioned by Ackman and Castell (28) and others (32, 33). It should be emphasized that, in this study, the t'_R were all determined using concentrations of FAME well below those that affect the retention time and, thus, represent the true retention time of the compound under the stated operating columns of the column and instrument.

Earlier failures to detect the nonlinearity phenomena probably are due to the use of packed columns for most GLC analysis of FAME. The relatively poor resolution of packed columns and their very broad base-widths make accurate determination of retention times difficult. Also, the large amount of stationary phase used in packed columns (10-20%) may mitigate the nonlinearity effect somewhat. However, the nonparallelism of the curves for various homologous series of FAME was and is readily observable on packed columns (17). Actually, it may be possible to detect the curvature of the t'_R/chain length plot for packed columns also. Haigh, et al., (34) reported that, on ethylene glycol adipate columns, linear log plots of t_R vs. equivalent chain

TABLE II

Calculated Carbon Chain Length Numbers for Saturated and Unsaturated FAME in Dolphin Red Blood Cells from Data Obtained with a SCOT-DEGS Column[a]

FAME	t'_R, min	Carbon chain length number	
		Linear fit	Quadratic fit
12:0	2.74	12.080	11.956
13:0	3.85	13.057	12.977
14:0	5.39	14.024	13.979
15:0	7.60	15.012	14.993
16:0	10.72	16.000	16.000
17:0	15.16	16.995	17.006
18:0	21.50	18.000	18.011
19:0	30.44	18.999	19.004
20:0	43.28	20.011	20.000
21:0	61.73	21.030	20.997
22:0	88.32	22.060	21.995
23:0	126.09	22.836	22.979
24:0	183.00	24.154	24.000
15:1ω9	8.68	15.051	15.044
16:1ω0	11.99	16.000	16.000
17:1ω9	16.81	16.993	16.995
18:1ω0	23.68	18.000	18.000
19:1ω9	33.60	19.028	19.021
20:1ω9	46.70	19.996	19.979
21:1ω9	66.01	21.013	20.981
22:1ω9	93.94	22.050	21.999
23:1ω9	133.82	23.090	23.015
24:1ω9	188.80	24.101	24.000
14:1ω7	6.26	13.991	13.956
16:1ω7	12.33	16.000	16.000
18:1ω7	24.21	18.000	18.000
20:1ω7	48.06	20.023	19.999
21:1ω7	67.78	21.052	20.990
22:1ω7	96.19	22.088	21.990
23:1ω7	137.20	23.142	22.997
24:1ω7	194.85	24.182	23.984
16:1ω5	12.83	16.000	16.000
18:1ω5	25.28	18.000	18.000
20:1ω5	50.04	20.014	20.000
22:1ω5	98.51	22.011	21.979
16:2ω6	14.63	16.000	15.947
18:2ω6	28.81	18.000	18.000
19:2ω6	40.24	18.986	19.003
20:2ω6	56.21	19.973	20.000
21:2ω6	78.86	20.971	21.004
24:2ω6	219.34	23.991	24.000

[a]Column conditions: 175 C, 18 psi; dimensions, 150 ft x 0.02 in. inside diameter.

length showed a marked break between the short and long chain saturated fatty acids. A linear fit with one slope was obtained for FAME between 12 and 20 carbons, but FAME with 20-28 carbons gave another line with a different slope. The authors could not explain this anomalous behavior. However, Figure 1 of this paper shows that two ca. straight lines can fit the solid line if it is broken in the C_{18}-C_{20} region. This may account for the observation of these authors; it should be noted, however, that their t_R for the longer chain FAME were shorter, rather than longer, than expected. We have observed a similar negative curvature in OTC columns (further discussion of OTC column behavior follows). Also the recent report by Castello, et al., (35) on the identification of branched chain hydrocarbons suggests that nonlinearity phenomenon described here is not limited to fatty acid methyl esters only.

Groenendijk (7) has discussed nonlinearity in capillary columns with particular reference to the problem of determining the true value of t_M. He was concerned primarily with hydrocarbons and steroids, but his conclusions are quite similar to those arrived at using FAME in this work, i.e. that the fit and identification of peaks on capillary columns can be improved significantly by the use of three reference standards rather than the two normally used in linear log plots. He introduced an additional concept, that of an adjusted t_M, designated ${}_ct_M$, and used ${}_ct_M$ to determine a carbon index, CI. Using these concept and two reference standards that bracket the unknown, an unambiguous CI can be assigned to the unknown which should be constant regardless of operating conditions. Groenendijk's approach seems a valid, if somewhat complex, method of treating the nonlinearity phenomen, and his thesis (36) contains a useful discussion of the nonlinearity problem as related to t_M and the identification of peaks using retention time data. It should be mentioned that for compounds with long retention times a corrected t_M is not likely to linearize a log plot because $\log x \approx \log(x + \Delta x)$ *if* x *is large and* Δx *is small*. Also, it may not always be practical to bracket an unknown of very long retention time with two reference standards.

Nonparallelism undoubtedly is a general feature of all GLC analyses of homologous series of closely related substances. Hence, it is advisable to exercise caution when applying methods, such as separation factors (2, 16, 37) and other elution time relationships based upon parallelism, to identify unknown peaks on GLC runs from samples of natural origin (20). It should be noted, however, that over short ranges of carbon-chain length, the linear fit is a good approximation (Table II). It is only when extrapolations are carried beyond 6-8 carbon chain differences in a homologous series that significant deviations from linearity are observed.

Some investigators prefer to use the frontal tangent intercept rather than the peak maximum to measure retention times (4). This approach does, indeed, have considerable merit for skewed peaks and also for peaks with short retention time. This is illustrated in Table I.

Inspection of Table I shows that a linear fit using the frontal tangent intercept yields carbon numbers closer to theoretical values than the linear fit using the peak maximum retention time and, indeed, is as good as the quadratic fit in the range of carbon numbers below 20. It does, however, begin to deviate at longer carbon numbers. The reason for this phenomenon has to do with the nature of the gaussian spreading of GLC peaks and is discussed in more detail in the Appendix to this paper. Hence, it would still seem that the quadratic fit described in this work is a better method for identifying FAME from capillary columns than a linear fit based upon the frontal tangent intercept because of the more fundamental nature of the peak maximum retention time, the failure of the frontal tangent intercept to fit FAME with long t'_R, and the increasing use of electronic digital instruments to determine peak retention times. Investigators who do use the frontal tangent intercept should certainly be aware of the factors influencing this parameter as discussed in the Appendix to this paper.

Most of the observations discussed here are from runs on DEGS or BDS polyester-coated SCOT columns. Apiezon L columns, which do not resolve polyunsaturated FAME, exhibited similar behavior at least for the saturated and monounsaturated FAME. The SCOT columns also exhibited somewhat different performance characteristics than OTC columns coated with the same stationary phase. However, it is difficult to operate an OTC column over as wide a chain length range as a SCOT column without markedly overloading the column for some of the components in the mixture. This causes skewed peaks and less accurate t'_R. With DEGS-coated OTC columns from which good data were obtained, the curvature of log t'_R vs. chain length was negative rather than positive. However, in all runs on both OTC and SCOT columns, no cases were observed in which the elution of FAME followed a strictly linear relationship between the log t'_R and carbon chain length over 12-24 carbons.

A disadvantage of the method using quadratic equations is the requirement of three reference FAME which may not always be available for each homologous series rather than the two required for the linear equations. Additionally, considerable caution must be used when selecting reference values to determine the coefficient of the quadratic equation. It is best to select three reference FAME extending over as wide as variation of chain lengths as practical. The quadratic curve is a very shallow parabola in the region of interest; hence, t'_R that are relatively close together will not produce the correct curvature over an extended range. Concurrently, it is important also to determine t'_R accurately, to within 0.1 min or less, which means that the correct reference point, the air peak, must be selected, as well as the true peak maximum in each case.

On the other hand, a major advantage of this method is that it lends itself to many purely mathematical manipulations of the GLC data and is well suited to processing by modern digital computers (38, 39). Computer analysis can remove much of the subjectivity currently found in the identification of GLC peaks when analyzing FAME mixtures. Furthermore, since the procedure is based primarily upon continuous standardization and analysis of each sample run, changes in the elution pattern due to column aging (2) have little effect upon the results.

The aging phenomenon in polyester columns causes fairly rapid changes in the coefficient of the quadratic equation. With a typical SCOT column, the coefficients usually changed significantly within a week or ca. 50 hr of high temperature operation. The BDS columns age less rapidly than DEGS columns, and Apeizon L columns, if not operated too close to their maximum temperature limits, change very little when operated as described here. Unfortunately, the aging properties of the column cause the coefficients to change in an unpredictable manner. For this reason, each run is treated independently, provided enough reference peaks can be identified. If this is not the case, then a standardized reference mixture can be chromatographed immediately before or after the sample under identical operating conditions. The values of the coefficients for the quadratic fit then can be calculated from the reference run and applied to the sample run with confidence.

Even using this technique, the identification of chromatographic peaks by t'_R alone never can be considered absolute (4). Nevertheless, it can be expected that much of the GLC data in the literature will continue to be based primarily upon retention time criteria simply because most investigators have neither the time or an adequate amount of sample to use more definitive procedures. Thus, any method that allows more accurate identifications of GLC peaks using retention time data should be extremely useful. Furthermore, while it is particularly hazardous to use GLC retention data to identify unknown substances, this procedure can be combined with silver-nitrate impregnated TLC (40, 41) of unsaturated FAME. The combination is, then, a powerful method for rapid and accurate identification of

saturated and unsaturated FAME from a wide variety of natural samples, using only minimum amounts of material.

Finally, while the discussion in this paper does not deal with fundamental GLC theory, it can be considered consistent with general thermodynamic theories of GLC. For further exposition of this matter, the reader is referred to theoretical texts on gas chromatography, such as that by Purnell (30).

APPENDIX

The basewidth and, hence, frontal tangent intercept of an ideal GLC peak is determined by the diffusional spreading of the peak in the gas phase, which is given by the expression derived from Fick's second law as follows:

$$N = N_{max}\, e^{-\sigma_t^2/4Dt} \qquad [1]$$

where N is the peak height at time, t, and N_{max} is the peak height at 0 time, σ_t is the peak half-width at the inflection points, and D is the diffusion coefficient of the substance in the gas phase.

This is a gaussian curve, therefore:

$$\sigma_e = (\sqrt{2})\,\sigma \qquad [2]$$

where σ_e is the half-width at the inflection points when N equals N_{max}/e and σ is the standard deviation.

Thus,

$$e^{-1} = e^{-\sigma_e^2/4Dt} \qquad [3]$$

or:

$$\sigma_e^2 = 4Dt \qquad [4]$$

substituting equation 2 into equation 4:

$$\sigma^2 = 2Dt. \qquad [5]$$

For any gaussian curve the basewidth, W_b, as determined by the distance between the intercepts of the tangents drawn through the inflection points, is related to σ by the expression:

$$W_b = 4\sigma. \qquad [6]$$

Now if we let $t = t_R$, be the peak maximum retention time and t_F, the frontal tangent intercept retention time, then:

$$t_F = t_R - (1/2\, W_b) \qquad [7]$$

substituting equation 6 into equation 7:

$$t_F = t_R - 2\sigma \qquad [8]$$

and equation 5 into equation 8:

$$t_F = t_R - 2\,(2Dt_R)^{1/2} \qquad [9]$$

and if we let $2(2D)^{1/2}$ equal α:

$$t_F = t_R - \alpha\,(t_R)^{1/2}. \qquad [10]$$

Hence, the frontal tangent intercept retention time is related to the peak maximum retention time by a function containing the square root of t_R. If t_M is the elution time of the air peak and t'_R the adjusted retention time ($t_R - t_M$), then:

$$t_F = (t'_R + t_M) - \alpha\,(t'_R + t_M)^{1/2} \qquad [11]$$

and if we define a new quantity t'_F as the adjusted frontal tangent intercept retention times as $t_F - t_M$, then:

$$t'_F = t'_R - \alpha\,(t'_R + t_M)^{1/2} \qquad [12]$$

Thus, t_F and t'_F are dependent upon the peak maximum retention time and σ, or the resolving power of the column, usually expressed as HETP. Whether t_F or t'_F improve or impair the linear fit of log of retention time vs. carbon number will depend upon the actual values to t_M and α. In any case, measuring t_F or t'_F seems one step removed from the actual peak maximum adjusted retention time, t'_R, which is not dependent upon σ.

If one plots some arbitrary values of t_R, t_M, and σ, one finds that, if log t'_R vs. carbon number is actually linear, neither t_F or t'_F will be linear but that all curves approach linearity at long retention times. In the case where t'_R follows a quadratic fit, which is what was found in this work, the log t'_F is a close to linear fit in the middle and short retention times. This explains why the frontal tangent intercept in practice, has yielded better fits than the peak maximum, simply because the linear relationship is not accurate. If it were, the frontal tangent intercept should yield a poorer fit.

Thus, if one does not want to do a quadratic fit to the data and is confined to the short and middle chain FAME, the adjusted frontal tangent intercept gives a close to linear fit. However, one must remember that the fit will vary depending upon the values of t_M and α.

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Phospholipids of Two Strains of Dermatophyte, *Arthroderma uncinatum*

ZOLTAN KISH and **R. CECIL JACK**, Department of Biology, St. John's University, Jamaica, New York 11439

ABSTRACT

The phospholipid composition of a mutant strain of the dermatophyte *Arthroderma uncinatum* was compared with that of the wild type. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, diphosphatidylglycerol, phosphatidylinositol, and phosphatidic acid were present, and there was marked variation in the amounts of phosphatidylcholine, phosphatidylserine, and phosphatidic acid in the two strains. Thus, the ratio of phosphatidylcholine (wild type) to phosphatidylcholine (mutant) was 2:1; the ratio of phosphatidylserine (wild type) to phosphatidylserine (mutant) was 3:1 and the ratio of phosphatidic acid (wild type) to phosphatidic acid (mutant) was 1:5. In both strains, the predominant fatty acid was 18:2, with 54.0% in the wild type and 46.7% in the mutant. Qualitatively, the same fatty acids, with the exception of $C_{20:0}$, were found in all of the phospholipid classes; $C_{20:0}$, both in the mutant strain (5.79%) and the wild type (trace amounts), was associated with phosphatidylserine. There was a significant difference in the rates of growth of the two strains; the mutant strain grew more rapidly than, and produced three times as much mycelium as, the wild type. The mutant strain also produced larger proportions of total lipid and phospholipid than the wild type; there was 20.5% total lipid and ca. 5% phospholipid in the mutant compared with 15.5% total lipid and 2.6% phospholipid in the wild type.

TABLE I

Composition of Wild Type and Mutant *Arthroderma uncinatum*[a]

	Wild type	Mutant
Mycelium (dry wt)	731	2,312
Total lipids	113	474
Phospholipids	19	114
Pigment	17	—

[a]Values expressed are mg of component/500 ml of culture.

INTRODUCTION

Arthroderma uncinatum (1) is a dermatophyte found commonly in the soil. This work concerns two strains of the dermatophyte: the wild type and a mutant strain. The wild-type has substantial amounts of a black, crystalline pigment; the phenotypic expression of the mutant is lack of pigment.

The phospholipids of dermatophytes have been investigated only to a limited extent (2), and the phospholipids of the genus *Arthroderma* have not been investigated at all; therefore, it was considered of interest to conduct a quantitative study of the phospholipids of the two strains of *Arthroderma*. Another point of interest was the implication that phospholipids, besides being involved in membrane function, also may be involved in allergic responses and in the pathogenicities of microorganisms (3); therefore, the phospholipids of dermatophytes may be presumed to bear a fundamental relation to the structure and metabolism of these fungi and to their abilities to cause dermatomycoses. Knowledge of dermatophyte phospholipids should be helpful in acquiring an understanding of the biochemical nature of dermatomycoses.

This report on *A. uncinatum* is the first comprehensive study of the phospholipids of any dermatophyte.

MATERIALS AND METHODS

Growth of Cultures

The cultures of *A. uncinatum* were maintained on bactopeptone agar slants. The slants were prepared from 15 g agar, 30 g bactopeptone, and 1 l tap water. After 10 days of growth the cultures were kept in the refrigerator until needed for inoculation. If they were not used for inoculation, the cultures were transferred to fresh slants every 2 months.

Mycelial mats were obtained by inoculating 500 ml Sabouraud dextrose broth in 2.5 liter Fernbach flasks. The flasks were inoculated with 3 ml mycelial and spore suspension derived from one slant culture and incubated at room temperature (23-25 C) for 4 weeks after which the mycelium was harvested. The pads were washed free of residual broth with tap water, and the bulk of the water was removed

TABLE II

Characterization of Phospholipids of Wild type and Mutant *Arthroderma uncinatum*

Phospholipid	Solvent[a] I (R_fx100)	Solvent[a] II (R_fx100)	Reaction with specific reagents[b] Iodine	MB	Nin	Drag	Deacylation[c] products (R_fx100)
PA[d]	7	82	+	+	–	–	32(GP)[d]
PS	15	50	+	+	+	–	30(GPS)
PI	22	47	+	+	–	–	19(GPI)
PC	46	71	+	+	–	+	86(GPC)
PE	68	88	+	+	+	–	68(GPE)
DPG	80	99	+	+	–	–	25(dGPG)

[a]Thin layer chromatography on silica gel H+10 percent $MgSiO_3$. Solvent I, $CHCl_3$:MeOH:NH_4OH (65:35:5); Solvent II, $CHCl_3$:CH_3COCH_3:MeOH:HOAc:H_2O (10:4:2:2:1). Identities of the phospholipids were confirmed by comparison with authentic standards. The R_f's are presented to indicate the location of spots on the chromatoplates; use of the R_f's as a means of identification is not implied.

[b]Abbreviations of color reagents for thin layer chromatography. MB = molybdenum blue, Nin = ninhydrin, and Drag = Dragendorff.

[c]Paper chromatography. Solvent, water-saturated phenol:EtOH:HOAc (100:12:10).

[d]PA = phosphatidic acid, PS = phosphatidylserine, PI = phosphatidylinositol, PC = phosphatidylcholine, PE = phosphatidylethanolamine, DPG = diphosphatidylglycerol, GP = glycerophosphate, GPS = glycerylphosphorylserine, GPI = glycerlphosphorylinositol, GPC = glycerylphosphorylcholine, GPE = glycerylphosphorylethanolamine, dGPG = diglyceryl-phosphorylglycerol.

by hand pressure.

Extraction and Treatment of Lipids

Total lipids were extracted with chloroform: methanol, 2:1 (v:v) according to Folch, et al. (4), and water-soluble nonlipid substances were removed from the lipids on Sephadex G-25 (5). The purified lipids were separated into phospholipids and neutral lipids on minus 325 mesh silicic acid (Bio-Sil HA, Bio Rad Laboratories, Richmond, Calif.) according to the procedure of Morgan, et al. (6).

The phospholipids obtained by silicic acid chromatography were fractionated further into classes by diethylaminoethyl (DEAE) cellulose column chromatography (7-9).

The fractions isolated on DEAE cellulose were analyzed by thin layer chromatography (TLC) on 0.5 mm layers of Silica Gel H + 10% magnesium silicate. Slurries of adsorbent for preparing the chromatoplates were made 1:2 (w:v) in 0.01M KOH. Immediately prior to use, the plates were heat activated at 105 C for 30 min, cooled, and the samples applied along with standards in a nitrogen atmosphere. The solvent systems were those of Rouser, et al. (10). All solvents were freshly distilled.

The completed chromatograms were air-dried and spryaed with specific reagents: ninhydrin to detect amino phosphatides (11), the Dragendorf reagent for choline phosphatides, and a molybdenum blue reagent to detect compounds containing phosphate ester bonds (12). In addition, phosphorus determinations were performed on spots obtained from the thin layer chromatograms and on aliquots of fractions from the separations on DEAE cellulose columns (13).

The phospholipid fractions from the DEAE cellulose columns also were subjected to deacylation; the resulting water-soluble phosphate diesters (glycerylphosphoryl compounds) then were chromatographed by ascending paper chromatography in a phenol (saturated with water): ethanol: acetic acid (100:12:10) solvent system (14).

Analysis of Fatty Acids

The phospholipids were saponified in methanolic KOH, and the recovered free fatty acids were esterified with boron trifluoride methanol according to Morrison and Smith (15).

The fatty acid methyl esters were analyzed by gas liquid chromatography (GLC) on two columns: on a 6 ft x 1/8 in. stainless steel column containing 10% (w/w) polydiethylene glycol succinate (DEGS) on 60-80 mesh Gas-chrom P, and on a 4 ft x 1/4 in. glass column containing 2.5% SE-30 on 100-120 mesh siliconized Gas-chrom Q. The instrument was a model 5750 F and M (Hewlett-Packard, Avondale, Pa.) gas chromatograph with a hydrogen flame detector. The DEGS and SE-30 columns were operated at 180 C and 200 C, respectively. The fatty acid methyl esters were identified by comparison of their retention times with the

TABLE III

Percentages of Phospholipid Classes in Wild Type and Mutant *Arthroderma uncinatum* Determined by two Methods[a]

Phospholipids[b]	Wild type[b]		Mutant	
	TLC	DEAE	TLC	DEAE
PC	39.4 ± 0.3	41.6 ± 0.3	26.2 ± 0.7	23.4 ± 0.2
PE	22.3 ± 0.5	19.8 ± 0.3	23.8 ± 0.7	26.4 ± 0.3
PS	15.6 ± 0.4	15.6 ± 0.6	6.3 ± 0.3	5.2 ± 0.4
PI	8.1 ± 0.2		10.2 ± 0.7	
PA	4.9 ± 0.5	23.0 ± 0.5 (PI, PA, DPG)	23.0 ± 0.5	44.8 ± 0.5 (PI, PA, DPG)
DPG	9.7 ± 0.5		10.4 ± 0.5	

[a]Each value is the mean of at least three determinations, ± average deviation.

[b]PC = phosphatidylcholine, PE = phosphatidylethanolamine, PS = phosphatidylserine, PI = phosphatidylinositol, PA = phosphatidic acid, DPG = diphosphatidylglycerol, TLC = thin layer chromatography, and DEAE = diethylaminoethyl cellulose column chromatography.

retention times of methyl ester standards (Supelco Co., Bellefonte, Pa., and Applied Science Laboratory, State College, Pa.) and by cochromatography of standards and samples.

Hydrogenation of the fatty acid esters was carried out with PtO_2 under 1 atmosphere of hydrogen in absolute ethanol for 60 min (16). This was followed by GLC of the hydrogenated samples.

The peak areas were calculated by summing up the spikes corresponding to each peak on a strip chart recorder (model 7127 A, Hewlett-Packard). Relative percentages were calculated for each peak from the ratio of its area to the sum of the areas of all the peaks.

RESULTS AND DISCUSSION

The total lipids of the wild type *A. uncinatum* amounted to ca. 15.5% mycelial dry wt, the phospholipids to 2.6% and Skellysolve B extractible pigment to 2.3%. The total lipids of the mutant strain amounted to 20.5% mycelial dry wt and the phospholipids to 4.9%. There was no extractible pigment in the mutant (Table I).

The total phospholipids of both strains, as well as the individual phospholipid fractions obtained by DEAE cellulose column chromatography, gave six major components on TLC. Table II shows that these major phospholipids were identified as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidic acid, phosphatidylinositol, and diphosphatidylglycerol in both strains. All of the criteria of identification that were applied to the phospholipid samples and corresponding standards, i.e. TLC in different solvents, reactions to specific color reagents, and paper chromatography of water-soluble phosphate diesters, were consistent with this identification (Table II).

Table III shows the amounts of each class of phospholipid quantitated in two ways: (A) by determining the phosphorus content of equal aliquots of the fractions from DEAE cellulose chromatography and (B) by determining the phosphorus content of spots resulting from TLC of total phospholipid. There was close correlation between the two methods both for the wild type and mutant strain. For example, fraction 9 recovered from DEAE cellulose chromatography contained three phospholipids: phosphatidylinositol, phosphatidic acid, and diphosphatidylglycerol; the sum of the percentages of the same three phospholipids, obtained individually by analysis of spots from thin layer plates, i.e. 22.7% wild type; 43.6% mutant, agreed with the value obtained by analyzing an aliquot of fraction 9 from DEAE cellulose (23.0% wild type; 44.8% mutant).

Tables IV and V summarize the fatty acid composition of the phospholipids of the wild type and mutant. The last two columns of Tables IV and V show that there was good agreement between the sum of fatty acids of individual phospholipid classes and the fatty acid profile of the total phospholipids. Qualitatively, the same fatty acids were found in all classes of the phospholipids, except phosphatidylserine. Nearly the entire complement of $C_{20:0}$ of the mutant strain (5.79%) was found in phosphatidylserine. $C_{20:0}$ which appeared in trace quantities in the fatty acids of the total phospholipids of the wild type also was found only in phosphatidylserine of the wild type. It seems, therefore, that $C_{20:0}$ was associated with phosphatidylserine. In both strains, the predominant fatty acid was 18:2 with 54.0% in the wild type and 46.7% in the mutant. Both strains also contained two, small unidentified peaks (UNKN 1 and 2, Tables IV and V). These

TABLE IV

Fatty Acid Composition of Phospholipids from the Wild type of *Arthroderma uncinatum*

Fatty acid methyl esters	Percentages of methyl esters in each fraction[a]				Sum of fractions[b]	Total PL[c]
	PC	PE	PS	PA, PI, DPG		
14:0	tr[a]	tr	tr	tr	tr	tr
15:0	2.0	3.2	2.4	2.6	2.6	2.4
16:0	16.8	12.5	19.7	14.9	16.0	17.1
16:1	1.5	1.7	1.6	tr	1.2	tr
17:0	0.8	2.5	2.9	1.4	1.9	1.4
17:1	0.6	tr	tr	tr	tr	tr
18:0	1.0	3.2	3.6	2.6	2.6	1.7
18:1	15.3	14.2	12.7	11.4	13.4	13.0
18:2	58.6	57.8	51.9	60.0	57.1	54.0
20:0	—	—	tr	—	tr	tr
UNKN[d] 1	0.8	1.7	1.8	1.4	1.4	1.4
UNKN 2	2.6	3.2	3.3	6.0	3.8	8.4

[a]Fractions from DEAE cellulose chromatography. PC = phosphatidylcholine, PE = phosphatidylethanolamine, PS = phosphatidylserine, PA = phosphatidic acid, PI = phosphatidylinositol, DPG = diphosphatidylglycerol, and Tr = trace.

[b]Sums of fatty acids of individual phospholipid fractions divided by the numbers of fractions containing at least a trace (Tr, less than 0.5%) of the given fatty acid.

[c]PL = phospholipid. This column gives fatty acid composition of the total phospholipids.

[d]UNKN 1 and 2: Unknown. May have been unusual C18 unsaturates since: %C18:0 after reduction of fatty acids of total PL equaled %(18:0 + 18:1 + 18:2 + UNKN 1 + UNKN 2) before reduction.

peaks may have been unusual C18 unsaturated fatty acids, since the percentage of C18:0 after reduction of fatty acid methyl esters from the total phospholipid equaled the sum of the percentages of (18:0+ 18:1 + 18:2 + UNKN 1 + 2) before reduction of the fatty acid methyl esters.

The previous work on the phospholipids of dermatophytes may be summarized as follows: Ghoshal (4) found 41% phosphatidylcholine and some sphingomyelin in *Tricophyton rubrum*, but the presence of other phospholipids could not be confirmed. *Microsporum cookei* (17) contained phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, and phosphatidylinositol. Except for phosphatidylcholine (4), quantitation was not reported in any of these previous studies.

In the present study, quantitative differences were found among six phospholipids that were identified in two strains of *A. uncinatum* (Table II). Quantitative differences in growth rate (amounts of mycelium) also were observed (Table I). The differences and similarities in the

TABLE V

Fatty Acid Composition of Phospholipids from the Mutant Strain of *Arthroderma uncinatum*

Fatty acid methyl esters	Percentages of methyl esters in each fraction[a]				Sum of fractions	Total PL[c]
	PC	PE	PS	PA, PI, DPG		
14:0	tr	tr	tr	tr	tr	tr
15:0	2.9	1.6	3.1	2.9	2.6	1.9
16:0	18.2	11.9	17.5	14.1	15.4	15.1
16:1	tr	tr	tr	tr	tr	tr
17:0	tr	tr	2.4	1.9	1.1	1.9
17:1	tr	tr	tr	tr	tr	tr
18:0	1.9	2.6	3.3	4.9	3.2	2.7
18:1	16.6	13.4	11.4	12.9	13.6	13.2
18:2	52.8	55.2	45.5	54.6	52.0	46.7
20:0	tr	—	5.8	—	2.9	4.6
UNKN 1	2.6	5.2	2.9	tr	2.7	3.9
UNKN 2	5.2	10.2	8.2	8.6	8.1	9.9

[a]See footnotes to Table IV.

two strains were as follows. (A) The amount of phosphatidylcholine (wild type) to phosphatidylcholine (mutant) was 2:1, of phosphatidylserine (wild type) to phosphatidylserine (mutant) 3:1, and of phosphatidic acid (wild type) to phosphatidic acid (mutant) 1:5. (B) The two strains contained similar proportions of phosphatidylethanolamine, phosphatidylinositol, and diphosphatidylglycerol. (C) $C_{18:2}$ was the predominant fatty acid in the two strains but there was slightly more of this fatty acid in the wild type. (D) Unknown fatty acids were present in relatively small proportions in both strains, but the mutant contained larger proportions of the unknowns than the wild type. (E) The wild type contained smaller percentages of total phospholipid and yielded ca. two-thirds less mycelium (had a much lower rate of growth) than the mutant; the basis of such a marked difference in growth is of interest but not yet understood. On the other hand, a tentative interpretation can be made of the data as a whole. The interpretation is that elucidating the role of phospholipids in the two dermatophytes requires an understanding of the need for specific phosphatides, as well as an understanding of the physiological effects of particular proportions of phosphatides on the properties of the organisms.

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Fatty Acid Desaturation by Mammary Gland Microsomes from Lactating Mice

G. ANANDA RAO and **S. ABRAHAM**, Veterans Administration Hospital, Martinez, California 94553 and Bruce Lyon Memorial Research Laboratory, Children's Hospital Medical Center of Northern California, Oakland, California 94609

ABSTRACT

Microsomal fractions obtained from lactating mouse mammary glands catalyze the desaturation of myristic, palmitic, and stearic acids in the presence of adenosine 5'-triphosphate, coenzyme A, and nicotinamide adenine dinucleotide, reduced form. The products of this reaction are the corresponding monoenoic fatty acids, myristoleic, palmitoleic, and oleic acids respectively. Desaturase activity was greater with stearate as substrate than with either palmitate or myristate. Palmitate was desaturated at a rate ca. 50% greater than that of myristate. Although both nicotinamide adenine dinucleotide, reduced form and nicotinamide adenine dinucleotide phosphate, reduced form served as electron donors, the former was superior in this regard. Desaturase activity was not influenced by the addition of glycerol 3-phosphate to the incubation medium. However, the presence of this compound did affect the type of lipid formed. In the absence of the added glyceride-glycerol precursor, the major products were phospholipids, whereas in its presence they were triglycerides.

INTRODUCTION

Stearoyl-CoA desaturase activity has been observed in the microsomal fractions obtained from the mammary glands of lactating cows (1,2), goats (3), and sows (3), although activity was not observed when these fractions were isolated from lactating rat (2) or rabbit (3) mammary glands. During our investigation of the utilization of various fatty acids by mammary gland slices from lactating mice, we detected appreciable amounts of radioactive palmitoleate and oleate in the triglycerides synthesized from 1-^{14}C-palmitate and 1-^{14}C-stearate (4). These observations suggested the presence of desaturase activity in this tissue. Here, we report studies with microsomal fractions isolated from lactating mouse mammary glands which demonstrate the presence of an active desaturase system in this tissue.

EXPERIMENTAL PROCEDURES

Mammary glands from mice which were lactating actively for 17 days were excised, sliced, washed, and homogenized in 5 volumes of 0.25 M sucrose with a Potter-Elvehjem homogenizer at 2 C as described previously (5). All subsequent preparative procedures were carried out at 2 C. The homogenate was centrifuged at 1,000 x g for 15 min to separate free floating fat and cell debris. The resulting supernatant fraction below the fat layer was centrifuged at 15,000 x g for 20 min, and pellet was discarded. The microsomal fraction was obtained as a pellet by additional centrifugation at 100,000 x g for 1 hr. The pellet was both washed and suspended in 0.154 M KCl. This suspension (5 mg protein/ml) was used as the enzyme source.

All experiments were carried out with the potassium salts of either 1-^{14}C-myristate, -palmitate, or -stearate. The incubation conditions were essentially those described by Raju and Reiser (6). Qualitative and quantitative determinations of the production of monoenoic fatty acids were carried out using gas liquid chromatography (7) and $AgNO_3$-impregnated silica gel thin layer chromatography (6). Protein was measured by the procedure of Lowry, et al. (8).

RESULTS AND DISCUSSION

Microsomes obtained from lactating mouse

TABLE I

Effect of Various Protein Concentrations upon Desaturase Activity of Mammary Gland Microsomes from Lactating Mice[a]

Microsomal protein mg	Myristate	Palmitate	Stearate
	n moles acid desaturated/5 min		
1	4.5	7.2	10.6
1.2	6.3	9.4	12.8
1.5	7.8	10.2	19.6

[a]Incubation medium contained potassium salts (0.2 mM) of either 1-^{14}C-myristic, 1-^{14}C-palmitic, or 1-^{14}C-stearic acids, coenzyme A (0.2 mM), adenosine 5'-tri-phosphate (5 mM), reduced glutathione (GSH, 10 mM), $MgCl_2$ (10 mM), nicotinamide adenine dinucleotide, reduced form (2 mM), DL-glycerol 3-phosphate (10 mM), and potassium phosphate buffer (pH 7.4, 0.1 M). Microsomal protein was added in the indicated amounts in a total volume of 1 ml. After 5 min incubation at 37 C with air as gas phase, the lipids were isolated and monoenoic fatty acid production was quantitated (6,7).

TABLE II

Specific Activity of Desaturase with Different Fatty Acids[a]

Additions	Myristate	Palmitate	Stearate
	n moles acid desaturated/min/mg protein		
GP, NADH	1.05	1.46	2.37
GP, NADPH	0.88	0.91	1.32
NADH	1.13	1.53	1.89
None	0.15	0.15	0.16

[a]Carried out in the presence of potassium salts of fatty acids, coenzyme A, adenosine 5'-triphosphate, reduced form of glutathione, $MgCl_2$, microsomal protein (1 mg) and potassium phosphate buffer as described in Table I. DL-glycerol 3-phosphate (GP, 10 mM) and nicotinamide adenine dinucleotide (phosphate) reduced form (2 mM) were added as indicated. Mean values from three different experiments are given.

mammary glands desaturated myristate, palmitate, and stearate in the presence of adenosine 5'-triphosphate (ATP), coenzyme A (CoASH), and nicotinamide adenine dinucleotide, reduced form (NADH) (Table I). The extent of desaturation was dependent upon concentration of microsomal protein. In the absence of added ATP and CoASH in the reaction medium, fatty acids were not desaturated. Desaturase activity proceeded linearly with time during a 10 min incubation period.

The specific activity of stearoyl-CoA desaturase (2.4 n moles/min/mg microsomal protein) with mammary gland microsomes from lactating mice (Table II) was similar to that reported for the liver enzyme of mice fed a stock diet when stearate was the substrate (9) and 5-6 times that reported for microsomes from lactating cows when stearoyl-CoA served as substrate (2). It is possible that the low activity observed for the cow mammary desaturase is due to the nature of the substrate used in the assay and the low activity of the original tissue.

Stearate was desaturated more rapidly than palmitate or myristate (Table II). The rate of palmitoleate production was ca. 50% greater than that of myristoleate. Although both reduced pyridine nucleotides served as electron donors for acyl desaturation, NADH was more effective than NADPH. Similar nucleotide specificity has been observed for the desaturase system in rat liver (10,11).

Both inhibitory and stimulatory effects of glycerol 3-phosphate upon the rate of stearoyl-CoA desaturation have been observed. Whereas Brenner and Peluffo (12) have reported that the desaturase activity in rat liver microsomes was not stimulated by glycerol 3-phosphate, Raju and Reiser (6) have shown that the presence of this glyceride-glycerol precursor will double the rate of desaturase activity. In addition, Bickerstaffe and Annison (3) reported that glycerol 3-phosphate stimulated the desaturase activity of mammary gland microsomes of lactating goat as much as threefold. However, McDonald and Kinsella (2) have observed that when the concentration of added glycerol 3-phosphate in the medium varied 0.2 mM-3.6 mM, there was a gradual decrease in desaturase activity in the lactating cow mammary gland microsome system with stearoyl-CoA as substrate. In our experiments with the lactating mouse mammary gland system with only one concentration of glycerol 3-phosphate (5 mM), we did not observe a stimulation of the conversion of myristate to myristoleate or palmitate to palmitoleate and detected only a small stimulation of the desaturation of stearate (Table II).

The microsomal fraction isolated from lactating mouse mammary gland produces complex lipids from added fatty acids and glycerol 3-phosphate (Table III). In the presence of the glyceride-glycerol precursor, almost all (94%) of the added myristate or palmitate was esterified. On the other hand, stearate was esterified (26%) to a more limited extent. In addition, ca. half of the esterified myristate and palmitate was found in the triglyceride fraction and ca. 18% in 1,2-diglycerides. Thus, the esterification system in the mouse mammary glands appears to be somewhat different from that described for the glands from lactating cows (2). In the latter tissue, the microsomal system in the presence of glycerol-3-phosphate incorporates a relatively larger amount of fatty acid into phospholipids than in its absence.

Kinsella (2,14) did not detect desaturase activity in the mammary glands of lactating rats, although such activity was shown in rat liver (2). However, one cannot infer that all lactating rodent mammary glands are devoid of desaturase activity, for, as shown here, the glands of lactating mice show a level of activity which is equal to that found in the livers of normal nonlactating mice (9).

TABLE III

Esterification of Fatty Acids by Mammary Gland Microsomes from Lactating Mice[a]

Fatty acid	GP	PL	MG	DG	TG	Fatty acid esterified
			n moles			
Myristate	–	30.8	3.9	8.5	4.5	47.7
Myristate	+	53.8	2.5	36.8	94.6	187.7
Palmitate	–	8	0.8	2.7	1.3	12.8
Palmitate	+	54.3	1.3	34.7	97.8	188.1
Stearate	–	13	0.7	3.9	1.3	18.9
Stearate	+	27	2.4	7.9	15	52.3

[a]Incubations were carried out as described in Table I with 1 mg microsomal protein in both the absence (–) or presence (+) of DL-glycerol-3-phosphate (GP) as indicated. Total lipids were isolated and the phospholipid (PL), monoglyceride (MG), 1,2-diglyceride (DG), and triglyceride (TG) fractions were separated by thin layer chromatography (13). Further analysis (13) revealed that in the presence of GP, 50-60% radioactivity of fatty acids in the phospholipid fraction were in the form of phosphatidic acid, whereas in the absence of GP, the ^{14}C-fatty acids in this fraction were found in phosphatidyl choline (85%) and phosphatidyl ethanolamine (10%).

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Sequential Gas Chromatographic Procedure for Microanalysis of Monoenoic Double Bond Position in Hydrogenated Oils[1]

E.A. EMKEN and H.J. DUTTON, Northern Regional Research Laboratory,[2] Peoria, Illinois 61604

ABSTRACT

Quantitative cleavage of epoxyoctadecanoates with periodic acid (HIO_4) has been demonstrated and the technique incorporated into an all gas chromatographic system for detailed lipid analysis. The overall procedure involves three sequential gas chromatographic separations interspersed by two microreactions. By this procedure, a complete analysis for *cis*- and *trans*-geometric isomers corresponding to each positional octadecenoate isomer is obtained. Total sample requirements are less than 10 mg, and the elapsed analysis time/sample is less than 10 hr. In this all gas chromatographic procedure, a lipid methyl ester sample is first separated by preparative gas chromatography, and the monoene fraction is collected and epoxidized. Next, the epoxidized sample is separated by gas chromatography into *cis*- and *trans*-epoxyoctadecanoate fractions. These epoxyoctadecanoate fractions are collected and cleaved with HIO_4 into aldehyde and aldehyde-ester fragments, which are quantitatively analyzed by gas chromatography. The double bond positions are determined from the aldehyde and aldehyde-ester cleavage data, which are stored and processed by a computerized on-line gas chromatographic data acquisition system. The procedure was tested on pure octadecenoate isomers, standard mixtures, and commercially hydrogenated vegetable oils. Analyses of hydrogenated vegetable oils are compared with data acquired by reverse-phase and argentation chromatography followed by reductive ozonolysis.

INTRODUCTION

Location of fatty acid double bond position is an important part in a wide variety of lipid researches. Double bond location is used widely in the analysis of hydrogenated vegetable oils, characterization of lipid extracts from biological sources, and for proving isotope and chemical purity of synthetic fatty acids. Lemieux and von Rudloff's original permanganate-periodate (1) oxidative cleavage method for double bond location was used for many years by lipid chemists (2,3) but has given way to ozonolysis procedures. Nickell and Privett used ozonolysis-pyrolysis to cleave octadecenoate standards (4) and Davison and Dutton (5) first applied ozonolysis-pyrolysis to isomeric mixtures of octadecenoates isolated from hydrogenated methyl linolenate. Stein and Nicolaides (6) demonstrated the reduction of ozonides to aldehydes with triphenylphosphine. Currently, ozonolysis followed by reduction with triphenylphosphine is used by many lipid laboratories for double bond location (7-9).

Recently, we described an epoxidation-gas chromatography (GC) technique for *cis*- and *trans*-analysis of octadecenoate isomers via the *cis*- and *trans*-epoxyoctadecenoate derivatives (10). We now are reporting upon the combination of this epoxidation-GC technique with a HIO_4 cleavage procedure (11,12) to provide for the first time an all GC approach for obtaining quantitative data on double bond location in *cis*- and *trans*-octadecenoate fractions from hydrogenated fats and oils. The combined epoxidation-HIO_4 procedure uses HIO_4 to cleave the epoxidized octadecenoate isomers into aldehyde and aldehyde-ester fragments. Subsequent analysis of these fragments allows the position of the oxirane ring to be determined.

Starting with the fatty acid methyl esters, the overall procedure involves three sequential GC separations and two microreactions. In this procedure, quantitative data are obtained for fatty acid composition, *cis*- and *trans*-configuration and double bond distribution.

The procedure has been demonstrated with pure octadecenoate isomers, octadecenoate mixtures of known composition, and commercial partially hydrogenated vegetable oil. The double bond distribution data are compared with data obtained by reductive ozonolysis.

EXPERIMENTAL PROCEDURES

Standards

Samples of methyl *cis*-6-octadecenoate were purchased from Hormel Institute; methyl *cis*-

[1]Presented at the AOCS Meeting, April 1973, New Orleans.

[2]ARS, USDA.

15-octadecenoate (13) and methyl *trans*-9-octadecenoate (14) were prepared by previously published procedures. A mixture containing methyl *cis*-9-octadecenoate, methyl *cis*-12-octadecenoate and methyl *cis*-15-octadecenoate was prepared by hydrazine reduction of linolenic acid and analyzed by capillary GC (13,15).

Commercially Hydrogenated Vegetable Oil

A commercially available salad oil and shortening manufactured from partially hydrogenated vegetable oil were previously studied as samples A and E (16). Methyl esters of these samples were prepared by transesterification with methanol containing sodium methoxide as a catalyst.

Periodic Acid (HIO_4)

Periodic acid ($HIO_4 \cdot 2H_2O$) assayed as 99.5% pure from Matheson Coleman & Bell, Elk Grove Village, Ill., was used in all of the experiments. The HIO_4 crystals were ground to ca. a 60 mesh powder for use in the HIO_4-Et_2O cleavage experiments. Since the powder is hygroscopic and photo unstable, exposure to atmospheric moisture and light must be kept at a minimum.

Diethyl Ether Purification

Commercial anhydrous reagent grade diethyl ether was found to require purification to prevent extraneous compounds from appearing during the HIO_4 cleavage reaction. The purification procedure consisted of shaking 200 ml ether with 10 g activated alumina (grade II) for 5 min, allowing the suspension to settle for 30 min and then decanting off the ether. The ether then was treated with a second portion of activated alumina and allowed to stand overnight at 3 C. The ether then was decanted off and treated with 5 g chromatographic grade silica gel and stored at 3 C until needed.

Periodic Acid-Methylene Chloride (HIO_4-CH_2Cl_2)

The HIO_4 used in the HIO_4-CH_2Cl_2 cleavage procedure was first dried for 16 hr at 100 C. The dried HIO_4 crystals were slightly yellow and easily ground to a 80-120 mesh powder. The methylene chloride was American Chemical Society reagent grade and did not require purification.

Periodic Acid-Diethyl Ether Reagent (HIO_4-Et_2O)

HIO_4-Et_2O reagent was used to cleave the epoxides directly. The HIO_4-Et_2O reagent was prepared by adding 2-3 ml purified ether to ca. 200 mg powdered HIO_4 and then shaking the mixture for 2-3 min to saturate the ether with HIO_4. The HIO_4-Et_2O mixture initially became cloudy and then cleared on standing at room temperature. The HIO_4-Et_2O reagent is stable for 24 hr, but best results were obtained when it was freshly prepared before each use.

Preparative GC

An octadecenoate fraction from commercially hydrogenated vegetable oil methyl esters was collected from an Aerograph Autoprep (model 600P), fitted with an aluminum 10 ft x 3/8 in. 10% EGSS-X column. The octadecenoate fraction was collected in glass tubes loosely packed with a 2 in. segment of glass wool. A single injection of 4-5 μliter sample generally provided enough octadecenoate (ca. 1.5 mg) for subsequent analyses.

Epoxidation

The collected octadecenoate sample was washed into a 2.0 ml vial with hexane, the solvent evaporated, and the residue (ca. 1.5 mg) epoxidized as previously described, using 100 μliter peracetic acid (10). The epoxidized octadecenoate mixture was diluted with 0.5 ml water and extracted twice with 0.2 ml portions of hexane. Combined hexane extracts were washed with an equal volume of water; the hexane layer was drawn off with a pipette, evaporated to dryness, and redissolved in 20 μliter hexane.

GC Separation and Collection of Epoxides

The *cis*- and *trans*-epoxyoctadecenoate isomers were separated and measured on an EGSS-X column as described previously (10), except that the Packard GC (model 6000) was modified with a 10:1 sample splitter at the exit of the column to allow collection of samples, since a flame ionization detector (FID) was used. The splitter assembly receiving the 10 parts of sample was heated to 230 C. Samples (0.2-0.3 μliter) of epoxidized octadecenoates dissolved in hexane were injected and the *cis*- and *trans*-epoxyoctadecanoate fractions collected in 3 in. segments of no. 17 gauge Teflon tubing attached to the sample splitter.

Epoxide Cleavage (HIO_4-Et_2O) (12)

Conical shaped vials were prepared by drawing out the bottoms of standard 0.5-dr screw-capped vials to 1-2 mm diameter. The collected epoxide fractions were washed from the Teflon tubing into these vials with 100 μliter hexane. The hexane was evaporated under a stream of nitrogen and the epoxides mixed with 50 μliter HIO_4-Et_2O reagent. The solution was allowed to stand at room temper-

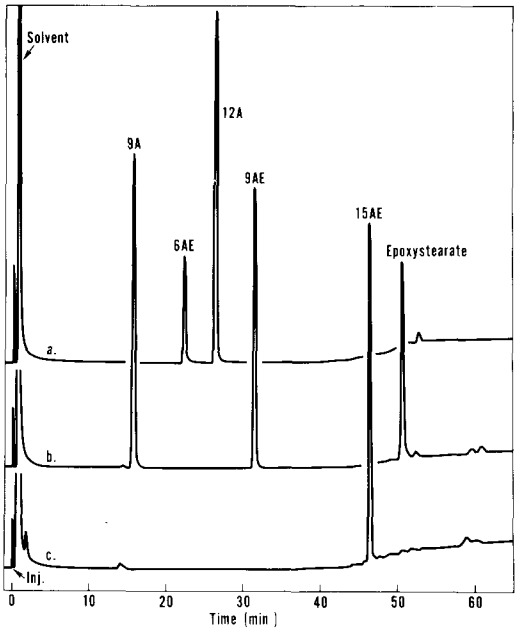

FIG. 1. Gas chromatography (GC) of HIO_4 cleaved standards (a) *cis*-6,7-epoxyoctadecanoate; (b) *trans*-9,10-epoxyoctadecanoate; (c) *cis*-15,16-epoxyoctadecanoate. GC conditions: temperature programed from 50-240 C at 4 C/min and held for 30 min; 180 cm x 4 mm glass column packed with 50:50 mixture of OV 17-225.

ature for 45 min during which time it became cloudy and a white iodic acid precipitate was deposited. A 5 μliter portion of sodium bicarbonate saturated water then was mixed with the reaction solution and allowed to stand for 10 min at room temperature. The aldehyde-aldehyde ester cleavage products then were analyzed by GC as described below.

Epoxide Cleavage (HIO_4-CH_2Cl_2) (11)

Redistilled hexane was used to wash the epoxide from the Teflon collection tubes into conical vials and the solvent evaporated. A 100 μliter portion of methylene chloride was added to the sample along with 2-3 mg dried HIO_4 powder. The reagents and sample were mixed well and allowed to stand at room temperature for 30 min. Mixing is important, since the oxidation occurs heterogeneously on the surface of the powdered HIO_4. Ca. 3 μliter portions of this reaction mixture were analyzed by GC.

GC Analysis of HIO_4 Cleaved Epoxyoctadecanoates

A Packard GC (model 6000) was used to analyze 2-4 μliter portions of the HIO_4 cleaved epoxyoctadecanoate mixtures. The GC was equipped with a 180 cm x 4 mm glass column

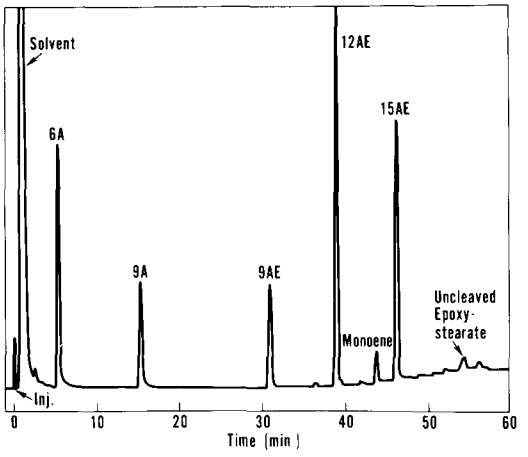

FIG. 2. Gas chromatography (GC) of HIO_4 cleaved epoxidized 9-, 12-, and 15-octadecenoate mixture. GC conditions are the same as in Figure 1.

and FID. The aldehyde and aldehyde-ester cleavage products were separated on a column packed with a 50:50 mixture of OV 17 and OV 225 on 100-120 mesh Chromosorb WHP (8). The GC was temperature programed from 50-240 C at 4 C/min and at a helium flow rate of 30 ml/min.

The programing conditions did not produce extensive baseline drift at moderate detector sensitivities. Standard dual column techniques can be used to compensate for baseline drift, if column bleed becomes a problem when high sensitivities are necessary.

Computerized Data Processing

An IBM 1800 computer was interfaced with the gas chromatographs to provide real time on-line data acquisition. The GC data were integrated and processed by typical GC computer techniques using a modified McCullough IBM 1800 GC monitoring program (17).

RESULTS

All the separations and quantitative analyses used in this all GC procedure are performed by GC techniques. The necessary derivatives are prepared quickly and easily. Total sample requirements ranged from ca. 10-20 mg. The only step in the procedure which had not previously proven to be a quantitative technique was the HIO_4 cleavage of the epoxyoctadecanoate isomers. Known epoxyoctadecanoate isomers were used to establish this point.

Quantitation with Standards

Cleavage of collected *cis*- and *trans*-epoxyoctadecanoate isomers to aldehyde and alde-

TABLE I

Periodic Acid Cleavage of Epoxyoctadecanoate Standards

Epoxyoctadecanoate	Cleavage fragment[b]	Percent[a] aldehyde	Percent aldehyde-[a] ester
cis-6,7-	12A	53.4	
	6AE		46.6
trans-9,10-	9A	53.3	
	9AE		46.7
cis-9,10-	9A	52.7	
	9AE		47.3
cis-12,13-	6A	54.6	
	12AE		45.4

[a]Percentages calculated from peak areas corrected for number of ionizable carbons.
[b]A = aldehyde and AE = aldehyde-ester

hyde-esters was carried out on several known standards. The GC curves in Figure 1 are representative of HIO_4 cleaved epoxidized octadecenoate standards. Both the HIO_4-Et_2O and HIO_4-CH_2Cl_2 reagents were used to cleave the epoxyoctadecanoates and found to produce good results. However, the HIO_4-CH_2Cl_2 reagent was preferred because it was easier to use. The HIO_4-Et_2O procedure (12) as modified and described in this article also produced lower yields of cleavage products and more extraneous peaks than the HIO_4-CH_2Cl_2 procedure (11). Furthermore, the HIO_4-Et_2O procedure tended to overoxidize the aldehydes slightly to the corresponding acids. These acids were removed by sodium bicarbonate treatment. The HIO_4-CH_2Cl_2 cleaved epoxides did not require this kind of treatment. Occasionally the HIO_4-Et_2O method produced an extraneous peak which had a retention time slightly less than the nonaldehyde peak. The problem of extraneous peaks was eliminated effectively by careful purification of the ether.

The GC analysis of a HIO_4-CH_2Cl_2 cleaved *cis*-epoxyoctadecanoate mixture containing known amounts of 9, 12, and 15 positional octadecenoate isomers (as determined by capillary GC) is shown in Figure 2. The GC data from a HIO_4 cleaved sample are first computer integrated and the peak areas corrected for the number of ionizable carbons. Percentages are calculated for each peak and the data printed out. The aldehyde-ester percentages are directly related to bond positions in the sample. The percentages listed in Table III and Table IV for commercial vegetable shortening and salad oil were calculated by this method and provide a major savings in time required for these calculations.

The GC curves in Figures 1 and 2 contain a small peak which has the same retention time as epoxyoctadecanoate. The size of this peak diminishes with time as the HIO_4 cleavage reaction proceeds.

Quantitation of the acetaldehyde and propanal peaks are difficult because of interference by the solvent peaks. The data in Table I summarize the corrected peak areas (7,9,18,19) obtained from the *cis*- and *trans*-epoxyoctadecenoate standards shown in Figures 1 and 2.

The HIO_4 cleavage data are compared to similar data obtained by reductive ozonolysis. Peak areas were integrated by using a computerized on-line data acquisition system and then converting the peak areas to area/ionizable carbon (7,9,18). Correction of peak areas is required because the FID does not respond equally to equimolar amounts of aldehyde and aldehyde-esters of varying chain lengths or mol wt. The correction is obtained by simply programing the computer to divide the total peak area by the appropriate number of ion-

TABLE II

Comparison of Methods for Double Bond Location in Octadecenoate Mixture

Octadecenoate	Ozonolysis,[a] %	Periodic acid,[a] %	Capillary GC, %
cis-9-	18.9	18.9	18.5
cis-12-	54.1	55.0	53.5
cis-15-	27.0	26.2	28.0

[a]Percentages calculated from peak areas corrected for number of ionizable carbons.

TABLE III

Double Bond Distribution in Commercial Shortening

Double bond position	Octadecenoate fraction						
	trans-(HIO_4)[a]	*cis*-(HIO_4)[a]	Total (HIO_4)[a]	Total (Cal)[b]	Total (O_3)[c]	*trans*-(O_3)[d]	*cis*-(O_3)[d]
7	1.5	—	0.9	0.6	1.0	3.5	—
8	6.6	2.4	5.5	4.2	4.9	8.5	3.5
9	21.5	67.0	47.4	47.9	49.1	20.0	74.0
10	26.0	5.9	14.4	14.3	14.0	30.0	3.5
11	22.7	8.0	14.5	14.2	14.2	22.5	6.0
12	10.5	13.1	11.8	12.0	12.4	8.5	10.0
13	4.3	1.7	3.3	2.8	2.6	4.5	3.0
14	2.2	1.8	1.7	2.0	1.1	2.0	—
15	3.8	—	0.5	1.6	0.7	—	—

[a]Octadecenoate fraction was epoxidized and then cleaved with HIO_4.

[b]Percentages calculated from *trans*-content (41.9%) of total octadecenoate fraction and percentage of double bond in each position as determined by HIO_4 cleavage.

[c]Total octadecenoate fraction was cleaved by reductive ozonolysis.

[d]These results were estimated from the bar graphs in reference 16.

izable carbons in the compound. This correction is only a first approximation (7,8,17,18), but it does provide reasonable areas for a homologous series of compounds. The usual discrepancies which have been observed previously for aldehyde and aldehyde ester data were found.

Table II compares the epoxidation-HIO_4 cleavage method with capillary GC analysis and reductive ozonolysis. The agreement between capillary GC analysis, reductive ozonolysis, and HIO_4 cleavage data in Table II is good. The mixture used in Table II was chosen because it can be separated by capillary GC which provides a good check on the other two procedures which are both cleavage methods.

Figures 1 and 2 and Tables I and II establish the quantitative accuracy of using HIO_4 cleavage of epoxyoctadecanoates as a means for determining double bond distribution in octadecenoate samples.

Commercially Hydrogenated Samples

The entire sequential procedure used in the all GC procedure was tested by determining the double bond distribution in *cis*- and *trans*-octadecenoate fractions isolated from commercially available salad oil and vegetable shortening. Results obtained by this procedure were compared with data compiled by a liquid chromatographic procedure (16). The all GC procedure used preparative GC to obtain a pure octadecenoate (monoene) fraction from the transesterified triglycerides. Small amounts (2-3%) of methyl stearate and methyl linoleate impurities occasionally were present in the monoene fraction, but these impurities do not interfere with subsequent reactions or separation, since they are separated readily after the sample has been epoxidized. The sample size required for the preparative GC step depends, of course, upon the octadecenoate content of the sample. Collection of 2-3 mg octadecenoate is usually more than sufficient sample for epoxidation and further analyses.

The data in Figure 3 and Table III were obtained from the analysis of a partially hydrogenated vegetable shortening. The GC curves in Figure 3 were obtained by HIO_4-Et_2O cleavage of epoxyoctadecanoates collected from a Packard GC equipped with a 10:1 splitter. Ca. 0.3-0.4 mg epoxyoctadecanoate is the maximum sample size which can be separated satisfactorily with a 4 mm x 300 cm 10% EGSS-X column. Larger samples cannot be separated completely, because they overload the column. Collection of *cis*- and *trans*-epoxyoctadecanoate fractions from one or two 0.3 mg injections easily provides at least 30-50 μg of each geometrical isomer needed for HIO_4 cleavage. Cleavage of these fractions yield enough fragmentation product for several subsequent GC analyses.

The data in Table IV are from a commercially available vegetable oil prepared by partial selective hydrogenation and winterization of soybean oil. The sample was analyzed by the all GC procedure. The GC analysis of the *trans*-fraction in Figure 3 shows the applicability of the method to samples containing a wide distribution of positional isomers. The peak following the methyl 16-formylhexadecanoate ($C_{16}AE$) peak is uncleaved epoxyoctadecanoate and will overlap partially the methyl 17-formylheptadecanoate peak.

The data in Table IV illustrate the analyses

TABLE IV

Double Bond Distribution in Commercial Salad Oil

Double bond position	Octadecenoate fraction					
	trans-(HIO_4)[a]	*cis*-(HIO_4)[a]	Total (HIO_4)[a]	Total (Cal)[b]	*trans*-(O_3)[c]	*cis*-(O_3)[c]
7	3.6	—	1.4	0.8	3.0	—
8	9.2	0.3	2.1	2.3	8.0	—
9	14.5	73.1	58.2	59.5	13.0	83.0
10	27.8	2.2	6.3	8.1	32.0	—
11	24.0	8.4	13.7	12.0	26.5	4.5
12	9.5	15.7	15.6	14.3	7.5	12.5
13	7.6	0.2	1.7	1.9	5.5	—
14	2.8	—	1.1	0.7	2.0	—
15	1.0	—	—	0.2	—	—

[a]Octadecenoate fraction was epoxidized and then cleaved with HIO_4.

[b]Percentages calculated from *trans*-content (23.1%) of total octadecenoate fraction and percentage of double bond in each position as determined by HIO_4 cleavage.

[c]These results were estimated from the bar graphs of reference 16.

obtained for total epoxidized monoenes from salad oil by HIO_4 cleavage. The HIO_4 cleavage of the separated *cis*- and *trans*-epoxide fractions also are shown in Table IV to illustrate the differences in double bond distribution in these fractions.

DISCUSSION

The HIO_4 cleavage of epoxidized octadecenoate isomers produced quantitative data which are as accurate as reductive ozonolysis data for determining double bond distribution in monoene fractions from partially hydrogenated vegetable oils. Ozonolysis is still the method of choice if only total double bond distribution of the monoene fraction is required. When double bond distribution data are needed for individual *cis*- and *trans*-monoene fractions (20), the epoxidation-HIO_4 cleavage procedure should be considered since the all GC procedure requires less total work and time. A comparison of the all GC procedure with an earlier method (16), both of which yield complete analytical information on geometric and positional octadecenoate isomers, is in order. Starting with the same methyl esters from hydrogenated samples, the earlier method (16) employed a rubber reverse-phase chromatographic column to separate the C_{18} isologues, i.e. octadecanoate, octadecenoates, octadecadienoates, and octadecatrienoates. Subsequently, a silver macroreticular ion exchange resin column was used to separate the *cis*-octadecenoates from *trans*-octadecenoates. Double bond positions in the *cis*- and *trans*-fractions then were determined by microozonolysis pyrolysis (5). In the present all GC procedure, an EGSS-X GC column separates the isologues. After epoxidation of the octadecenoates, the *cis*- and *trans*-epoxides are separated by an EGSS-X column and the collected fractions are cleaved by HIO_4. The cleavage products (aldehyde-esters and aldehydes) are analyzed with an OV 17-225 GC column.

The results in Tables III and IV and Figure 3 are compared with analysis of samples A and E in Figures 4 and 5 in reference 16. Note that

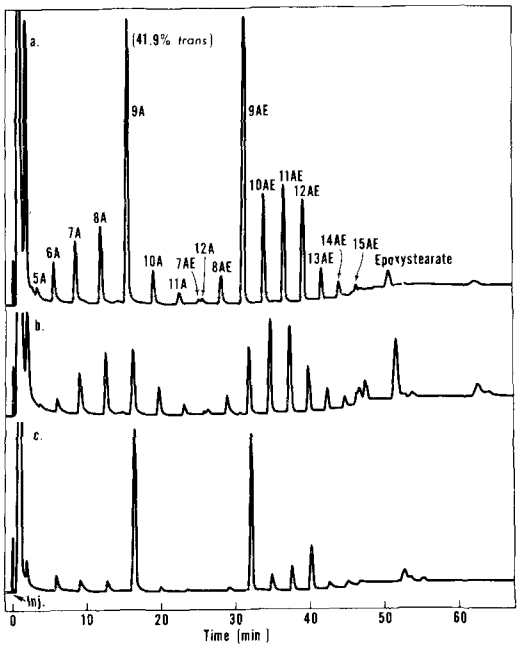

FIG. 3. Analysis of octadecenoate fraction from partially hydrogenated vegetable shortening (a) total octadecenoate sample, (b) *trans*-octadecenoate fraction and (c) *cis*-octadecenoate fraction. GC conditions are the same as used in Figure 1. Periodic acid cleaved.

both methods give a similar pattern of results, but there are fairly large variances between the values for some individual positional isomers. Since the individual values under the columns total (HIO_4), total (Cal), and total (O_3) in Table III agree quite well, we feel the HIO_4 cleavage data are more accurate than the ozonolysis-pyrolysis results given in reference 16. However, this increased accuracy may be due to better GC instrumentation and integration methods, rather than experimental procedural or separation techniques. Also the values listed in Tables III and IV were estimated from bar graphs in reference 16 and may contain appreciable errors. In addition to being more accurate, the all GC system reduces the total sample requirement from ca. 4 g for the liquid chromatographic ozonization procedure (16) to ca. 10 mg or less, and the elapsed analysis time/sample from 3 days to 10 hr. This decrease in analysis time and sample size is possible, because separation of the monoene fraction by preparative GC requires only 30-40 min and 10 mg sample compared to ca. 8 hr and 4 g sample for a rubber column separation. Subsequent separation into *cis*- and *trans*-fractions requires ca. 1 hr by GC compared to 8 hr by silver resin column chromatography (16,21).

Both the HIO_4-Et_2O and HIO_4-CH_2Cl_2 methods give similar results; but the HIO_4-CH_2Cl_2 method is simpler, more reliable, produces no extraneous peaks, and the yield of aldehyde and aldehyde-ester cleavage products is higher. The HIO_4 cleavage and reductive ozonolysis procedures both require the FID response to be corrected (7,8). Correcting the peak areas by dividing peak area by the number of ionizable carbons results in relatively poor agreement between the corrected aldehyde and aldehyde-ester peak areas. The FID response must be calibrated with standards or known mixtures (18) to obtain correction factors which will provide good agreement between equimolar amounts of aldehydes and aldehyde-esters. Accurate detection of acetaldehyde and propionaldehyde is difficult because these peaks overlap the methylene chloride or ether solvent peak. This problem is not serious, since only the aldehyde-ester peak areas need to be used for double bond location in monoenes. Since each aldehyde-ester peak must have a corresponding aldehyde partner, the aldehyde peaks are used qualitatively here to aid in identifying aldehyde-ester peaks and for detecting extraneous peaks.

The epoxidation HIO_4 cleavage sequence has been demonstrated for octadecenoate standards and for monoene fractions isolated from partially hydrogenated fats. The procedure should be applicable to any biological or synthetic octadecenoate sample which can be gas chromatographed.

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Sterols, Aliphatic Hydrocarbons, and Fatty Acids of a Nonphotosynthetic Diatom, *Nitzschia alba*

T.G. TORNABENE[1], Department of Microbiology, Colorado State University, Ft. Collins, Colorado 80521, **M. KATES**, Department of Biochemistry, University of Ottawa, Ottawa, Canada, KIN 6N5, and **B.E. VOLCANI**, Scripps Institution of Oceanography, University of California, La Jolla, California 92037

ABSTRACT

The unsaponifiable lipids and total fatty acids of a nonphotosynthetic diatom, *Nitzschia alba*, have been examined. The major fatty acids were found to be 14:0, 16:0, 18:1, and 20:5; small amounts of 15:0, 16:1, 18:0, 18:2, 18:3, and 20:4 acids also were present. The unsaponifiable lipids consisted mostly of sterols, with only traces (<0.1%) of hydrocarbons (chiefly C_{16}, C_{18}, and C_{28} normal olefins). The sterols contained brassicasterol (major) and clionasterol (minor), as well as traces of an unidentified sterol; clionasterol was present only in glycosidically bound form.

INTRODUCTION

Recent studies have indicated the widespread occurrence of sterols in both procaryotic and eucaryotic photosynthetic microorganisms (1-3). For example, β-sitosterol (24-ethyl cholesterol) and cholesterol have been identified in blue-green algae (4,5); cholesterol, desmosterol, and 22-dehydrocholesterol have been found in red algae (6); and phytosterols with 24S – configuration occur in green algae (7) and phytoflagellates (8). Hydrocarbons also are widespread, having been identified in fresh water and marine algae (9-18), including blue-green algae (10-13). In particular, a C_{21}-hexaene hydrocarbon has been identified in marine and fresh water algae, such as photosynthetic diatoms, dinoflagellates, and chrysophytes (13-18), but not in nonphotosynthetic dinoflagellates or diatoms, nor in blue-green algae (13,14,18). Fatty acid patterns in marine and fresh water algae are also quite unique, characteristically high proportions of C_{16}, C_{18}, C_{20}, and C_{22} polyunsaturated acids being present (19-26).

In connection with studies on silica shell formation in a colorless, nonphotosynthetic diatom, *Nitzschia alba* (27), we undertook a study of the lipids of this organism, for comparison with those of related photosynthetic diatoms (20) and other photosynthetic microorganisms. A preliminary examination revealed the presence of a rather high proportion of unsaponifiable material (ca. 10% total lipids), consisting largely of sterols with traces of hydrocarbons. Appreciable amounts of C_{20} polyunsaturated fatty acids, found in photosynthetic diatoms (20-23), also were observed.

This article deals with the composition of fatty acids and hydrocarbons in *N. alba* and the identification of its major sterol components.

MATERIALS AND METHODS

Cells of *N. alba* Lewin and Lewin (strains LTP-1 and 3-2) were grown in 9 liters of an enriched sea water medium (20,27), containing 0.5% or 0.7% of Bacto-Tryptone peptone (Difco, Detroit, Mich.) and 20 ppm of Si, at 30 C under forced aeration (11-14 liters of air/min). Cultures were harvested by centrifugation after 24 hr (exponential phase) and 40 hr (stationary phase) of growth. The cell paste was washed three times with sea water and immediately extracted by a modification (20) of the Bligh and Dyer procedure (28).

To a suspension of 10 g packed wet cells (9 ml packed wet cell volume; 2.2 g dry cells) in 32 ml sea water was added 100 ml methanol and 50 ml chloroform, and the mixture was shaken and left at room temperature for 1-2 hr. After centrifugation, the supernatant extract was decanted; the residue was reextracted with a mixture of 20 ml water, 50 ml methanol, and 25 ml chloroform. The mixture was centrifuged and the combined supernatants were diluted with 75 ml each of chloroform and water; the phases were left to separate in a separatory funnel. The lower chloroform phase was withdrawn, diluted with an equal volume of benzene, and evaporated just to dryness in a rotary evaporator (30-35 C). The residue was dissolved immediately in 5 ml chloroform; yield of total lipids on a cell dry wt basis: for LTP-1 strain, 3.7% and 5.3% in exponential and stationary growth phases, respectively; for strain 3-2, 4.4% and 6.0% in exponential and stationary growth phases, respectively.

^{14}C-Labeled cells of *N. alba* were obtained by growing the organism for 30 hr in 50 ml Rila Marine Mix (29) containing 0.5% Tryptone and

[1]National Research Council of Canada Postdoctorate Fellow, 1967-68.

TABLE I

Fatty Acid Composition of *Nitschia alba*[a]

Fatty acid	Strain 3-2		Strain LTP-1	
	Exponential[b]	Stationary[c]	Exponential[b]	Stationary[c]
14:0	30.0	26.1	15.0	18.9
15:0	1.5	tr	tr	1.2
16:0	20.9	20.0	30.9	19.6
16:1	8.5	7.8	2.9	2.3
18:0	tr	tr	tr	tr
18:1	23.6	19.8	32.2	20.2
18:2	4.7	4.0	2.3	4.4
18:3	tr	tr	tr	1.5
20:4	tr	tr	tr	2.0
20:5	10.8	21.8	16.6	29.1

[a]Analysis on butanediolsuccinate polyester at 178 C; tr = trace (<1%). Results are given in area %.

[b]Cells were harvested after 24 hr growth.

[c]Cells were harvested after 40 hr growth.

0.5 mC_i of $Na_2{}^{14}CO_3$. The cells were centrifuged, resuspended to 1.6 ml in unlabeled growth medium, and extracted with 6.0 ml methanol-chloroform (2:1, v/v) overnight at room temperature. The mixture was centrifuged; the supernatant was removed by Pasteur pipet; and the residue was suspended in 1.9 ml methanol-chloroform-water (2:1:0.8, v/v). After centrifugation, the supernatant was removed, and the combined extracts were diluted with 2.5 ml each of chloroform and water. The mixture was centrifuged, and the upper methanol-water phase was withdrawn; the lower chloroform phase was washed twice with 1 ml portions of methanol-water (10:9), then diluted with 4 ml benzene and concentrated to dryness in a stream of nitrogen. The residual ^{14}C-labeled lipids were dissolved in 1 ml chloroform and stored at -20 C.

Preparation of Unsaponifiable Material and Fatty Acids

Samples of total unlabeled lipids (25-40 mg) were hydrolyzed in 4 ml 2.5% methanolic-HCl under reflux for 1 hr followed by addition of 0.5 ml 7N NaOH and further refluxing for 1 hr, as described previously (30). After extraction of the total unsaponifiable material with 5 ml portions of petroleum ether (bp 30-60 C), the hydrolysate was acidified with 6N HCl, and the free fatty acids were extracted with petroleum ether and converted to methyl esters by treatment with 2.5% methanolic-HCl under reflux for 1 hr (30). Unsaponifiable material also was obtained by heating the sample of total lipids in 5 ml 2N NaOH in 85% methanol-water under reflux for 1-2 hr; the mixture was extracted at the centrifuge with several 5 ml portions of petroleum ether (bp 30-60 C), and the combined extracts were brought to dryness in a stream of nitrogen. The material obtained by the alkaline procedure contained the free sterols, together with those derived from alkali-labile sterol derivatives, e.g. sterol esters, but not those from alkali-stable derivatives, e.g. sterylglycosides.

To remove traces of alkylamines from the unsaponifiable material, the latter was dissolved in 4.0 ml methanol, the solution was acidified with 0.5 ml 1N HCl, and the mixture was extracted with several 5 ml portions of petroleum ether (bp 30-60 C).

The total unsaponifiable material obtained after methanolic-HCl hydrolysis amounted to 10-11% and 11-13% of the total lipids in strains LTP-1 and 3-2, respectively; unsaponifiable material obtained after alkaline hydrolysis alone amount to 2-3% and 4-5% of the total lipids in strains LTP-1 and 3-2, respectively.

The total unsaponifiable material was fractionated by preparative thin layer chromatography (TLC) on Silica Gel G in the solvent systems of Freeman and West (31): first, ethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2); then, hexane-ethyl ether (94:6) in the same direction. Zones were visualized with iodine vapor and then eluted with chloroform-methanol (3:2).

Gas Liquid Chromatographic (GLC) Analyses

Fatty acid methyl esters were analyzed by GLC on 1.2 m x 0.4 cm glass columns of 10% Apiezon L on Gas-Chrom P (at 195 C) or 10% butanediol succinate polyester on Gas-Chrom A (at 180 C) using a Pye Argon or an F and M 5750 gas chromatograph. Chain length and degree of unsaturation of the fatty acids were verified by chromatography before and after

TABLE II

Aliphatic Hydrocarbons of *Nitschia alba*

Probable identity	Retention time (min)[a]	Composition, area %	
		Unhydrogenated	Hydrogenated
16:0	15.0	3.2	38.4
16:1	15.4	1.3	—
16:2	17.0	33.8	—
Pristane	17.6	0.7	0.7
17:0	19.2	5.5	5.4
Phytane	22.8	1.9	2.1
18:0	24.5	5.3	25.6
18:2	26.8	20.4	—
19:0	29.2	3.1	2.9
20:0	34.5	2.7	5.0
20:2	37.0	2.7	—
21:0	40.0	1.9	2.0
22:0	45.0	0.9	1.0
26:0	104.0	2.0	2.2
27:0	127.0	2.3	2.3
28:0	158.0	3.0	12.4
28:2	173.0	9.3	—

[a]Gas liquid chromatography analysis on 91 m x 0.76 mm column coated with Igepal CO-990 programed from 110-200 C at 2 C/min and held isothermally; N_2 flow rate, 15 cc/min.

hydrogenation in methanol with platinum catalyst and by comparison of their relative retentions with those of authentic samples of palmitoleic, oleic, *cis*-vaccenic, linoleic, linolenic, eicosatetraenoic, and eicosapentaenoic acids (Hormel Institute, Austin, Minn.).

Hydrocarbons were analyzed before and after hydrogenation in methanol-chloroform (2:1, v/v) with platinum catalyst, by GLC on a stainless steel capillary column (0.76 mm x 91 m) coated with Igepal CO-990 (32). Peaks were identifed by comparison of their retention times with those of authentic normal, saturated hydrocarbons (C_8-C_{34}) (Applied Science Laboratories, College Park, Pa.). GLC analyses of the sterol fraction were carried out on 1.9 m x 0.3 cm glass columns packed with 11% OV-17 plus QF-1 (silicones) on 80/100 Gas-Chrom Q for free sterols or with 3% HI-EFF-8BP (cyclohexanedimethanol succinate polyester) on 100/120 Gas-Chrom Q for trimethylsilyl (TMS)-ether derivatives. The major sterol GLC peaks were collected, and their mass spectra were recorded repetitively on an AEI MS-12 mass spectrometer connected to a small computer data system (33).

RESULTS AND DISCUSSION

Fatty Acids

Total fatty acid compositions of the two strains of *N. alba* were generally similar and showed considerable increases in the proportion of 20:5 acid in cells grown to the stationary phase (Table I). The major acids in both strains were found to be 14:0, 16:0, 18:1, and 20:5 acids. Small quantities to trace amounts (less than 1%) of 15:0, 16:1, 18:0, 18:2, 18:3, and 20:4 acids also were detected. The fatty acid composition of *N. alba* was qualitatively similar to those of photosynthetic diatoms (20-23), but the colorless diatom had higher proportions of 14:0 and 18:1 acids and a much lower proportion of 16:1 acid. The increase in proportion of the 20:5 acid in the late stage of growth also has been observed for photosynthetic diatoms (23).

The presence in *N. alba* of the polyunsaturated fatty acids 20:4 and 20:5 characteristic of photosynthetic diatoms (20-23) is of particular interest. Such acids are believed to be involved in the functioning of the photosynthetic apparatus in eucaryotic algae. The presence of these acids in the nonphotosynthetic diatom, *N. alba*, suggests that the latter may be an apochlorotic mutant derived from a photosynthetic precursor, a conclusion supported by the presence of proplastids in this colorless diatom (27). On the other hand, it is possible that 20:4 and 20:5 acids may not be involved in the functioning of the photosynthetic apparatus at all but are merely components of diatom membranes. Further studies with isolated organelles will be necessary to settle this question.

Unsaponifiable Material

TLC and autoradiography of the unsaponifi-

TABLE III

Gas Chromatographic Analysis of Sterols of *Nitschia alba*[a]

Peak	Retention time (min)[b]	Composition, % (peak areas)[c]	
		Total sterols	Esterified + free sterols
1	53.0	80	90
2	65.0	15	0
3	74.5	5	10

[a]Sterol fractions were isolated from the unsaponifiable material by preparative thin layer chromatography (see text) and analyzed in free sterol form by gas liquid chromatography on a glass column (1.9 m x 0.3 cm) packed with 11% OV-17 and QF-1 on GC-Q;N_2 flow rate at 60 cc/min; temperature, 240 C.

[b]Retention time values for authentic standards were: cholestane, 14.5; cholesterol, 39.0; ergosterol, 51.0; stigmasterol, 56.0; β-sitosterol, 65.0.

[c]Peak areas were determined as the product of peak height x peak width at one-half the peak height.

able material obtained from cells labeled with ^{14}C, using the solvent systems of Freeman and West (31), revealed the presence of two main ^{14}C-labeled fractions: a major component (R_f 0.39) corresponding to authentic sterols, and a trace component (R_f 0.90) corresponding to aliphatic hydrocarbons. Both strains of *N. alba* had essentially the same hydrocarbon and sterol compositions which were unaffected by the stage of growth. It should be emphasized that the hydrocarbons, although present in trace amounts, are not contaminants derived from the growth medium but true cellular constituents, since they were labeled with ^{14}C.

The hydrocarbon fraction, isolated by preparative TLC of unlabeled lipids, amounted to only ca. 0.1% of the total lipids and was found by GLC to contain predominantly C_{16}, C_{18}, and C_{28} olefins; traces or low proportions of even and odd carbon numbered normal saturated hydrocarbons from 16:0-28:0, as well as pristane and phytane, also were present (Table II). On the basis of their relative retention times and the fact that hydrogenation yielded the corresponding normal saturated hydrocarbons, the major olefin components were identified as the straight chain dienes 16:2, 18:2, and 28:2 (Table II). The location of the double bonds, however, has yet to be determined. No C_{21}-hexaene hydrocarbon was detected, agreeing with the previous finding (13,14,18) that this hexaene is present only in photosynthetic diatoms and eucaryotic algae.

The presence of traces of long chain (C_{16}-C_{28}) normal alkanes, as well as pristane and phytane, in the nonphotosynthetic diatom *N. alba* is not unusual, since these are found universally in algae, including photosynthetic diatoms (13-15). However, the predominance in *N. alba* of even carbon numbered olefins, as compared to the high content of odd carbon numbered olefinic hydrocarbons found in other algae species (13-18), is unique and suggests that *N. alba* may have an unusual biosynthetic pathway for hydrocarbons.

The total sterol fraction isolated by preparative TLC of the total lipids in the solvent systems of Freeman and West (31) gave a positive Salkowski test for sterols and on analysis by GLC (Table III) was found to contain one major (peak 1) and two minor components (peaks 2 and 3). The mass spectrum of the major sterol (Fig. 1) had a molecular ion at m/e 398 and other ions at m/e 383 (M^+-CH_3), m/e 380 (M^+-H_2O) and m/e 365 [M-(CH_3+H_2O)]. Ion peaks at m/e 300 and m/e 271 can be ascribed (7,8) to M^+-(part of side chain), and M^+- (total side chain), respectively. The mass spectrum of peak 1 is in accord with that expected for the structure 24-methyl-5,22-cholestadien-3-β-ol (brassicasterol or dehydrocampesterol) (8). One of the minor components (peak 2, Table III) had GLC retention data on both OV-17 + QF-1 and HI-EFF-8BP liquid phases corresponding to those of authentic β-sitosterol. Since 24R and 24S steroisomers of sterols were not separable in the GLC system employed, it is not possible to identify this component or the major component (peak 1) unambiguously. However, on the basis of the observation that sterols in diatoms and green algae so far studied have the 24S configuration, while those in higher plants have the 24R configuration (1,7,8), the major (peak 1) and minor (peak 2) components tentatively may be identified as brassicasterol and clionasterol, respectively. The other minor component (peak 3, Table III) has not yet been identified. Analysis of the alkali labile sterols (esterified + free) showed that clionasterol was absent from this fraction (Table III), indicating that it was present only in glycosidically-bound

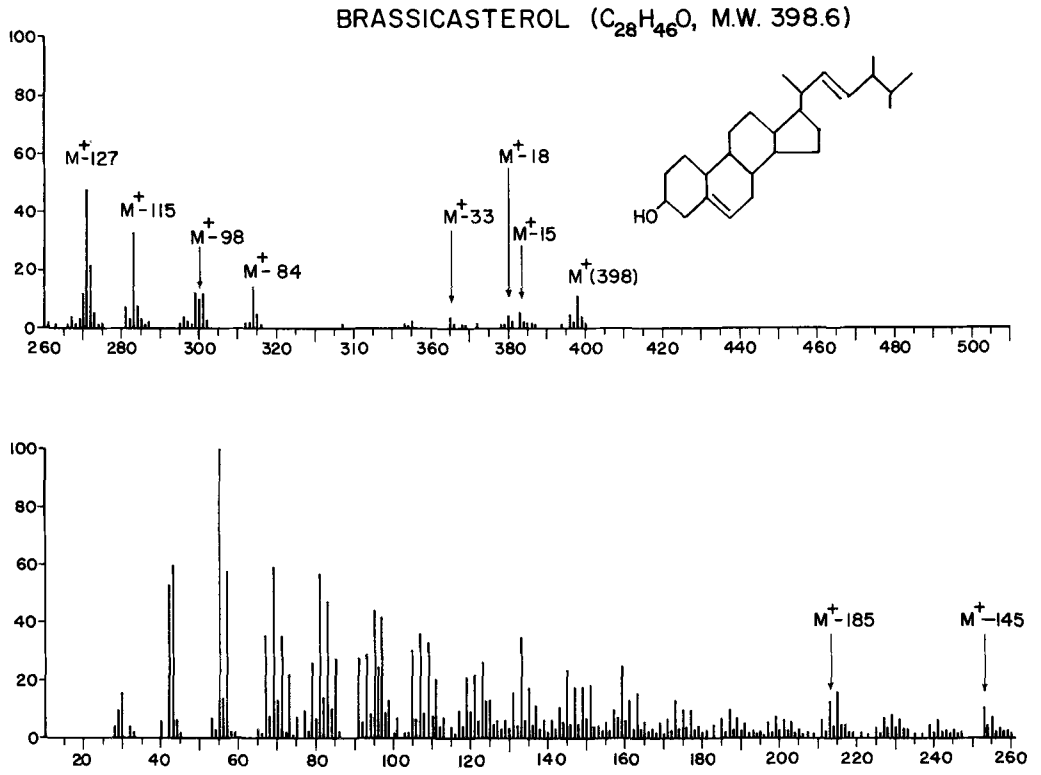

FIG. 1. Mass spectrum of major sterol component (peak 1, Table III) isolated from *Nitzschia alba*.

form. Chromatographic studies of the intact lipids of *N. alba* (to be reported separately) confirmed the presence of free sterols, sterol esters, and sterol glycosides.

Brassicasterol and clionasterol have been found previously in algae (1-3,7); however, the present results show that brassicasterol in *N. alba* probably occurs both as sterol esters and in free sterol form, whereas clionasterol occurs only in a glycosidically-bound form. Studies on the identification of the steryl glycoside components are in progress.

ACKNOWLEDGMENTS

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Effect of Oxygen Levels on the Fatty Acids and Lipids of *Mucor rouxii*[1]

S. SAFE[2] and **J. DUNCAN**, Atlantic Regional Laboratory, National Research Council of Canada, Halifax, Nova Scotia, Canada.

ABSTRACT

The effect of aerobic and oxygen limiting (anaerobic) growth conditions upon the fatty acid and lipid composition of *Mucor rouxii* has been examined. The aerobic cells contained a range of fatty acids typical of phycomycetes, i.e. γ-linolenic acid, with an unsaturation index of 1.20, whereas the anerobic cells contained relatively high levels of shorter chained fatty acids and very low concentrations of unsaturated acids (unsaturation index = 0.025). The unsaturated compounds were monoolefinic tetra-, hexa-, and octadecenoic acids; and closer examination of their di-trimethylsilyl derivatives by gas chromatography and mass spectrometry showed that all three acids contained the double bond in the Δ^9 position. These results were consistent with a microaerobic biosynthetic pathway. In addition, there were major quantitative differences in the lipid composition of the two types of cells; and it was evident that the differences in growth environment markedly affected the cellular lipid and fatty acid compositions.

INTRODUCTION

It has been reported that *M. rouxii* (1-3) and other *Mucor* species (4) were capable of growth under both aerobic and anaerobic conditions. The aerobic cells were filamentous with considerable chlamydospore formation, whereas the anaerobic cells were yeast-like. This article describes the growth of a *M rouxii* isolate (5) aerobically (under helium) and shows that there are major differences in the lipids and fatty acid composition of the filamentous and yeast-like cells. Comparisons also are made between the lipid and fatty acid composition of the above cells and spores. The structures of the unsaturated fatty acids produced under anaerobic conditions also have been examined in detail to determine whether these compounds are produced microaerobically or via another pathway.

MATERIALS AND METHODS

Cultivation and Growth of *M. rouxii*

The isolation of *M. rouxii* (Calmette) Wehmer (HLX 1093) from sheep rumen contents has been described (5). Inocula of *M. rouxii* were prepared by adding sterile water (75 ml) to a culture maintained on defined medium to which agar (2%) had been added. The resulting spore suspension was decanted, and 3 ml (ca. 10^7 spores) used to inoculate 2 liters of medium contained in a 4 liter thick walled conical flask. The medium, described by Bartnicki-Garcia and Nickerson (6), was supplemented with Difco yeast extract (2%) and used for both aerobic and anaerobic growth. Aerobic growth was carried out without agitation at 25 C for 6 days. A stream of helium was bubbled through the medium used in the anaerobic experiments for 2 hr prior to inoculation (5). The flasks used in this study are shown (Fig. 1); opening the stopcock A permits evacuation and refilling of the flasks with helium (5). After refilling with helium, the clamp B is removed and a positive pressure of helium flushes both the flask and the deoxygenation trap which is

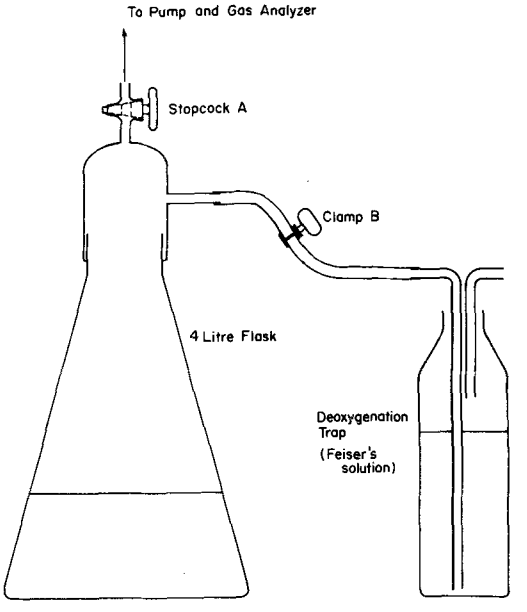


FIG. 1. Flasks used in the study.

[1]Issued as NRCC No. 13935.

[2]Present address: Department of Chemistry, University of Guelph, Guelph, Ontario, Canada.

TABLE I

Fatty Acid Composition of Anaerobically Grown[a] *M. rouxii* at Specific Incubation Times

Incubation time (hr)	Mycelial[b] wt	Fatty acid composition									Unsaturation index
		8:0	10:0	12:0	14:0	14:1	16:0	16:1	18:0	18:1	
20	0.08	4.0	20.5	30.5	23.5	tr[c]	10.0	1.0	9.5	0.5	.015
30	0.26	6.5	48.0	21.0	13.1	tr	5.5	1.5	4.0	0.5	.020
40	0.49	4.0	34.0	26.5	21.2	tr	7.5	1.0	5.0	1.0	.020
50	0.57	tr	24.5	32.5	27.5	tr	8.0	2.0	2.0	1.5	.025
120	0.65	1.0	24.0	25.0	25.0	tr	14.0	1.0	8.0	1.5	.025

[a]Grown at 25 C.

[b]Expressed in terms of g/liter of media.

[c]tr = Trace.

filled with Fieser's solution (7). The excess gas pressure which develops during growth then can be released through the trap. Gas in the flasks was analyzed as previously described (5); and, in all cases, the anaerobic flasks were grown under an atmosphere containing less than 0.01% oxygen. The cells (both aerobic and anaerobic) were collected by filtration and then freeze-dried; spore suspensions were collected by centrifugation followed by freeze-drying.

Extraction of Fatty Acids and Lipids

The freeze-dried cells (or spores) were macerated with chloroform/methanol (2:1) (8) in a Waring blender and the mixture allowed to stand for 12 hr. A lipid extract was obtained by filtration to remove the cell debris. Fatty acids were obtained by concentrating the extract to dryness and saponifying the residue with 8% methanolic potassium hydroxide solution for 2 hr under reflux. The alkaline solution was diluted with water and the nonsaponifiable material removed by ether extraction; the aqueous layer was acidified with hydrochloric acid and extracted with ether to give the fatty acid fraction.

Lipid Analysis

The lipid fraction was purified by preparative thin layer chromatography (TLC) on Silica Gel $HF_{254+366}$ (Merck Darmstadt, Germany) using the solvent system, petroleum spirit/ether/methanol (90:30:15) as described by Sallee and Adams (9). The appropriate bands were visualized readily by long wave UV light and extracted with chloroform/methanol (2:1) to give the various lipid fractions.

Fatty Acid Analysis

The lipid and fatty acid fractions were transmethylated by refluxing for 90 min in benzene/methanol/sulfuric acid (20:10:1). The resultant fatty acid methyl esters then were purified by TLC (petroleum spirit/ether [97:3] as solvent system). Further purification of the fatty acid methyl esters according to degree of unsaturation was carried out using Silica Gel $HF_{254+366}$ plates impregnated with silver ni-

TABLE II

Fatty Acid Composition of Aerobically-Grown[a] *M. rouxii* at Specific Incubation Times

Incubation time (hr)	Mycelial[b] wt	Fatty acid composition									Unsaturation index
		8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	
24	0.01	tr[c]	5.00	11.4	18.4	21.4	17.9	3.0	20.9	1.40	0.40	0.43
48	0.20	0.60	13.2	22.2	26.5	15.9	6.9	2.6	8.50	1.60	1.00	0.22
72	0.31	0.40	12.0	19.6	24.0	16.8	6.4	4.0	9.50	3.20	4.00	0.33
96	0.38	2.70	12.5	14.2	16.9	12.9	4.50	8.50	17.6	4.80	5.50	0.48
120	0.56	0.70	8.10	12.1	17.0	12.5	4.70	5.90	20.7	7.20	11.0	0.73
144	0.58	0.15	6.30	10.6	15.6	11.9	4.30	7.00	22.8	8.60	12.1	0.81

[a]Grown at 25 C.

[b]Expressed in terms of grams/liter of media.

[c]tr = trace.

TABLE III

Fatty Acid and Lipid Composition of Spores of *M. rouxii* Grown at 25 C[a]

Lipid	Percent (by wt)	Fatty acid composition									Unsaturation index
		10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	
Triglycerides	22.0	4.5	8.5	12.0	34.5	6.5	10.5	15.0	5.5	2.0	0.39
Diglycerides	5.4	5.0	16.0	7.7	32.0	3.5	15.0	16.5	3.5	tr[b]	0.27
Polars	69.5	—	2.5	5.0	40.5	10.5	11.0	19.0	5.0	tr	0.40
Sterols	3.1										

[a]2-3 Week growth time.

[b]tr = trace.

trate (12% by wt) and eluted in benzene. The appropriate bands were visualized using long wave UV light.

The ester fractions were analyzed by gas liquid chromatography (GLC) on a stainless steel column (6 ft x 1/8 in.) packed with 8% HIEFF-1BP on Gas-Chrom Q (Applied Science) at a temperature of 140 C. The fatty acid methyl esters were identified by their relative retention times and by mass spectrometry. The unsaturated fatty acids were isolated by preparative GLC, converted into their di-trimethylsilyl derivatives, (10) and analyzed by mass spectrometry.

RESULTS AND DISCUSSION

The phycomycete, *M. rouxii* was capable of growth under both aerobic and anaerobic conditions and the differences in the cell growth environment were accompanied by changes in morphology. The aerobically grown cells were filamentous with considerable chlamydospore formation, whereas the anaerobic cells were yeast-like. The aerobic and anaerobic cells were harvested at various times from early to late log-phase growth and a summary of the mycelial wt and their fatty acid compositions is given in Tables I and II. The anaerobically grown cells readily adapted to growth at the low oxygen levels with a maximum growth rate occurring between 20-40 hr after inoculation. Similarly the growth rate of the aerobic cells was at a maximum between 24-48 hr after inoculation.

The fatty acid composition of the aerobic cells varied over the growth period, particularly with respect to the concentration of the 18:2 and 18:3 components. Their concentrations both increased with increasing age of the cells and not unexpectedly was accompanied by an increase in the unsaturation index of the fatty acids. The identity of the 18:3 component was shown to be γ-linolenic acid by comparative GLC and mass spectrometry with an authentic γ-linolenic acid standard. This acid commonly is found in phycomycetes, and Shaw (11) has proposed that γ-linolenic acid is a useful phylogenetic marker which distinguishes phycomycetes from the other orders of fungi which produce α-linolenic acid as the sole 18:3 species. The anaerobic cells contained only trace quantities of monoenoic acids (14:1, 16:1, and 18:1) and no dienoic or trienoic components and the unsaturation index was relatively constant over the entire growth period. There were

TABLE IV

Fatty Acid and Lipic Composition of *M. rouxii* (Aerobic)[a]

Lipid	Percent (by wt)	Fatty acid composition									Unsaturation index
		10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	
Triglycerides	22.6	8.0	19.0	16.5	9.0	6.0	4.0	14.0	7.5	16.5	0.84
Diglycerides	0.6	tr[c]	12.5	17.5	10.0	25.0	tr	25.0	10.0	tr	0.70
Free fatty acid	2.5	tr	2.0	9.5	20.5	7.0	7.5	28.0	10.5	13.5	0.96
Polars	60.5	4.0	3.0	8.0	13.5	10.0	1.0	22.0	14.5	25.0	1.36
Sterols	13.4										

[a]Grown at 25 C.

[b]Number of double bonds/mole fatty acid.

[c]tr = trace.

TABLE V

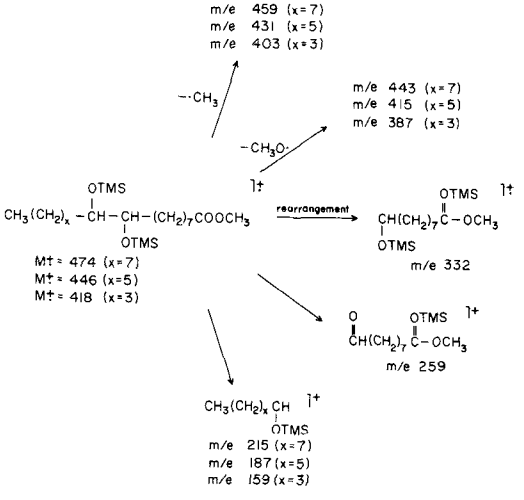
Fatty Acid Composition of Aerobically-Grown[a] *M. rouxii* at Specific Incubation Times

Lipid	Percent (by wt)	Fatty acid composition									Unsaturation index
		8:0	10:0	12:0	14:0	14:1	16:0	16:1	18:0	18:1	
Triglycerides	6.4	1.0	12.5	36.0	31.5	tr[a]	7.5	tr	11.0	0.5	.005
Diglycerides	1.8	—	2.0	26.0	29.0	tr	26.0	tr	13.5	2.5	.025
Polars	91.0	0.5	12.0	16.0	20.0	tr	19.5	0.5	27.0	2.0	.025
Sterols	0.8										

[a]tr = trace.

variations in the relative amounts of the short chained saturated fatty acids, i.e. 8:0, 10:0, 12:0, and 14:0, present in the anaerobic cells; but their overall concentration was 75-85% of the total fatty acids at 20, 30, 40, 50, and 120 hr after inoculation. In contrast, the aerobic cells contained only 32.6-62.3% short chain saturated acids. Similar results also have been observed for the fungus *Mucor genevensis* (4).

Since it is possible that the formation of the unsaturated compounds could occur microaerobically (via oxygen-dependent desaturases) or anaerobically (as in bacteria [12]), the position of the double bond in the above monounsaturated fatty acids was investigated. The olefinic esters were separated readily from the saturated acids by argentation TLC, and the R_f values indicated that the esters were *cis*-monoenes; further separation of the three components could be affected by preparative GLC. The monoenes were treated with $0_s\,0_4$/pyridine and the resulting diols converted into the ditrimethylsilyl (di-TMS) derivatives (10). The fragmentation pattern of all three di-TMS derivatives exhibited a common fragmentation pathway as shown in the equation; intense *m/e* 332 and 259 ions were observed as well as the expected $[M\text{-}CH_3O]^+$ species. In addition, an intense ion also was observed at *m/e* 215 for the 18:1 compound, at *m/e* 187 for the 16:1 ester, and at *m/e* 159 for the 14:1 ester. These results indicated that the structures of the olefinic fatty acids were 18:1ω9 (oleic acid), 16:1ω7 (palmitoleic acid), and 14:1ω5 (myristoleic acid); and these acids are typically produced by specific oxygen-dependent desaturation of the corresponding saturated fatty acid precursors. Thus, the samll quantities of unsaturated fatty acids were biosynthesized microaerobically, rather than by an anaerobic pathway.



The lipid and fatty acid compositions of the chloroform-methanol extracts of the aerobic and anaerobic cells and the spores are shown in Tables III-V. There were significant variations in the compositions of these extracts with relatively high levels of triglycerides (22.0 and 22.6%) and polar fatty acids (69.5 and 60.5%) in the spore and aerobic cell extracts. In contrast, the anaerobic cells contained only 6.4% triglyceride, with the polar fatty acid fraction being the major component (91.0%). The unsaturation indices for the aerobic cell lipids were higher than those observed for the spore lipid fractions, and this was primarily due to higher levels of the γ-linolenic acid. These results agreed with the data reported by Sumner and Morgan (13) for a number of mesophilic *Mucor* species. The sterol levels in all 3 extracts also varied considerably; the anaerobic cell extract contained only 0.8% sterols, whereas the aerobic cells and spores contained significantly larger quantities (13.8 and 3.1%) of extractable sterols.

Thus, the fatty and lipid composition of *M. rouxii* was markedly affected by the growth environment, particularly with respect to the concentrations of chloroform-methanol extractable lipids, as well as the relative amounts of short chained and unsaturated fatty acids present.

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Age Dependent Structural Changes in the Diol Esters of Uropygial Glands of Chicken[1]

ABSTRACT

The chain lengths and diastereoisomer composition of alkane-2, 3-diol diesters of the uropygial glands of chicken changed significantly as the birds became physiologically mature. The C_{24} diol decreased, and C_{22} and C_{23} diols increased. Threo isomer content of the diols decreased. In the acyl portion, the shorter (C_{12}-C_{15}) acids decreased, and the longer (C_{17}-C_{20}) acids increased.

[1]Scientific paper 4168, Project 2001, Agricultural Research Center, College of Agriculture, Washington State University, Pullman, Wash.

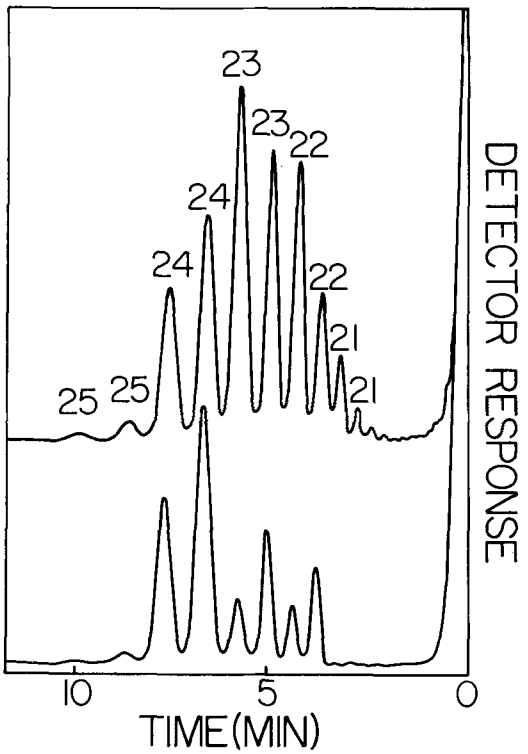

FIG. 1. Gas liquid chromatograms of the isopropylidine derivatives of alkane-2,3-diols of 3 month old (lower) and 13 month old (upper) chickens. The number on each peak represents the chain length.

INTRODUCTION

Uropygial glands of birds produce a variety of unusual lipids presumably evolved for the protection of the birds. Diesters of alkane-2,3-diols constitute the major components of the uropygial glands of chicken (1), turkey (2), green pheasant (3), ring-necked pheasant, quail (4), and a variety of other birds (5). Chain lengths of the diols in chicken and turkey are C_{20}-C_{25} in which C_{22}, C_{23}, and C_{24} predominate, while pheasants produce C_{18} diol as the major component. Heretofore, composition of uropygial gland lipids had not been reported to change significantly with the physiological state of the bird. In this communication, we report that the chain length distribution and the diastereoisomer composition of alkane-2,3-diols change significantly with the age of the chicken. Significant changes occur also in the chain length distribution of the acyl portion of the wax.

EXPERIMENTAL PROCEDURES

Clear oil was squeezed at specified intervals from ca. 6 birds randomly selected from a group of 20 birds which were raised on a commercial diet. The lipid samples were kept at -20 C until analysis. Isolation of diol diesters from the uropygial excretions and methanolysis, followed by isolation of the methylester and diol fractions, were done as described before (4). Isopropylidine derivatives were prepared as described by Hansen, et al. (2). The isopropylidine derivatives and methyl esters were subjected to gas liquid chromatography (GLC) on a 6 ft x 0.25 in. stainless steel column packed with 5% SE-30 on 80-100 mesh Gas Chrom Q with a 23 lb inlet pressure of He as carrier gas. Column temperatures for methyl esters and isopropylidine derivatives of diols were 190 C and 250 C, respectively. Identifica-

tion of methyl esters was done by comparison of the retention times with those of authentic compounds and by mass spectrometry of each component. The structures of diols were determined by mass spectrometry.

RESULTS AND DISCUSSION

Thin layer chromatographic examination of the uropygial excretion of chickens showed that diol diesters constituted the major component in all ages examined. GLC analysis of the diols, as their trimethyl silyl derivatives, indicated that in young (2 months) chickens C_{24} diols predominated, while in older birds (10 months) C_{23} diol was the major component. Therefore, a systematic analysis of the effect of age upon uropygial gland diols was done.

GLC analysis of isopropylidene derivatives of the diols gave two peaks for each diol (Fig. 1), and the structure of each was determined by mass spectrometry. The isomer with the lower retention time was assigned threo configuration for the reasons described by Hansen, et al. (2). Results of such analyses are shown in Table I. C_{22}, C_{23}, and C_{24} were the major alkane diols with smaller amounts of C_{21}, C_{25}, and C_{20} in the order of decreasing amounts. The most significant change brought about by the age of the bird was that C_{24} content decreased dramatically between 3 and 5 months of age, with a substantial increase in C_{23} and C_{22}. As the birds matured, C_{23} became the dominant diol. The results in Table I also show that the content of threo isomer in the major diols decreased with age. While threo isomers dominated in 3 month old birds, in mature birds erythro isomers became the major components.

Significant changes in the chain length distribution of acyl chains of the diol diesters also occurred with the age of the birds (Table II). The major changes were a decrease in the shorter (C_{12}-C_{15}) acids and an increase in the longer (C_{17}-C_{20}) acids. The results presented here show that significant changes in the structure of the uropygial gland excretions can occur as a bird matures and changes thereafter appear to be minor. Such changes have not been reported heretofore. The biosynthetic and functional implications of such changes are not understood. However, it is clear that factors, such as age, should be considered when uropygial gland excretions are used for taxonomic purposes (6, 7).

TABLE I

Chain Length Distribution of Alkane-2,3-diols of the Uropygial Glands of Chickens

Date	C_{20}		C_{21}		C_{22}		C_{23}		C_{24}		C_{25}	
	Percent of diols	Threo %	Percent of diols	Threo %	Percent of diols	Threo %	Percent of diols	Threo %	Percent of diols	Threo %	Percent of diols	Threo %
10-8-72 (3 months)	T	—	T	—	14.6	60.9	21.4	65.4	60.1	61.2	3.94	75.4
11-5-72	1.81	40.9	5.19	33.3	24.5	51.0	27.8	53.2	39.0	60.8	3.17	58.4
12-19-72	3.78	47.9	10.43	41.8	27.4	39.6	32.7	49.9	23.3	61.9	2.34	79.9
1-21-73	1.14	29.4	8.14	20.4	27.0	28.6	39.7	32.3	21.2	54.3	2.75	74.9
2-5-73	1.89	29.6	10.4	27.6	28.5	34.7	34.4	41.3	22.4	57.6	2.31	63.2
4-4-73	1.64	33.5	8.35	25.6	26.5	28.7	39.8	37.2	21.0	54.8	2.63	52.1
5-9-73	2.54	16.1	9.94	21.7	25.9	31.7	36.2	38.7	22.9	53.7	2.51	57.8
5-26-73	1.1	28.2	8.77	25.3	26.9	28.9	39.9	38.6	21.4	53.3	1.74	63.8
8-20-73	1.84	43.5	5.52	30.4	22.1	38.8	42.5	42.6	24.8	56.8	3.36	66.7

[a]T = trace.

P.E. KOLATTUKUDY
W.N. SAWAYA
Department of Agricultural Chemistry
Washington State University
Pullman, Washington 99163

TABLE II

Chain Length Distribution of the Acyl Portion of Diol Diesters of the Uropygial Glands of Chickens

Date	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{17}	C_{18}	C_{19}	C_{20}
10-8-72 (3 months)	15.2	4.2	36.0	4.9	18.0	3.0	3.5	T[a]	T
11-5-72	10.6	4.3	22.2	5.9	12.6	5.9	11.7	8.7	T
12-4-72	17.0	4.1	28.3	4.1	20.6	4.1	8.1	2.9	T
1-8-73	2.6	0.74	6.4	2.5	19.0	10.8	16.9	20.6	10.5
2-5-73	3.4	T	5.6	2.2	21.0	9.9	23.0	19.0	13.6
5-9-73	2.8	T	5.5	2.1	17.6	10.0	26.1	20.4	10.9
5-26-73	2.3	T	5.8	2.7	18.0	12.5	24.0	18.2	10.9
8-20-73	4.1	1.1	7.3	3.6	22.5	8.4	20.4	14.3	10.7

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[Received December 6, 1973]

Metabolism of Actinic Skin Tumors: Incorporation of ^{14}C-Acetate into Lipids

ABSTRACT

Biochemical parameters of normal and actinically induced tumorous skin were compared. Similar respiratory rates and respiratory quotients were observed. However, both quantitative and qualitative differences occur in these tissue's ability to incorporate ^{14}C-acetate into lipids.

INTRODUCTION

In 1960, Winklemann, et al., (1) reported that hairless mice, when exposed to UV radiation, developed skin tumors. Subsequently, the hairless mouse has proved to be a useful model system in the study of actinic skin cancer. However, little is known of the basic skin biochemistry. Here, we report observations on respiratory rates, $^{14}CO_2$ evolution, and ^{14}C-acetate incorporation into lipids.

MATERIALS AND METHODS

Female hairless mice (Smith-Meyers strain) were irradiated with a General Electric UA3 mercury arc lamp 5 days a week. After 6 months' irradiation, nearly 100% of the animals bore tumors. Tumors were diagnosed as squamous cell carcinomas. Detailed procedures for tumor production have been outlined previously (2,3).

Tumor tissue was harvested by decapitation of the animal after which a flap of dorsal skin bearing the tumor was removed. Subcutaneous tissue was scraped away and a 4 or 6 mm diameter punch biopsy taken of the tumor bearing site. Control tissue was obtained from similar dorsal anatomical sites of nonirradiated animals. The tissue was weighed and placed in manometric flasks containing 2 ml Krebs-Ringer phosphate buffer, pH 7.4, 3 mM glucose, and 1 μC^{14}C-1-acetate. Standard manometric procedures were followed, and the tissue

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Date	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{17}	C_{18}	C_{19}	C_{20}
10-8-72 (3 months)	15.2	4.2	36.0	4.9	18.0	3.0	3.5	T[a]	T
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was incubated at 37 C for 2 hr. Each flask contained 50-100 mg tissue. At the termination of the incubation period, the tissues were removed, thoroughly rinsed in several changes of water, and immediately frozen at -20 C prior to subsequent analysis. Samples of the KOH in the center wells were taken for $^{14}CO_2$ determinations.

The tissues were homogenized in 10 ml H_2O with a model 10 Polytron equipped with saw-toothed generator. Total lipids were obtained by multiple extraction with chloroform-methanol (2:1 v/v) and washed (4). The total lipid extract was dried in vacuo, made to desired volume, and aliquots removed for liquid scintillation counting. Total ^{14}C-acetate uptake levels were determined by digesting an aliquot of tissue homogenate in NCS reagent (Nuclear-Chicago) and measurement of radioactivity.

Thin layer chromatography (TLC) was accomplished using Mallinckrodt silica TLC-7GF coated glass plates. Aliquots of the total lipid extracts from analagous wt of tumor and control tissues were streaked on plates which were divided into two lanes. The plates were developed in 1,2-dichloroethane. One lane of each plate was sprayed with 50% H_2SO_4 and the plates charred. TLC-densitometric scans of the plates were obtained, and 1 cm bands from the adjacent lane were scraped into counting vials and radioactivity determined.

RESULTS AND DISCUSSION

In the present study, skin tumor respiratory rates were not significantly lower than those of controls, yet the $^{14}CO_2$ evolved was only ca. one-half that of controls (Table I). Others have reported lower rates of $^{14}CO_2$ production from radiolabeled fatty acids in liver tumor tissues and have suggested that this results from lower

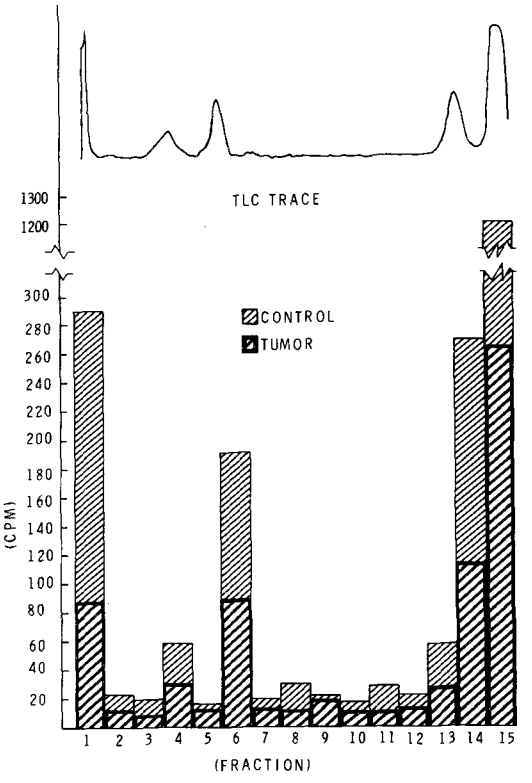

FIG. 1. ^{14}C-Acetate incorporation into lipids by control and tumorous skin tissues. Thin layer chromatography (TLC) trace represents separation of total lipid extract into various lipid classes. From left to right are phospholipids, fatty acids, free sterols, glycerides, and sterol esters, respectively. The TLC trace is typical of extracts from both control and tumorous tissues. Histogram represents radioactivity found in corresponding 1 cm fractions. The values shown are typical of those from three experiments. The mean incorporation level of acetate into lipids by tumorous tissue was ca. 25% that of controls. Standard deviation of the percentage difference between experiments was ±3.8.

TABLE I

Comparison of Skin Biochemical Parameters

Treatment	Respiratory rate[b]	$^{14}CO_2$ Evolution[c]	^{14}C-Acetate uptake[c]	^{14}C-Acetate incorporation to lipid[d]
Control[a]	410	3.38	1.87	6088
Tumor[a]	362	1.52	0.93	1324
Average difference	-11%	-55%	-50%	-78%
Standard deviation[e]	±7.2	±2.4	±3.6	±8.8

[a]Mean of three experiments.
[b]μl O_2/g tissue/hr.
[c]10^4 counts/min (CPM)/100 mg tissue.
[d]CPM/100 mg tissue.
[e]Standard deviation of % differences.

oxidative rates and a lower percentage of $^{14}CO_2$ in the total CO_2 evolved (5). The latter is indicative of a greater dependence of tumor tissue upon endogenous substrates for oxidation. Obviously, the oxidative rates could not account for the current observations. Furthermore, the respiratory quotients for control and tumor tissues were 0.59 and 0.64 respectively, indicating similar substrate utilization.

Numerous workers have reported the ability of tumors to synthesize lipids from acetate; but considerable disagreement concerning tumor activity, in comparison to normal tissues, exists (6). As seen in Table I, the levels of ^{14}C-acetate incorporated into skin tumor lipids are only ca. 25% that of control tissues. Lower ^{14}C-acetate uptake, possibly due to increased thickness of the tumor specimen, could not alone account for this marked difference. Indeed, when the major classes of lipids are separated by TLC and radioactivity determined for each class, both quantitative and qualitative differences in acetate incorporation can be seen (Fig. 1). Incorporation of acetate into phospholipids and sterol esters is affected most. It has been reported previously that marked effects upon ^{14}C-acetate incorporation into lipid occur in human skin exposed to UV radiation (4). Inhibition of acetate activation is the suspected site of action (7). It is conceivable that chronic exposure to UV, resulting in actinic tumors, evokes a similar response in the skin of hairless mice. If such were the case, UV induced inhibition of ^{14}C-acetate incorporation into skin lipids would represent a biochemical lesion expressed at a very early stage in the development of actinic tumors.

HOMER S. BLACK
J. SCOTT JONES
WAN-BANG LO
Veterans Administration Hospital and Departments of Dermatology and Biochemistry
Baylor College of Medicine
Houston, Texas 77031

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[Received October 5, 1973]

Cholanoic (Bile) Acids in Hepatic and Nonhepatic Tissues of Miniature Swine

ABSTRACT

Preliminary information is given indicating that all tissues of the miniature swine examined thus far contain cholanoic (bile) acids. Thin layer and gas chromatographic analyses suggest that the cholanoic acids are conjugated with glycine and taurine. The amounts appear to be substantial and should be assessed in regard to studies of cholesterol turnover.

INTRODUCTION

Liver is considered to be the only tissue in which bile acids are synthesized from cholesterol (1,2). This view has been held on the basis of indirect evaluation of bile acid origin in the rat (3). One of the early studies showed substantial conversion of cholesterol-26-^{14}C to $^{14}CO_2$ by kidney and lesser amounts by spleen, lungs, and brain in vitro (4).

In two studies where 4-^{14}C-cholesterol was administered to rats and the acidic lipid fraction in carcass assayed after several days, substantial quantities of ^{14}C have been observed (5,6). This fraction was not characterized further, however.

EXPERIMENTAL PROCEDURES

In the present study, young adult female

oxidative rates and a lower percentage of $^{14}CO_2$ in the total CO_2 evolved (5). The latter is indicative of a greater dependence of tumor tissue upon endogenous substrates for oxidation. Obviously, the oxidative rates could not account for the current observations. Furthermore, the respiratory quotients for control and tumor tissues were 0.59 and 0.64 respectively, indicating similar substrate utilization.

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HOMER S. BLACK
J. SCOTT JONES
WAN-BANG LO
Veterans Administration Hospital and Departments of Dermatology and Biochemistry
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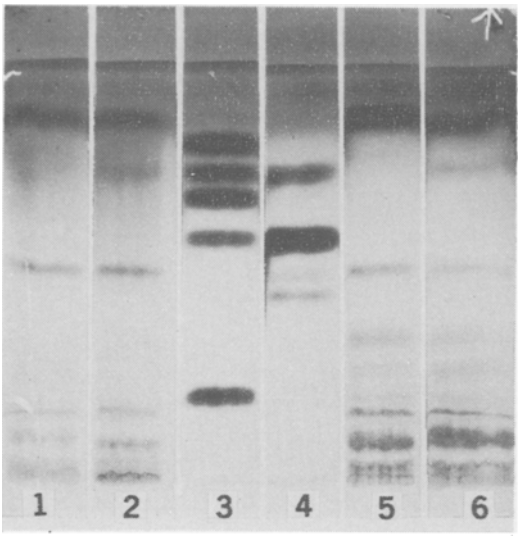

FIG. 1. Thin layer chromatography on Silica Gel G developed with propionic acid:isoamyl acetate:water: n-propanol (3:4:1:2, ref. 11) and visualized with 10% phosphomolybdic acid in ethanol. Channel 1 = liver; 2 = liver plus hyodeoxycholic acid; 3 = (bands from bottom) taurodeoxycholic acid, glycochenodeoxycholic acid, cholic acid, hyodeoxycholic acid, and deoxycholic acid; 4 = glycochenodeoxycholic acid; 5 = kidney; and 6 = kidney plus hyocholic acid.

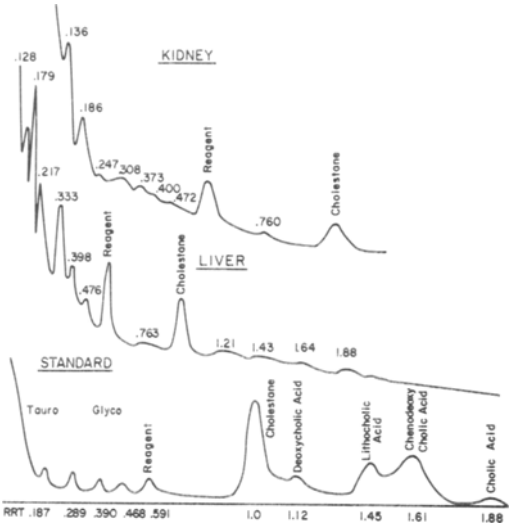

FIG. 2. Diagram of tracings of gas liquid chromatography analysis of liver and kidney cholanoic acids. The tauro- and glyco-derivatives were purchased as pure standards, but each gave several peaks. Column was 6 ft x 4 mm packed with 3% OV-210 on 80-100 mesh Supelcoport (8); carrier gas, helium; temperatures, column, 275 C, detector, 290 C, injection port, 320 C. Relative retention time (RRT) is given in relation to 5-α-cholestane.

miniature swine were slaughtered, and tissues were dissected and frozen. Representative amounts (0.5-10 g) of each tissue were weighed, minced, and heated at 80 C with 10 volumes of 10% ethanolic KOH (2-5 hr, depending upon the tissue). The samples were cooled and nonsaponifiable material extracted 4 times with petroleum ether (30-60 C) and discarded.

The aqueous-ethanol fraction was acidified to pH 2 with HCl and extracted four times with ethyl ether. The extract was dried and taken up in methanol. An aliquot was spotted on a Silica Gel G thin layer plate which had been precleaned by running in benzene. Bile acids and fatty acids were separated in a solvent system of benzene:isopropyl alcohol:glacial acetic acid, 30:10:1 (7). The bile acid bands were collectively scraped and eluted with methanol. Trifluoroacetic anhydrides of the methyl esters were prepared (8) and chromatographed with a Hewlett-Packard model 402 gas liquid chromatograph (GLC) equipped with dual hydrogen flame detectors and an electronic integrator.

Other samples of the acidic extract were separated into fatty acid and bile acid fractions by mixing in heptane:ethyl ether:water:ethanol (1:1:1:1) which separates into two phases (9). The organic layer was discarded and the aqueous layer dried, the residue dissolved in metha-

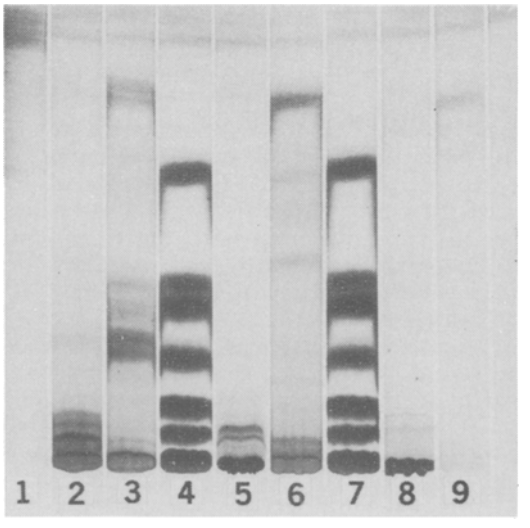

FIG. 3. Thin layer chromatography on Silica Gel G developed with trimethylpentane:ethyl acetate:acetic acid (5:5:1, ref. 7) and visualized with 10% phosphomolybdic acid in ethanol. Channel 1 is blank (note impurities); 2 = liver; 3 = liver after deconjugation; 4 = (bands from bottom) taurodeoxycholic acid + glycocholic acid, glycochenodeoxycholic acid + glycine conjugated cholic acid, glycine conjugated chenodeoxycholic acid, cholic acid, hyodeoxycholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid; 5 = kidney; 6 = kidney after deconjugation; 7 = same as 4; 8 = skeletal muscle; 9 = skeletal muscle after deconjugation.

TABLE I

Relative Proportions of Cholanoic Acids in Tissues of Miniature Swine (Identification of Individual Components is Tentative)[a]

Tissue	Tauro				Glyco				Free
	1[b]	2	3	4	1	2	3	4	
	Percent of total								
Liver	24.3	15.5	16.7		18.8	9.2	1.0		14.4
Bone marrow	20.7	16.6	—		8.7	52.7	tr		1.1
Pancreas	38.5	44.1	tr[c]		4.7	9.3	3.5		tr
Spleen	35.8	26.8	9.7		11.4	10.0	6.3		tr
Lung	—	8.4	37.9		46.4	tr	2.5		4.8
Kidney	40.0	41.7	11.1		1.1	1.1	5.0		1.1
Heart	8.0	15.0	13.7	16.7	16.7	11.9	6.8	7.6	4.1
Muscle	tr	44.1	3.7	37.3	1.9	3.7	—	3.7	5.6

[a]Gas liquid chromatography analysis. Gas liquid chromatography column same as in Figure 2.

[b]Numbers refer to consecutive gas liquid chromatography peaks in the regions of tauro- and glyco-conjugated cholanoic acids. Concentrations of the free bile acids, being much lower, were pooled for quantitative estimation.

[c]tr = trace.

nol and spotted for thin layer chromatography (TLC) as described. Some samples were deconjugated by hydrolysis in 20% KOH in ethylene glycol at 220 C for 20 min (10). The ethyl ether extract was dried, dissolved in methanol, and run on TLC plates.

RESULTS AND DISCUSSION

Results of TLC to separate individual cholanoic acids from a liquid extraction (9) are illustrated by Figure 1. Liver and kidney are seen to have a number of bands which ran in the range of the conjugated standards used. The poor quality of standards is illustrated by glycochenodeoxycholic acid which had 4 bands on TLC and numerous peaks on GLC. Two bands occurred with R_f higher than any primary bile acids and appear to be cholesterol (R_f .77) and fatty acids (R_f .88) (Fig. 1).

A diagram of tracings found by GLC is shown in Figure 2. The peaks are predominantly in the region of conjugated cholanoic acids. Relative proportions of the cholanoic acids found in various tissues by GLC are given in Table I.

Comparison of tissues prepared with and without the deconjugation procedure is shown in Figure 3. Bands corresponding to tauro- and glyco-conjugates diminished or disappeared, and bands corresponding to free cholanoic acids appeared. Three of the major bands from deconjugated liver samples corresponded to hyodeoxycholic acid (HDC), chenodeoxycholic acid, and deoxycholic acid. A fourth major band migrating faster than HDC did not move with any standards we tested. Deconjugated kidney samples had a faint band corresponding to HDC; however, other unidentified bands were more prominent. Deconjugated samples from skeletal muscle had only one significant band, having an R_f of 0.76. The same band also occurred in liver and kidney.

Because of impurity of standards and unknown compounds and some uncertainty as to quantitative extraction procedures, concentration data are not given. Most tissues appear to contain cholanoic acids within the same range of concentration as liver. The amount is also in the same range as cholesterol concentration. Work is being continued to verify quantitation and to extend identification to mass spectrometry.

JACQUELINE DUPONT
SUK-YON OH
LOUISE A. O'DEEN
SIGMUND GELLER
Department of Food Science and Nutrition
Colorado State University
Fort Collins, Colorado 80521

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Composition of the Lipids in Cabbage[1]

ANDREW C. PENG, Department of Horticulture, Ohio Agricultural Research and Development Center, Wooster, Ohio 44691

ABSTRACT

Cabbage leaves contain 0.16% total lipids of which 51.02% are neutral lipids, 40.78% glycolipids, and 8.18% phospholipids. The predominant fatty acids in the total lipid analysis are linolenic, linoleic, oleic, palmitic, and stearic acids. Linolenic, palmitic, tridecanoic, and oleic are the principal components in the neutral lipid fraction while glycolipids are composed mainly of linolenic, palmitic, lauric, myristic, and tricosanoic acids. Phospholipids are high in palmitic, linolenic, and linoleic acids. Both glucose and galactose were observed in the glycolipid fraction.

INTRODUCTION

Lipids are a major constituent of foods. Their presence, quantity, and composition are, not only important to organoleptic satisfaction, but also significant to nutrition and keeping quality.

Several studies have provided the quantity of lipids and their fatty acid composition in cabbage, but none of them covered all lipid classes. Wheeldon (1) studied cabbage leaf phospholipids. Nichols (2) separated phospholipids and glycolipids qualitatively from cabbage leaf and stalk. Vorbeck, et al., (3) reported fatty acids in acetone-soluble and -insoluble fractions of cabbage; and Laseter, et al., (4) determined the chemical characteristics of selected fatty acids from cabbage leaf.

This report was to isolate, separate, and identify cabbage lipids into three classes: neutral lipids, glycolipids, and phospholipids and to analyze the fatty acid composition of each class as a part of an inclusive investigation of lipid changes of selected vegetables and fruits, as related to the storage stability and shelf life after processing.

MATERIALS AND METHODS

Preparation of Sample

Golden Acre Yellows Resistant cabbage (*Brassica oleracea* var. *capitata* L.) was obtained from the Ohio State University Horticultural farm, Columbus. Following removal of the outside leaves, the head was cut into small pieces and thoroughly mixed to avoid the possibility of uneven distribution of lipids. Samples (200 g) were placed in a plastic bag and frozen until used. The moisture content was determined by wt difference after heating in a Precision-Thelco recirculating oven at 100-105 C for 20 hr.

Extraction of Lipids

Duplicate frozen samples were blended with 200 ml distilled water in a Waring blender for 3 min. The slurry was mixed thoroughly with 20 g silicic acid (SilicAR cc-7, Special, 100-200 mesh, Mallinckrodt Chemical Works, St. Louis, Mo.) and 10 g Celite (Johns-Manville, New York, N.Y.). The mixture was filtered with Whatman no. 1 paper in a Buchner funnel under reduced pressure until no continuous water drop was observed.

The sample pad was extracted in a Waring blender with 200 ml Folch reagent (5) consisting of chloroform-methanol (2:1 v/v) for 3 min at room temperature and filtered by a Buchner funnel as previously described. The residue was reextracted with another portion of 200 ml solvent, filtered, washed 2 times with 25 ml solvent/washing and 25 ml chloroform.

The combined extract was transferred quantitatively to a separatory funnel and allowed to stand for 5-10 min. The lower chloroform phase was collected; the upper alcohol phase was extracted with 30 ml chloroform and combined with the lower phase. The extract was left in a refrigerator overnight for complete separation. Only a small amount of an aqueous layer was formed. This was removed by siphoning. The extract then was concentrated by a rotary evaporator at reduced pressure at 45 C and stored in a vacuum desiccator until a constant wt was obtained.

Column Chromatography

Cabbage lipids were fractionated into three classes by two column separations. Polar lipids were separated from nonpolar lipids by silicic acid (6,7) at a sample loading ratio of 2-100 g adsorbent in a 1.1 cm diameter glass column with a 250 ml reservoir flask. Neutral lipids were eluted by chloroform and polar lipids by methanol at an elution ratio of 25 ml solvent/g adsorbent with a flow rate of 0.5 ml/min. Each fraction was collected in bulk, its solvent was

[1]Journal article 112-73, the Ohio Agricultural Research and Development Center, Wooster, Ohio.

TABLE I

Fatty Acid Composition of Cabbage Lipids (%)

Fatty acid	Total lipids	Neutral lipids	Glycolipids	Phospholipids
11:1[a]	—	—	—	4.5
12:0	1.6	5.1	7.9	2.5
13:0	0.6	2.1	6.0	1.5
13:1	2.0	3.7	7.1	3.9?
14:0	2.9	5.4	7.1	2.2
14:1	1.2	2.5	2.6	1.2
15:0	2.3	3.7	2.4	1.6
15:1	1.4	1.5	0.7	0.3
16:0	7.5	13.3	17.1	26.2
16:1	2.6	2.3	1.1	1.4
17:0	0.6	1.1	1.2	Trace
17:1	1.0	1.0	1.5	Trace
18:0	5.4	3.6	3.5	3.4
18:1	10.8	7.0	4.2	10.1
18:2	14.0	6.7	4.1	14.2
20:0	1.1	—	—	2.0
18:3	16.9	24.0	26.4	16.3
21:1	0.9	4.9	—	2.5
22:0	0.5	1.0	2.9	3.2
?	2.1	—	—	—
23:0	0.4	9.9	3.7	—
24:0	21.1?	0.3	—	—
24:1	1.9	—	—	—
U/S[b]	1.2	1.2	0.9	1.1

[a]Carbon number: number of double bonds.

[b]Ratio of unsaturated to saturated fatty acids.

removed, and stored as previously described.

The polar fraction was redissolved in 5 ml chloroform and transferred quantitatively onto a Florisil column (8-10). Glycolipids were eluted by acetone (7) at 40 ml/g adsorbent ratio with the same flow rate as before, and phospholipids were recovered by methanol at 25 ml/g adsorbent.

Thin Layer Chromatography (TLC)

TLC was used to monitor the purity of each lipid class. Glass plates (20 x 20 cm) were coated with Silica Gel G (Brinkmann Instruments, Westbury, N.Y.) 250 μ thickness. The developing solvent system for neutral lipids was chloroform, and the system was sprayed with phosphomolybdic acid. Chloroform-acetone-methanol-acetic acid-water (65:20:10:10:3 v/v) (11) was for polar lipids. Molybdenum blue reagent was used for detecting phospholipids, and diphenyl amine solution was used to identify glycolipids (12). The results were confirmed qualitatively by phosphorus analysis (13) and anthrone determination (28).

Gas Liquid Chromatography (GLC)

All analyses of fatty acid composition were carried out in a Packard model 409 Becker gas chromatograph (Packard Instrument, Downers Grove, Ill.) equipped with a flame ionization detector, Bristol's Dynamaster recorder, and Disc chart integrator. Methyl esters were prepared according to Metcalf, et al., (14) by using boron-trifluoride methanol. A coiled stainless steel column (8 ft x 1/8 in. outside diameter) was packed by Applied Science Labs., State College, Pa., with 15% by wt, diethyleneglycol succinate (DEGS) and 1% by wt phosphoric acid on acid-washed Chromosorb W, 80-100 mesh as the support phase. The operating conditions were: column temperature, 190 C; detector temperature, 210 C; injection port temperature, 210 C; carrier gas, nitrogen; carrier gas flow rate, 20 ml/min; and chart speed, 1 min/in. Identifications of the fatty acids on the chromatogram were made by comparing the retention time of reference compounds and by plotting retention time vs. carbon number on semilog paper for supplementing those other than reference compounds run on the same column under the same conditions. The fatty acids were expressed as area percentage of the total area from all methyl esters.

RESULTS AND DISCUSSION

The advantages of blending the frozen sample immediately with solvent were to eliminate the problem of heat generated by the blender;

the frozen tissue was disorganized readily resulting in a more homogeneous slurry which facilitated the extracting process; and the enzyme activity was slowed.

The moisture content of the cabbage, 92.57% was close to that reported in the U.S. Department of Agriculture, 92.40% (15); and the total lipid content, 0.16%, was in the range of Pederson and Albury's finding (16), 0.15-0.20%, but less than that found by Wheeldon, 0.21% (1).

The lipids isolated from the cabbage leaf were predominantly neutral lipids, 51.02%; followed by glycolipids, 40.78%, which are typical of photosynthetic tissue; and phospholipids, 8.18%, the least. Sugar residue in the glycolipids was determined by means of boron TLC (17). Two single spots were found on the plate which agreed with Kean's pattern (17). These were identified as glucose and galactose.

Data in Table I reveal that cabbage lipids contained higher unsaturated fatty acids than saturated as shown by the unsaturation-saturation ratios, 1.2 for total lipids and neutral lipids and 1.1 for phospholipids, except glycolipids which was 0.9. The fatty acid composition of the total lipids was also qualitatively similar to those studied on other vegetable crops: cucumber and pepper (18), sweet potato, (19), potato tubers (20-22), tomato seeds (23,24), spinach (25), pea (26), and turnip root (11). These data also agreed with the pattern of selected cabbage fatty acids found by Laseter, et al. (4). The distribution of the fatty acids were mainly linolenic, linoleic, oleic, palmitic, and stearic acids.

The neutral lipids predominantly were constituted by linolenic acid, 24.0%; followed by palmitic acid, 13.3%; and also contained 9.9% tricosanoic acid. The TLC of glycolipids developed three spots after being sprayed with diphenylamine in sulfuric acid. Identification by R_f values compared to those reported in the literature (17,27) and reference compounds indicating they were monogalactosyl and digalactosyl diglycerides and cerebrosides. Palmitic acid was the major saturated fatty acid which comprised 17.1%; followed by lauric, 7.9%; myristic, 7.1%; and tridecanoic, 6.0%. The predominant components in the unsaturated portion were linolenic, 26.4%; linoleic, 4.1%; oleic, 4.2%; and tridecenoic acid, 7.1%, which agreed with Vorbeck, et al., findings in his acetone-soluble fraction (3), except for tridecenoic acid.

The phospholipid fraction was present in the least quantity in the cabbage lipids. Their fatty acids were palmitic, 26.2%; linolenic, 16.3%; linoleic, 14.2%; oleic, 10.1%; and undecanoic acid, 4.5%. The higher amount of unsaturation and high palmitic acid content were characteristic of the cabbage phospholipids (1).

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Pentane Production by Peanut Lipoxygenase[1]

HAROLD E. PATTEE, JOHN A. SINGLETON, and **ELIZABETH B. JOHNS,** Mid-Atlantic Area, Southern Region,[2] and Department of Botany, North Carolina State University, Raleigh, North Carolina 27607

ABSTRACT

Pentane and hexanal were the major volatile end-products of a peanut lipoxygenase and linoleic acid model system and were produced by both crude and purified enzyme preparations. The enzyme system did not require an anerobic condition for the production of pentane and hexanal, thus distinguishing it from other reported systems. A 122-fold purification of the enzyme was achieved.

INTRODUCTION

Lipoxygenase has been isolated from many plant sources and studied extensively in soybeans. Recent attention has been focused upon the end-products of the enzymic reaction and the mechanism involved (1,2). Pentane first was postulated to be an enzymic product of lipoxygenase activity from studies on peanut volatiles (3) and direct evidence came from soybean studies (4). A recent publication reported that hexanal was the only major volatile reaction product of the peanut lipoxygenase-linoleic acid system and did not indicate that pentane was produced by enzymic oxidation of linoleic acid (5). The apparent lack of production of pentane by this system thus suggested that pentane was not a product of the peanut enzymic reaction. The present study was undertaken to clarify whether or not pentane is a product of the peanut lipoxygenase reaction, as well as to determine the effects of selected parameters upon the production of volatile

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[2]ARS, USDA.

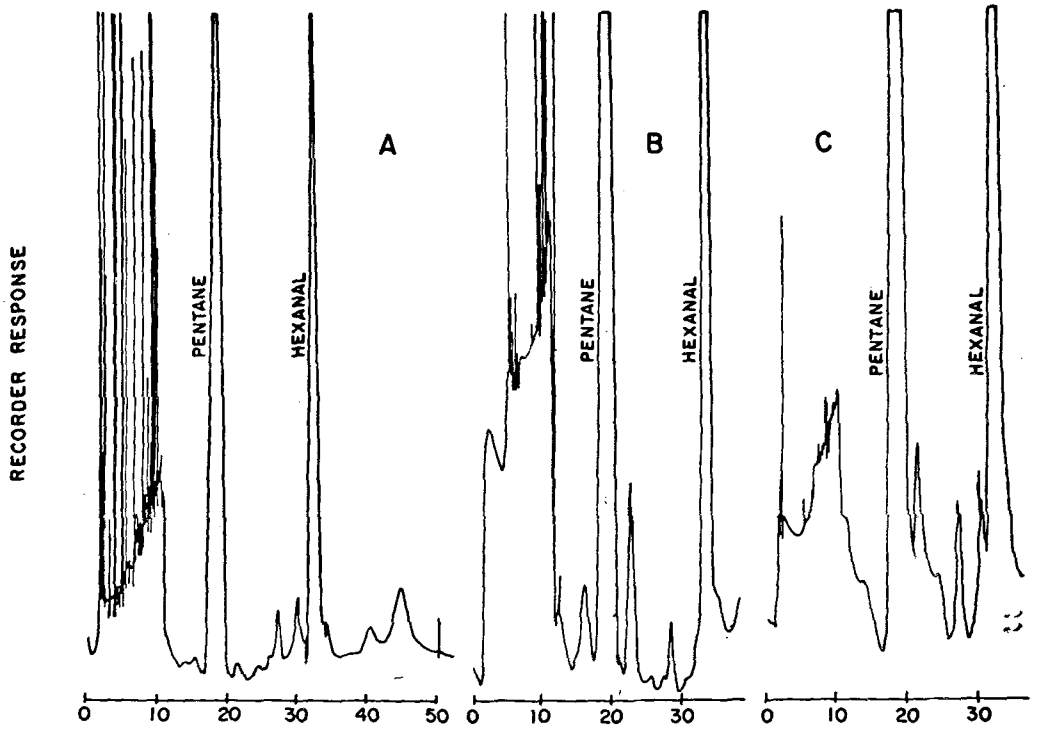

FIG. 1. Effect of O_2 on the volatiles produced from peanut lipoxygenase-linoleic acid system. Chromatogram A = no O_2 incorporation; Chromatogram B = substrate oxygenated for 1 hr prior to enzyme addition; Chromatogram C = substrate oxygenated (1 hr) prior to enzyme addition and O_2 incorporation during reaction period.

components by peanut lipoxygenase.

EXPERIMENTAL PROCEDURES

Sources of Materials

Peanuts of the Virginia 56R variety were obtained from Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md., and of the NC-2 variety from the North Carolina Central Crops Research Station, Clayton, N.C. Linoleic acid was purchased from Hormel Institute, Austin, Minn., and Sephadex G-150 and DEAE-Sephadex A-50 from Pharmacia Fine Chemicals, Piscataway, N.J.

Enzyme Assay

Hexanal determinations were performed as previously described (5), except that only a Porapak Q column was used for gas liquid chromatography (GLC) analysis. Lipoxygenase fractions were assayed by measuring O_2 consumption and pentane production at room temperature (25 ± 2 C) in a glass reaction apparatus fitted with a Clark oxygen electrode and a serum stopper as described by Johns, et al. (6). The vessel contained 11.87 μmoles linoleic acid, 2.6 ml 0.1% Tween 20 in 0.05 M phosphate buffer pH 6.5, 0.025-0.4 ml enzyme solution, and distilled water to a total volume of 3 ml. Values for O_2 concentration (7) were assumed to be 260 nmoles/ml and were not corrected for the effect of ionic solutes. Lipoxygenase activities were calculated from initial reaction rates. At a given time interval, a 5 ml volume of gas was withdrawn with an air-tight syringe and injected into a model 1840 Varian Aerograph gas chromatograph. A Chromosorb 102 column operated isothermally at 140 C was used to determine pentane. Peak areas were integrated using an Infotronics CRS-100 digital readout system, and pentane data are presented as integrator area units. Protein content was determined spectrophotometrically as described by Layne (8).

Enzyme Purification

Acetone powders prepared from mature peanuts, as described by Pattee and Swaisgood (9), were used as the enzyme source. Acetone powders (35 g) were extracted with 700 ml 0.1 M Tris-HCl buffer (pH 7.0) by stirring for 1 hr at room temperature. The slurry was filtered through Whatman no. 4 filter paper, centrifuged for 15 min at 11,700 g, the pellet discarded, and the supernatant taken to 40% saturation with solid $(NH_4)_2SO_4$. After standing for 45 min at 4 C, the suspension formed was centrifuged as above. The pellet was dissolved in 0.1 M Tris-HCl buffer (pH 7.0), analyzed for activity, and discarded. The supernatant was taken to 60% $(NH_4)_2SO_4$ saturation, allowed to stand for 45 min at 4 C, then centrifuged as above. The supernatant was discarded, the pellet dissolved in 46.5 ml 0.1 M Tris-HCl buffer (pH 7.0), and enough 95% ethanol (EtOH) added to make a 20% solution. This solution was placed at -20 C for 1 hr, centrifuged for 15 min at 25,300 g, and the pellet discarded. The supernatant was dialyzed against 4 liters of 0.1 M phosphate buffer (pH 7.0) at 4 C overnight. After dialysis, the supernatant was taken to 80% $(NH_4)_2SO_4$ saturation, centrifuged as above, and the pellet dissolved in 10 ml 0.1 M phosphate buffer (pH 7.0). This solution was placed on a Sephadex G-150 column (2.5 x 80 cm), equilibrated with 0.1 M phosphate buffer, and eluted with the same buffer. Ca. 4 ml fractions were collected. Fractions containing lipoxygenase activity were pooled (37 ml) and placed on a DEAE-Sephadex A-50 column (1.5 x 75 cm) previously equilibrated with 0.1 M phosphate buffer (pH 7.0). The column was eluted with a 0.0-0.4 M NaCl gradient using 600 ml same buffer. Fractions of ca. 5 ml were collected. Fractions containing lipoxygenase activity were pooled and used for further experimentation.

RESULTS

Chromatograms of volatiles produced by the 40% $(NH_4)_2SO_4$ precipitate from Virginia 56R peanuts using linoleic acid as the substrate indicated that several volatile components were produced (Fig. 1A). When the substrate solution was oxygenated thoroughly (Fig. 1B) prior to the addition of the enzyme, the two major volatile components increased in concentration. These two components have been identified as pentane and hexanal by GLC on two different columns and by mass spectrometry (10). Oxygenation of the substrate before enzyme addition and during the reaction period (Fig. 1C) increased the levels of both pentane and hexanal. The chromatograms in Figure 1 show that pentane and hexanal were produced in the presence of linoleic acid by the 40% $(NH_4)_2SO_4$ precipitate and that the amounts of the products were increased by saturating the system with oxygen.

In using the direct GLC method proposed by St. Angelo, et al. (5), interruption of the carrier-gas flow rate masked the early part of the chromatogram (Fig. 1) and simultaneously caused an increase in the standing current of the flame ionization detector (FID). Without carrier gas flowing through the column and

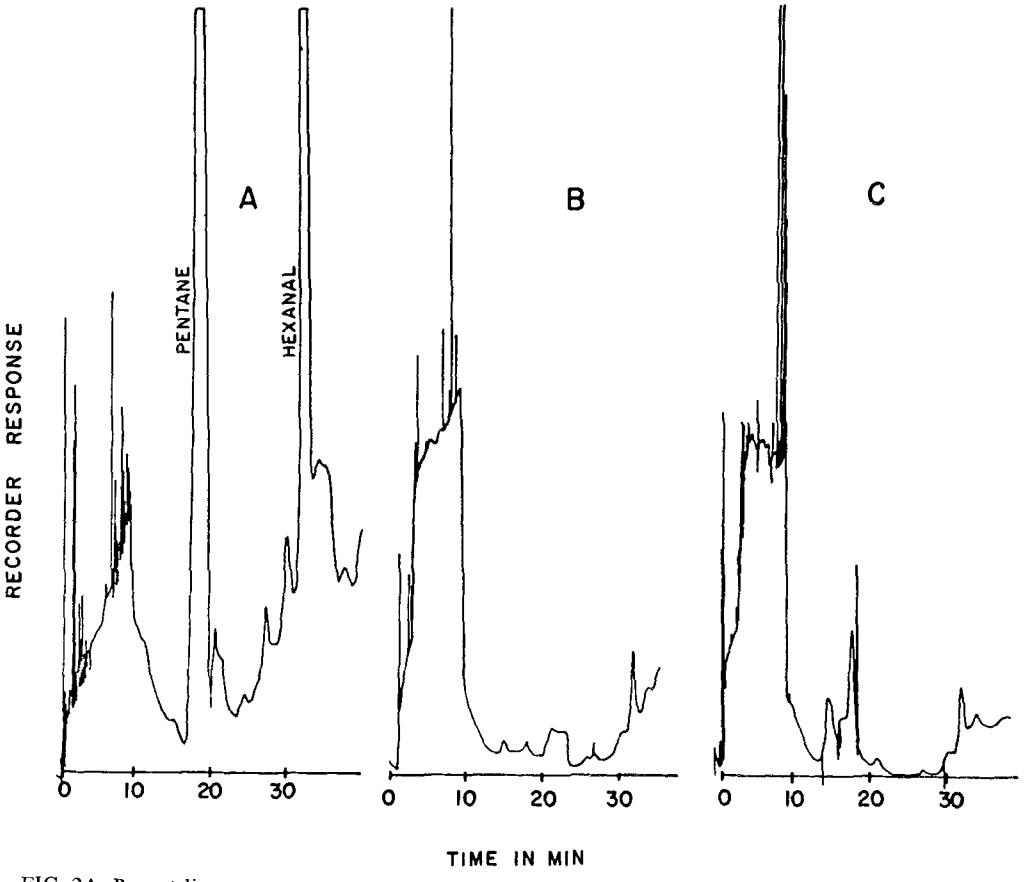

FIG. 2A. Peanut lipoxygenase system oxygenated and monitored by a Clark oxygen electrode. 2B. Control reaction with substrate omitted from reaction mixture. 2C. Control reaction with enzyme omitted from reaction mixture.

FID, the temperature of the flame tip rises and operation of the GLC becomes difficult, due to spiking and column bleed, until equilibrium between the carrier gas and column has been reestablished. Also, water from the aqueous solution must pass through the column before a useful chromatogram can be obtained. Further, because of interrupted gas flow, the direct GLC method does not lend itself to the more sensitive operating ranges. Electrometer setting for the chromatograms shown in Figure 1 was 32 x 10^{-12} amps/mv.

To confirm that pentane was produced under strictly aerobic conditions, the oxygen level of the reaction mixture was monitored by inserting a Clark oxygen electrode into a specially designed 1 liter reaction flask. Oxygenation during the reaction period was maintained at the saturation level. The chromatogram in Figure 2A shows that both pentane and hexanal were produced under aerobic conditions by the model system. Control chromatograms of separate substrate and enzyme buffer solutions indicated that volatiles in the controls were negligible (Figs. 2B and 2C).

A high level of peanut lipoxygenase purification was achieved by extraction of acetone powders with 0.1 M Tris buffer pH 7.5, $(NH_4)_2SO_4$ precipitation, 20% EtOH treatment, and column chromatography. Data presented in Table I show a 122-fold purification after DEAE-Sephadex column chromatography. Purification of the enzyme resulted in a 28-fold increase in pentane production.

DISCUSSION

The experiments reported here show that both pentane and hexanal, along with several minor components, were produced by the action of peanut lipoxygenase on linoleic acid. Purification of the enzyme did not separate or eliminate the production of pentane or hexanal

TABLE I

Summary of Enzyme Purification

Fraction	Total protein, mg	Total activity nmoles O_2/min	Specific activity nmoles O_2/min/mg	Pentane production area units/mg
Crude	8435	4528	.54	152,485
40% $(NH_4)_2SO_4$ Ppt	741	558	.75	5,548
60% $(NH_4)_2SO_4$ Ppt	2098	4011	1.91	17,191
20% EtOH[a] supernatant	1236	1677	1.35	
Dialysis	888	1572	1.77	256,121
Sephadex G-150	96.4	1505	15.61	341,645
DEAE-Sephadex	6.3	4.3	65.61	4,290,414

[a]EtOH = ethanol.

from the lipoxygenase activity. These observations support the report by Johns and coworkers (11,12) that pentane production could not be separated from soybean lipoxygenase even using isoelectric focusing with a 0.5 pH gradient and Smith and Lands' postulation (1) of substrate- and product-binding sites on lipoxygenase. The results also confirm the postulation by Pattee, et al., (3) that pentane is a product of peanut lipoxygenase and extend the observations by St. Angelo, et al., (5) which indicated that hexanal was the only volatile end-product of peanut lipoxygenase.

Garssen, et al., (4) has shown that soybean lipoxygenase (pH 9) catalyzes the peroxidation of linoleic acid to produce initially ca. 95% 13-hydroperoxy linoleic acid. With subsequent onset of anaerobic conditions, pentane and dimeric compounds were produced enzymatically. Our results during characterization of peanut lipoxygenase indicated that pentane could be produced by an aerobic reaction mechanism. Pentane production by peanut lipoxygenase under continuous aerobic conditions was confirmed by using a Clark oxygen electrode to verify O_2 saturation levels. These results differed from those from the soybean pH 9 enzyme characterized by Garssen, et al., (4) in that, in Garssen's system, production of pentane required a lag period and began only after oxygen had been depleted. However, the peanut enzyme, with a single pH optimum between pH 6 and 7 (13), does resemble the pH 7 isoenzyme of soybeans which Johns (11) found did not require a lag period when absorbance of the reaction was measured at 285 nm.

The initiation reactions for both aerobic and anaerobic production of pentane by lipoxygenase from linoleic acid probably involve the same intermediates. As oxygen becomes a limiting factor, the terminal steps of the enzyme-radical mechanism may change. Incorporation of oxygen (Fig. 1C) into the reaction mixture increased the concentration levels of the volatile end-products and suggested that oxygen is a limiting factor under static conditions.

A separate pentane-producing enzyme could not be isolated from the crude preparation using $(NH_4)_2SO_4$ and column chromatography on Sephadex G-150 and on DEAE Sephadex. A 122-fold purification of the enzyme was achieved (Table I) and pentane and hexanal were produced at each purification step. Intermediate steps of the purification procedure resulted in some loss of pentane-production activity. The nature of this activity loss is not known at the present time but appears to result from ion binding at the product reaction site, since the purification step on DEAE Sephadex relieved the inhibitory action.

The peanut lipoxygenase used in this study probably reacted with linoleic acid in an enzyme controlled radical mechanism similar to that proposed by Garssen, et al. (2). Production of pentane and hexanal by the peanut enzyme is evidence that the 13-hydroperoxy isomer is produced, and both compounds probably are derived from the 13-isomer via structural rearrangement of the peroxy compound by a radical mechanism.

Other minor components, probably carbonyls, were produced by peanut lipoxygenase (Fig. 1). Grosch and Schwenchke (14) reported the presence of several volatile and nonvolatile carbonyls from the soybean lipoxygenase-linoleic reaction. Similar types of components probably are produced by the peanut lipoxygenase. Additional experimentation will be needed to elucidate the system further.

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Lipid Composition of Rat Superior Cervical Ganglion[1]

D.T.ORGANISCIAK[2] and **J.D.KLINGMAN**, Department of Biochemistry, State University of New York at Buffalo, Buffalo, New York 14214

ABSTRACT

Excised rat superior cervical ganglion were incubated in Krebs-Ringer solution, freeze-dried, and lipids extracted with chloroform:methanol (2:1) v/v. Chromatography of lipid extracts in three separate thin layer chromatography solvents was accomplished on a single thin layer chromatography plate coated with acid-washed Silica Gel H. Individual lipids were eluted from the silica gel using a modified Swinny filter holder technique and transesterified with methanolic-BF_3. Fatty acid methyl esters were separated and quantitated by gas liquid chromatography, while phospholipids were determined with a Malachite green dye organic phosphorus assay. Quantitation of neutral lipids was accomplished with a micro Liebermann-Burchard technique and sphingolipids estimated colorimetrically as free sphingosine. The composition of lipids from freeze-dried rat ganglia resembles the lipid composition of rat brain. Phosphatidylcholine and phosphatidylethanolamine represent 76% of the glycerolphosphatides. The sphingolipids were comprised primarily of sphingomyelin with moderate levels of cerebroside and sulfatide. Cholesterol was the predominant neutral lipid. The major phospholipid fatty acids included palmitic, stearic, and oleic acids.

INTRODUCTION

The identification and subsequent quantitation of lipids from rat brain (1) and peripheral nerve (2) have been performed using conventional techniques. Generally, a preliminary column chromatographic separation is employed to separate the extracts into lipid classes. This usually is followed by thin layer chromatography (TLC) of the column eluants on 20 x 20 cm silica gel plates. Without pooling samples, the high degree of sophistication in conventional lipid chromatography rarely permits the quantitation and characterization of lipids from tissues weighing less than 5 mg.

Using conventional techniques, the lipids of the rat superior cervical ganglia have been identified partially and characterized (3). The size of the rat ganglia (1 mg wet wt), however, has inhibited the analysis and characterization of lipids. Quantitative microtechniques for the determination and analysis of the lipid composition of a single rat superior cervical ganglion would be desirable for metabolic studies. We report here on the separation and quantitation of lipids from rat ganglion and the utilization of methods which permit the analysis of individual lipids on a microscale.

MATERIALS AND METHODS

Excision, Incubation, and Freeze-Drying

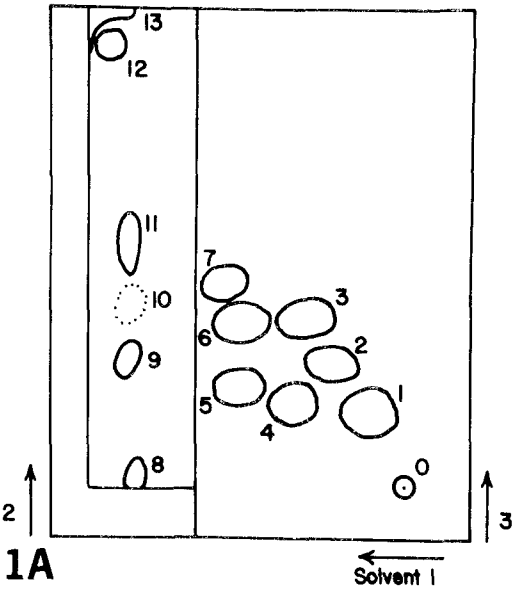
Ganglia were excised from Sprague-Dawley rats (140-180 g) under urethane anaesthesia and the connective tissue sheaths removed in Krebs-Ringer bathing solution aerated with 95% O_2-5% CO_2 (pH 7.2-7.4). Desheathed ganglia were mounted on vertical bipolar platinum electrodes and incubated in specially designed cells constructed in such a fashion as to permit removal and replacement of bathing solutions without disturbing the ganglia (4). Incubations were performed at 37 C in aerated Krebs-Ringer solution (pH 7.2-7.4) modified to contain 2 mM pyruvic acid. Ganglion viability was checked initially and at 20 min intervals by stimulation with a Grass S-4 stimulator at 5.0 volts 5 Hz with 0.5 millisecond duration and the action potentials viewed on a Tektronix 503 cathode ray oscilloscope. Lipid analyses were performed on ganglia which maintained at least 75% of their original action potential during the 20 min incubation. Immediately after incubation ganglia were plunged into liquid N_2 and then freeze-dried at -50 C in aluminum tissue holders (5) for 48 hr. Freeze-dried tissue wt were determined on an electrobalance (model G, Cahn Instrument Co., Paramount, Calif.) in a room with less than 50% relative humidity (6).

Extraction, TLC

Dried ganglia were extracted at 0 C with a tissue to solvent ratio of (1:100) w/v using 20 μliter redistilled methanol in glass angular microhomogenizing tubes fashioned from 5 ml conical centrifuge tubes (3). An additional 10 μliter methanol was used to wash adhering

[1]Submitted in part as a partial requirement for the Ph.D. degree, State University of New York at Buffalo, Buffalo, N.Y.

[2]Present address: Neurosensory Laboratories, 2211 Main Street, State University of New York at Buffalo, Buffalo, N.Y. 14214



1A

FIG. 1A. Thin layer chromatographic separation of neutral lipid and phospholipids from rat superior cervical ganglion: O = Origin, gangliosides; 1 = sphingomyelin, 2 = phosphatidylcholine, 3 = phosphatidylserine, 4 = phosphatidylinositol, 5 = sulfatide, 6 = phosphatidic acid, 7 = phosphatidylethanolamine, 8 = cerebroside, 9 = cholesterol, 10 = diglyceride, triglyceride (not detectable in freze-dried ganglia), 11 = free fatty acids, 12 = cholesteryl esters, and 13 = unidentified.

tissue from the pestles into the mortars. Homogenizers then were capped and centrifuged at 3000 x G for 5 min at room temperature. After centrifugation, the supernatants were removed and placed in siliconized (Siliclad, Clay-Adams, New York, N.Y.) 10 x 75 mm test tubes under N_2 gas also at 0 C. A second extraction at 0 C was performed with 20 μliter chloroform:methanol (2:1) v/v, the pestle rinsed, homogenizers capped and centrifuged as before. Two extractions at room temperature were performed comprising extractions three and four. During the fifth extraction the homogenizers were maintained at 55 C for 5 min, centrifuged, and the supernatants pooled with the previous supernatants. The heating process helped solubilize lipids which may have been sparingly soluble at 0 C (7). The volume of the combined supernatants was adjusted to 100 μliter under N_2 gas. Each extract (95 μliter) was spotted on a 3 1/2 in. x 4 in. micro TLC plate under N_2.

Silica Gel H (Brinkman Instrument, Great Neck, N.Y.) was acid-washed according to Parker and Peterson (8), and a 500 μ thick slurry was spread on 3 1/2 in. x 4 in. glass plates and dried. The slurry was prepared by mixing 30 g Silica Gel H and 60 ml H_2O in a Waring blender for 3 sec. Prior to use, TLC plates were activated at 100 C for 30 min and then cooled in a desiccator. Solvents were allowed to equilibrate in their respective tanks for 45 min prior to use. TLC plates spotted with lipid extracts were desiccated in vacuum 10 min and then developed in solvent 1, chloroform:methanol:water (65:35:4) v/v/v (3 in. direction 8-9 min). The plates then were removed, air-dried 10 min, turned 90°, and run in solvent 2, petroleum ether (bp 36-51 C):diethyl ether:acetic acid (70:30:1) v/v/v, (8 min), and briefly dried. A line then was scratched through the silica gel 1 in. from the left edge of the plate; an area 5/8 in. from the bottom and 1 in. from the left edge was cleared of silica gel (Fig. 1A). This permitted solvent 3 to separate the phospholipids and yet not affect the previously separated neutral lipids. After development in solvent 2, excess solvent and water was removed from the partially developed TLC plates by subjecting them to vacuum (250 μ) for 30 min. The development of TLC plates in solvent 3, chloroform:methanol:diisobutylketone:acetic acid:water (45:15:30:20:4) v/v/v/v/v was in the same direction as solvent 2 and was complete in 20-22 min.

The three solvent chromatography system employed permitted the separation of ganglionic lipids without the prior use of partitioning chromatographic columns and resulting losses of material. Initially, lipid spots were visualized with an assortment of analytical detection sprays, including Rhodamine 6-G, ninhydrin, phosphomolybdate, and 50% sulfuric acid charring. The R_f values of ganglionic lipids were compared with those of pure standard lipids. When experimental ganglia lipid extracts were chromatographed, only 0.005% Rhodamine 6-G spray was used for detection.

To ensure that the lipids identified on TLC plates of ganglion extracts were chromatographically pure, a separate chromatography system was employed. An extract of two ganglia was prepared, chromatographed, eluted, and individual lipids dried with N_2 gas. Lipid elutes were resolubilized with chloroform:methanol (2:1) v/v, spotted on 250 μ Silica Gel G TLC plates (Quantum Industries, Fairfield, N.J.), and developed 15 cm with chlorform:methanol:water (65:25:4) v/v/v. After charring, all rechromatographed lipids appeared as single spots, except phosphatidylethanolamine, which contained two spots. The second slower migrating spot which cochromatographed with phosphatidylethanolamine in our solvent systems may have been a phosphatidylethanolamine plasmalogen or a diglycerolphosphatide, both of which have been identified in nervous tissue

(9, 1). Lysophosphatidylcholine, which upon TLC migrates with an R_f lower than that of sphingomyelin, was not detected in extracts of freeze-dried ganglia. While minor amounts of lysophosphatidylcholine may have been present in ganglia in vivo, the treatment of tissues with liquid N_2 immediately after incubation presumably prevented the degradation of phosphatidylcholine by enzymatic or oxidative means. Phosphatidylinositol also rechromatographed as a single spot. Both di-and tri-polyphosphoinositides have been identified in rat ganglia; however, extraction at 0 C with acidic solvents was necessary to preserve their identity (10). Since polyphosphoinositides deteriorate rapidly at room temperature (11), extraction at the elevated temperatures used in our studies would most certainly cause their degradation.

Aspiration and Elution

After chromatography and visualization, individual lipids were aspirated and eluted from the TLC plates using a modified Swinny filter holder technique (12). A series of 5 ml Luer-lock glass syringes were maintained vertically in a rack by spring clamps. Metal Swinny filter holders were prepared with 13 mm teflon filters (Mitex LSWPO 1300, Millipore Corp., Bedford, Mass.), locked into position on the syringes, and washed with 6 ml chloroform:methanol (1:4) v/v. The washed Swinny holders were removed and vacuum applied so that individual lipids could be aspirated into the hub of the filter holders. The filter holders then were locked on the syringes and eluted with 6 ml chloroform:methanol (1:4) v/v followed by 6 ml absolute methanol. Phosphatidylcholine and sphingomyelin required 18 and 24 ml solvent, respectively, to ensure quantitative elution. The elution of nonpolar neutral lipid materials, such as cholesterol, was initiated with 1 ml chloroform followed by the normal elution solvents. Flow rates were determined by gravity, and elutants were collected in acid-washed 16 x 75 mm glass (teflon lined screw cap) culture tubes under nitrogen. The teflon filters contributed negligably to the phosphate and gas liquid chromatography (GLC) sample blanks, because they contained only 0.1% organic solvent extractable material. In the absence of filtration with Millipore filters, considerable phosphate and GLC blank values were found when BF_3 tranesterification was performed.

Transesterification

Before transesterification, an internal standard, heneicosanoic acid, (2.2 nmoles) was added to each lipid sample. Lipid eluates were

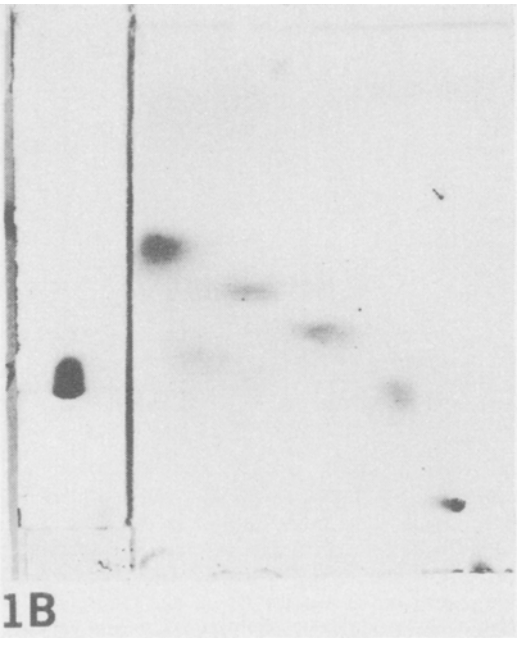


FIG. 1B. Actual charred thin layer chromatography plate illustrating a similar chromatographic separation.

dried with N_2 gas and transesterified with 250 μliter of 14% w/v methanolic-BF_3 solution (13). After addition of the BF_3 reagent, tubes were purged with N_2 gas, capped, and the transesterification completed by heating at 100 C for 20 min (sphingomyelin required 40 min). After heating, tubes were cooled to 0 C; and 250 μliter distilled water and 500 μliter redistilled n-hexane were added, tubes vortexed, and the phases allowed to separate. The upper, or water, methanol saturated n-hexane phase, which contained the fatty acid methyl esters, was removed and placed in acid-washed 10 x 75 mm test tubes. The tubes were placed in a Biodryer (Virtis, Gardiner, N.Y.) and the n-hexane phase dried under vacuum (less than 5 min). GLC analysis of standard fatty acid methyl ester mixtures revealed that losses of less than 2% short chain methyl esters occurred when n-hexane solutions were dired under vacuum for periods of up to 5 min. The dried fatty acid methyl esters then were quantitatively transferred with two 0.2 ml portions of redistilled n-hexane to 3 ml conical centrifuge tubes and similarly dried. Polar contaminants from the transesterification reaction, which were soluble in the water, methanol saturated n-hexane phase remained on the side walls of the test tubes when dry n-hexane was used for the transfer. This transfer step avoided the additional TLC step recommended by Morrison

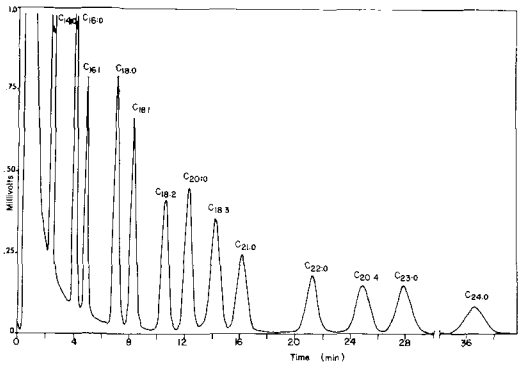


FIG. 2. Gas liquid chromatographic separation of an equal molar mixture of fatty acid methyl esters.

and Smith (13) for the removal of polar contaminants from transesterified samples of fatty acids. The fatty acid sample tubes were then flushed with N_2 gas, capped, and stored at -30 C until analysis by GLC. The recovery of fatty acid methyl esters from the transesterification reaction was based upon the recovery of the internal standard. To determine recoveries, replicate samples of heneicosanoic acid (2.94 nmoles) were added to 16 x 75 mm reaction vials along with samples of synthetic dipalmitol-L-α-lecithin (6.17 nmoles). The samples were transesterified and prepared for GLC injection as previously described. The mean recovery ± standard deviation of heneicosanoate was 2.84 ± 0.06 nmoles, n=4, or 96.7 ± 1.8%, while 12.23 ± 0.41 nmoles or 97% ± 2.0, n=4, of palmitate was recovered. Determination of palmitate using the internal standard and calculating results according to Gehrke and Stalling (14) gave a value of 12.91 ± 0.41 nmoles, n=4.

GLC

Fatty acid methyl esters were separated on a GLC (MT-220, MicroTek Instrument Corp., Austin, Tex.) equipped with dual flame ionization detectors. All glass 1/4 in. outside diameter, 8 ft analytical columns packed with 6% diethyleneglycol succinate on Diatoport S 80-100 mesh were operated isothermally at 185 C, detector 220 C, inlet 195 C, and transfer line 218 C (Fig. 2). The carrier gas was helium 60 cc/min, detector hydrogen 55 cc/min, oxygen 0.1 cfm. Columns and flame sensitivities were calibrated with National Institutes of Health mixture F to which equal molar amounts of fatty acids 16:1, 21:0, 22:0, 23:0, and 20:4 (Supelco, Belfonte, Pa.) were added. Recalibration of GLC columns and detectors was performed after every eight samples. Tentative identification of fatty acid methyl esters was based upon retention time, and their quantification was achieved with the aid of an electronic intergrator CRS-11-HSB (Infotronics Corp., Houston, Tex.).

Water Soluble Products, Neutral Lipid, and Sphingolipid Analyses

Water fractions remaining after the transesterification were dried and the glycerol phosphate bases resolubilized with 100 μliter methanol. A 50 μliter aliquot was dried under vacuum and digested at 185 C for 20 min with 70% perchloric acid. Lipid phosphorus was determined with a Malachite green dye assay (15) and optical density read at 660 nm. The linear range of the assay from 1-10 nmoles permitted the quantitation of low concentrations of lipid phosphorus quickly and reliably.

Cholesterol and cholesteryl ester elutes were dried with N_2 gas and extracted from water five times with equal volumes of diethyl ether. A micro Liebermann-Burchard reaction was performed on the dried ether extracts. Optical densities were determined on a Beckman Du Spectrophotometer (Beckman Instruments Corp., Fullerton, Calif.) at 660 nm in 450 μliter reaction volumes 30 min after initiating the assay. The extraction procedure, which eliminated water soluble contaminants, was over 98% effective based upon the recovery of ^{14}C-cholesterol.

Cerebrosides and sulfatides were estimated colorimetrically as sphingosine with a trinitrobenzene-sulfonic acid assay described by Siakotos (16). Rhodamine 6-G spray used in the visualization of TLC lipids was a contaminant in this assay; and, therefore, estimations, rather than determinations, have been reported. As mentioned by Siakotos (16), it is difficult to spray TLC plates uniformly with visualization reagents which lead to variable errors in this assay. In our laboratory, determinations of free sphingosine with previously reported tri-nitrobenzene-sulfonic acid assays (16, 17) were not reliable; therefore, sphingomyelin was quantitated by assaying for lipid phosphorus.

RESULTS AND DISCUSSION

A typical TLC separation of ganglionic lipids is depicted in Fig. 1B. The separation of phospholipids in the first solvent is initiated, while a class separation of neutral lipids and cerebrosides occurs. Development of the TLC plate in the second solvent separates the neutral lipids along the left edge of the plate but does not cause the migration of either the phospholipids or cerebrosides. The cerebrosides remain isolated on the neutral lipid portion of the TLC plate, because development in the

TABLE I

Concentration of Rat Superior Cervical Ganglionic Lipids[a]

Lipid	Nmoles p/mg tissue dry wt		
Phosphatidylethanolamine	24.4	31.8	33.7
Phosphatidic acid	8.0	8.2	5.3
Phosphatidylserine	10.0	9.9	8.4
Phosphatidylcholine	39.3	51.7	48.5
Phosphatidylinositol	9.0	8.9	7.1
Sphingomyelin[b]	29.9	23.4	29.1
Cerebroside[c]	2.1		
Sulfatide[c]	2.4		
Cholesterol[d]	57.1	56.3	53.7
Cholesteryl esters[d]	11.5	12.2	10.8

[a]Results are expressed as nmoles p/mg tissue dry wt for ganglia incubated 20 min in Krebs-Ringer buffer.

[b]Assayed as a phospholipid.

[c]Mean estimate as free sphingosine by the method of Siakotos (16).

[d]Determination by Liebermann-Burchard assay.

third solvent is accomplished only on the right hand portion of the plate. Lipid extracts from 1 or 2 ganglia (ca. 50-100 nmoles) separate well on micro TLC plates as long as the diameter of the origin spot remains small. Overloading these plates with excessive lipid material, however, results in trailing of lipid spots and their incomplete separation.

The lipid composition of rat superior cervical ganglia is presented in Table I. As in rat brain (1), the most predominant phospholipids are phosphatidylcholine and phosphatidylethanolamine, with lower concentrations of phosphatidylserine and phosphatidylinositol. Phosphatidic acid is present in the lowest concentration of the glycerophospholipids in both rat ganglia and rat brain (1). Sphingomyelin is the most predominant sphingolipid and represents 53% of that class. Estimations for both cerebrosides and sulfatide indicate that they are present in moderate concentrations in rat ganglia. As previously determined, the mean concentration of gangliosides, which remain at the origin in our TLC system, is low; 0.3 ± 0.02 nmoles/mg wet wt (18). The neutral lipids were found predominantly as cholesterol with lesser

TABLE II

Tentative Fatty Acid Composition of Lipids in Rat Ganglia[a,b]

Fatty acid[a]	PE	PA	PS	PC	PI	Sph
14:0	7.98	11.09	6.89	7.12	6.75	11.38
15:0	1.85	4.98	2.79	3.82	3.01	8.87
16:0	28.86	43.45	33.28	41.45	26.71	31.98
16:1	5.06	7.75	6.58	7.22	18.36	14.05
17:0	2.99	1.59	1.21	0.80	1.41	0.34
18:0	27.29	15.61	21.85	10.46	11.85	14.40
18:1	15.46	7.85	15.22	20.78	17.30	4.35
18:2	3.56	3.59	2.32	1.64	3.47	5.47
20:0	1.78	0.72	0.52	0.61	0.32	4.74
18:3	0.42	1.38	1.10	1.17	3.32	0.30
21:0[c]	—[d]	—	—	—	—	—
22:0	—	2.05	2.79	0.11	1.94	1.85
20:4	4.70	1.54	1.54	3.16	3.60	—
23:0	—	0.10	0.10	—	0.32	—
24:0	—	3.39	3.95	1.64	1.59	2.24

[a]Results are expressed as a wt % methyl ester value from three pooled ganglia incubated for 20 min. Fatty acids are listed by carbon number in order of elution from gas liquid chromatography.

[b]PE = phosphatidylethanolamine, PA = phosphatidic acid, PS = phosphatidylserine, PC = phosphatidylcholine, PI = phosphatidylinositol, and Sph = sphingomyelin.

[c]Internal standard.

[d]— = not detected, above trace levels.

TABLE III

Recovery of Phosphatidylinositol from Aspriation and Elution Procedures[a]

	Experiment	Disintergrations/min. ± standard deviation, n=4	Average recovery %
1.	Sample ^{14}C PI[b] placed in vial and counted	2435 ± 100	100.0
2.	Sample ^{14}C PI applied to TLC plate scrapped into vial and counted	2479 ± 80	100.1
3.	Sample applied to thin layer chromatography plate aspirated and eluted with 6 ml C:M (1:4) v/v	2146 ± 110	88.5
	a) Sample then eluted with 6 ml MeoH	112 ± 25	4.6
	b) Sample then eluted with another 6 ml MeOH	66 ± 6	2.7
	c) Silica gel and filter	67 ± 14	2.7
		Recovery 3,a,b,c	98.5

[a]Recovery for the aspiration and elution of phosphatidylinositol was determined as follows: replicate samples of ^{14}C-phosphatidylinositol were applied to a thin layer plate. An area of silica gel, including the radioactive samples, then was aspirated and eluted into scintillation vials. The elution solvent then was dried, scintillation fluid added, and radioactivity determined in a Nuclear Chicago Mark I liquid scintillation counter. In addition, the eluted silica gel and teflon filter were placed in a separate vial and 15 ml 4% solution of thixotropic gel, Cabosil (Packard Instrument Co., Downers Grove, Ill.) was added to disperse the material for proper counting.

[b]PI = phosphatidyl inositol.

amounts of cholesteryl esters. Free fatty acids identified in Fig. 1A were not found in freeze-dried tissue above trace levels.

The tentative fatty acid composition of ganglionic phospholipids can be found in Table II. Palmitic, stearic, and oleic acids were present in the highest concentrations. Palmitic acid was the most predominant saturated fatty acid with lesser amounts of stearic and myristic acids. The unsaturated fatty acids were composed primarily of oleic acid with lower concentrations of polyunsaturated C:18 fatty acids. Moderate amounts of palmitoleic acid were found in most samples, while phosphatidylinositol and sphingomyelin contained higher levels. The fatty acid composition of two samples of cerebrosides and sulfatides proved to be similar to that of sphingomyelin.

The concentrations of the lipids determined from freeze-dried rat ganglia resemble those of rat brain (1) and wet weighed ganglia (3). When approximations from dry to wet weighed ganglionic tissue are made, dry tissue representing 19.23% wet wt, our values for phosphatidylcholine and phosphatidylethanolamine are lower than those previously determined (3). Analysis of our techniques revealed that the esterification procedure resulted in the production of 2 moles fatty acid methyl ester/mole of phosphorus and was 97% complete as judged by the recovery of the internal standard. The recovery of radioactive lipids eluted after chromatography on TLC plates was 92-96% complete for all phospholipids. A summary of the elution methodology, using phosphatidylinositol as an example, can be found in Table III. The label (88%) was eluted with 6 ml chlororform:methanol (1:4) v/v (19, 20). Lesser amounts of radioactivity were eluted with succeeding volumes of methanol until 97% recovery occurred. After elution, only 3% label remained on the silica gel and filter. Similarly, the elution of phosphatidylethanolamine was 96%, while that of phosphatidylcholine was 95% following elution with 18 ml solvent.

The observed differences in lipid concentration may be due to alterations in the extractability of lipids from wet and freeze-dried tissues. Svennerholm (21) extracted lypholized tissues with boiling chloroform:methanol (2:1) for 2 hr and achieved 100% recovery. When we compared the extraction of lipids from wet and freeze-dried ganglia, we found only 70% lipid phosphorus was extracted from dried tissues by our method. Based upon the extractability of wet tissues, the addition of 2% water to the chloroform:methanol extraction solvent resulted in essentially complete extraction of freeze-dried ganglia. Some autolysis of lipid samples probably occurred during the extraction and chromatography procedures. While N_2 gas was used when possible, the use of butylated hydroxytoluene in extraction solvents has been reported (10) and may help to

minimize the oxidation of lipid samples during extraction. However, when lipids were eluted from TLC plates with solvents containing butylated hydroxytoluene and then esterified, an extraneous peak appeared in GLC chromatograms. The artifactural peak had a retention time corresponding to methyl heptadecanoate. Finally, species variations may account for some of the observed differences since we used an inbred strain of Sprague-Dawley rats raised in our laboratory.

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Effect of Subacute Toxic Levels of Dietary Cyclopropenoid Fatty Acids upon Membrane Function and Fatty Acid Composition in the Rat[1]

J.E. NIXON, T.A. EISELE, J.H. WALES, and **R.O. SINNHUBER,** Department of Food Science and Technology, Oregon State University, Corvallis, Oregon 97331

ABSTRACT

The effect of subacute toxicity levels of dietary cyclopropenoid fatty acids upon several physiological parameters was determined in the rat. Diets containing 2% corn oil, 2% *Sterculia foetida* oil or 2% hydrogenated *Sterculia foetida* oil were fed. *Sterculia foetida* oil (50% cyclopropenoid fatty acids) fed rats exhibited retarded growth, elevated organ to body wt ratios, increased saturation of tissue lipid, and abnormal histopathology when compared to corn oil and hydrogenated *Sterculia foetida* oil fed rats. Growth was retarded 50%, liver/body wt doubled, and the percentage of saturated fatty acids in adipose tissue increased 2.5-fold for *Sterculia foetida* oil vs. corn oil comparisons. Three membrane systems were examined in corn oil and *Sterculia foetida* oil fed rats. Erythrocyte hemolysis rate in 0.3 M glycerol was increased by 30%; induction of mitochondrial swelling by reduced glutathione was inhibited completely and microsomal codeine demethylase activity was depressed nearly 50% in *Sterculia foetida* oil fed rats. The ability of cyclopropenoid fatty acids to inhibit fatty acyl desaturase and influence tissue and membrane lipid composition is discussed. Most of the detrimental effects observed in cyclopropenoid fatty acids fed rats may be associated with alteration of normal lipid metabolism and membrane function.

INTRODUCTION

Dietary cyclopropenoid fatty acids (CPFA) are responsible for a variety of biological effects in several species of animals. This subject, reviewed by Phelps, et al. (1), included retarded growth in rats and chicks, delayed sexual development in female rats, altered fatty acid metabolism, and pink discoloration to avian egg whites. Recently Sinnhuber and coworkers (2-4) demonstrated a cocarcinogenic activity for CPFA when fed with aflatoxin to rainbow trout. Miller, et al., (5) reported a high incidence of prenatal and postnatal mortality in progeny of CPFA fed rats. They found degenerative changes in several organs, but hemorrhage in lung alveoli was the immediate cause of infant mortality. They suggested that altered membrane permeability and increased capillary fragility was responsible for the detrimental effects of CPFA. The discoloration in egg white was attributed to increased permeability of the vitelline membrane (6).

Several groups (7-9) have shown that dietary CPFA inhibit acyl desaturase causing the stearate to oleate ratio to rise. Since variations in lipid composition alter permeability to membrane systems (10-12) and CPFA change the ratio of saturated to unsaturated fatty acids in tissue, CPFA may influence the composition and function of membrane structure.

In the hope of elucidating the mode of action of CPFA, experiments were set up to examine the effect of subacute levels of dietary CPFA on three membrane systems in the young rat. Hemolysis of erythrocytes was used to assess changes in membrane permeability and fragility. Swelling of liver mitochondria was used to evaluate detrimental changes in mitochondrial membranes. The activity of codeine demthylase, a membrane-bound drug metabolizing enzyme found in liver microsomes, was assayed to evaluate microsomal membrane integrity. Fatty acid composition of adipose tissue and liver subcellular fractions was measured to determine the general effect of CPFA upon fatty acid composition in these rats.

MATERIALS AND METHODS

Experimental Animals

Weanling, Wistar strain rats (24), 12 of each sex, were fed a semipurified diet containing either 2% corn oil (CO) or 2% *Sterculia foetida* oil (SFO) for 6 months. The diet consisted of 24.0% casein, 67.5% glucose, 4.3% mineral, 2.2% vitamin mix, and 2% oil. A premix of vitamin-free casein, glucose, and mineral mix 4164 was purchased from Nutritional Biochemical Corp. (NBC) Cleveland, Ohio. Vitamin mix was NBC vitamin fortification mixture. Diet

[1]*Technical paper 3638, Oregon Argricultural Experiment Station, Oregon State University, Corvallis, Oregon 97331*

consumption of these rats was measured. Another group of 10 rats, 5 of each sex, were fed a diet containing 2% mildly hydrogenated SFO for 4 months.

Hemolysis Studies

Hemolysis of erythrocytes in 0.3 M glycerol and thiourea and diethylene glycol was measured in a recording spectrophometer (11). Fresh blood (20 μliter) from the tail vein was hemolyzed in 3 ml solution at 25 C. The maximum rate of hemolysis was determined from the slope at the inflection point of the sigmoid hemolysis curve obtained from the spectrophometer.

Tissue Preparation

Rats were fasted for 12 hr and sacrificed by decapitation. Body and organ wt were recorded, and tissue was saved for histology, lipid analysis, and liver subcellular preparations. Mitochondria and microsomes were prepared from 4 g liver homogenized (Potter-Elvehjem tube) in 16 ml 0.44 M sucrose containing 2.3% 0.1 M citric acid. The homogenate was diluted with 16 ml 0.44 M sucrose and centrifuged at 500 x g for 20 min to remove the nuclei. Mitochondrial sediment, obtained by centrifugation of the 500 x g supernatant at 15,000 x g for 10 min, was washed in 25 ml 0.44 M sucrose and resedimented at 15,000 x g. The washed pellet was suspended in 6 ml 0.44 M sucrose and used for mitochondrial swelling assays within 3 hr. The 15,000 x g supernatant (10 ml) was centrifuged at 105,000 x g for 60 min and the resultant microsomal pellet was suspended in 2 ml 0.15 M KCl. The nuclei and 105,000 x g supernatant fractions and unused mitochondrial and microsomal preparations were saved for lipid analysis.

Mitochondrial Swelling

Swelling of liver mitochondria was measured as a decrease in absorption at 520 nm at 30 C in a recording spectrometer equipped with a temperature controlled cell chamber (13). Enough mitochondrial solution ($\simeq$ 0.10 ml) to give an initial absorption of 0.7 was allowed to swell in 3 ml 0.05 M Tris HCl, pH 7.4, 0.25 M, sucrose or Tris-sucrose buffer plus 0.005 M reduced glutathione (GSH).

Codeine Demethylase Assay

A modified procedure of Cochin and Axelrod (14) was used to measure demethylation of codeine by formaldehyde formation. The assay system consisted of: 100 μmoles Tris HCl, pH 7.4; 6 μmoles codeine; 50 μmoles nicotinamide; 25 μmoles $MgCl_2$; 50 μmoles neutral semicarbazide; 0.3 μmoles nicotinamide adenine dinucleotide phosphate oxidized form (NADP); 4.0 μmoles glucose-6-phosphate; 0.3 units glucose-6-phosphate dehydrogenase; 5.0 μmoles nicotinamide adenine dinucleotide, reduced form (NADH); and 4 mg microsomal protein in a final volume of 3.0 ml. The assay system was incubated for 1 hr at 38 C in a metabolic shaker. The reaction was stopped by addition of 0.2 ml 20% $ZnSO_4$; 0.5 ml saturated $Ba(OH)_2$ was added, and the solution was centrifuged to sediment the protein. Clear supernatant (1 ml) was mixed with 1 ml Nash reagent (15) and heated at 50 C for 20 min. The solution was cooled and read at 412 nm to determine the amount of formaldehyde present. Results were expressed as μmoles of formaldehyde released/hr/mg protein or g liver.

Protein Analysis

Protein was assayed by the biuret method of Gornall, et al., (16) using bovine serum albumin (BSA) as a standard.

Lipid Extraction

SFO was extracted with hexane from ground *S. foetida* seeds obtained from the Phillipines. The SFO oil contained ca. 50% CPFA. Lipid was extracted from rat tissue by the method of Folch (17). Subcellular fractions from liver were extracted by the Bligh and Dyer procedure (18).

Lipid Analysis

CPFA were assayed by the Halphen reaction as described by Hammonds et al. (19). When CPFA and fatty acids were measured together in a sample, the NMR method of Pawlowski, et al., (20) or the gas liquid chromatography (GLC) procedure of Schneider, et al., (21) was used. Fatty acid composition was determined by GLC of boron trifluoride or sodium methoxide derived methyl esters on a 15% DEGS, Chromosorb column at 185 C. SFO was hydrogenated mildly to reduce the cyclopropene ring with a minimum hydrogenation of other unsaturated bonds. The oil was heated under N_2 in a Brown hydrogenator at 100-110 C overnight using Rainey nickel (22) as a catalyst. This treatment reduced the CPFA content from 49.2 to 1.5% while the iodine number decreased from 86.0 to 54.3. The corn oil (CO) iodine number was 116.

Histopathology

Liver, lung, and kidney tissues were fixed in Bouin's solution. Tissue sections were cut at 4 μ and stained with hematoxylin and eosin.

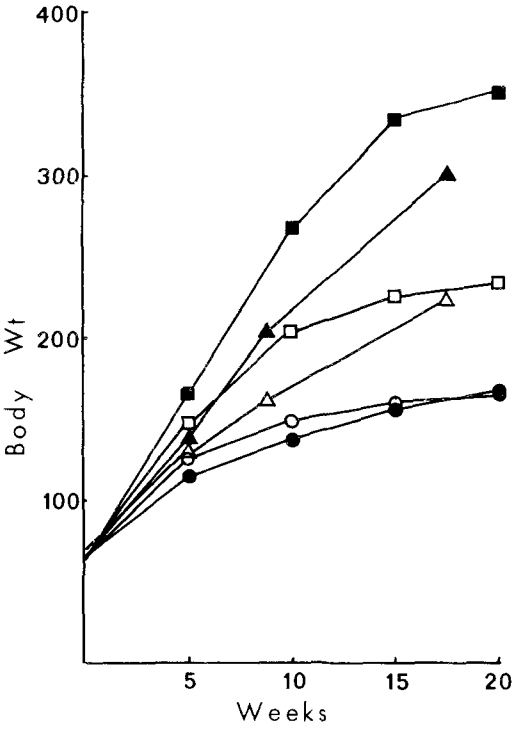

FIG. 1. Growth curves of rats are designated as follows: corn oil females □, males ■; hydrogenated *Sterculia foetida* oil females △, males ▲; *Sterculia foetida* oil females ○, males ●.

RESULTS

Growth and General Health

Growth was retarded sharply in SFO fed rats (Fig. 1), while the hydrogenated *Sterculia foetida* oil rat growth pattern resembled the CO fed group. The normal difference in growth between males and females occurred in the HSFO rats but not the SFO rats. Apparently the SFO diet was more toxic to male than female rats. Diet consumption was depressed in the SFO rats during the first 12 weeks. The consumption was the same for CO and SFO males or females thereafter to 24 weeks. Low feed intake was not the only factor in the poor growth of the SFO rats during the first 12 weeks. The average amount of feed required to produce a g of wt gain was 10.6 g for SFO rats compared to 5.7 g for CO rats. SFO rats were not using the diet efficiently. The SFO rats had an unthrifty general appearance in contrast to the healthy slick coat appearance of the CO and HSFO rats.

Red Blood Cell (RBC) Hemolysis

After 6 months, CO and SFO rats were subjected to hemolysis studies on 1 day and sacrificed for subsequent studies on the following day. Groups of 3 CO and 3 SFO rats were processed on each 2 day period. Table I shows the results of erythrocyte hemolysis studies. The RBC from SFO rats hemolyzed 32% ($p < 0.05$) faster than RBC from controls in glycerol. The relatively large standard deviation of these assays was caused by day to day variation. On a given day, differences between groups were consistent. Differences in hemolysis rates between control and SFO rats for thiourea were not apparent. Control RBC hemolyzed significantly faster ($p < 0.05$) than RBC from SFO rats in diethylene glycol.

TABLE I

Hemolysis of Rat Red Blood Cell in Nonelectrolytes

Nonelectrolyte	Δ OD/min[a]	
	CO[b]	SFO[b]
0.3M glycerol	5.9 ± 2.0[c]	7.8 ± 2.4
0.3M thiourea	3.2 ± 0.3	3.2 ± 0.5
0.3M diethylene glycol	11.6 ± 1.5[c]	10.1 ± 1.9

[a]ΔOD (600 nm)/min at 25 C at inflection point of hemolysis curve of 20 μliter blood in 3 ml solution. Mean ± standard deviation. p-Values refer to significance of differences between corn oil (CO) and *Sterculia foetida* oil (SFO) rats for the same treatment using the *t* test.

bControl (CO) is the mean of 24 rats; cyclopropenoid fatty acids (SFO) is the mean of 21 rats.

[c]$p < 0.05$.

Body and Organ Wt

Body wt, organ wt, and organ wt expressed as a percentage of body wt are tabulated in Table II. Except for liver, organ wt of SFO rats were generally less than CO and HSFO rats. In contrast, the percentage organ wt for SFO rats was higher than the other groups for all organs. The average percentage organ wt of SFO liver, kidney, spleen, and heart was higher than control tissue by 114, 47, 40 and 53%, respectively. The organ wt data are the same for male and female in the SFO rats, while CO and HSFO rats exhibited the usual sex difference. HSFO wt data were similar to the CO data, even though the HSFO rats were 2 months younger.

Mitochondrial Swelling

The ability of freshly prepared mitochondria to swell in 0.05 M Tris HCl, 0.25 M sucrose, pH 7.4 buffer or in Tris-sucrose buffer containing 0.005 M GSH was measured. Table III shows that swelling in buffer was nearly the same for CO and SFO mitochondria. When swelling was done in buffer plus GSH, the swelling increased

TABLE II

Body and Organ Wt

Diet[a]	Body wt	Liver		Kidneys		Heart		Spleen	
	g	g	%[b]	g	%	g	%	g	%
Females									
CO	248	5.84	2.35	1.42	0.57	0.81	0.33	0.45	0.18
HSFO	222	6.83	3.08	1.43	0.64	0.79	0.36	0.55	0.25
SFO	177	8.65	4.89	1.45	0.82	0.72	0.41	0.44	0.25
Males									
CO	353	7.91	2.24	1.90	0.54	1.10	0.31	0.66	0.19
HSFO	301	6.96	2.31	1.75	0.58	0.89	0.30	0.67	0.22
SFO	176	8.67	4.93	1.43	0.81	0.67	0.38	0.47	0.27

[a]CO = corn oil, HSFO = hydrogenated *Sterculia foetida* oil, and SFO = *Sterculia foetida* oil.

[b]Percent of body wt.

31% in the control and decreased 14% in the SFO mitochondria. Consequently, SFO mitochondrial swelling induced by GSH was only 60% ($p < 0.05$) of control. The protein concentration of the SFO mitochondrial preparation was 40% ($p < 0.05$) below the control and probably is a reflection of the enlarged liver in the SFO rats.

Codeine Demethylation

The activity of codeine demethylase, a microsomal membrane-bound enzyme, is shown in Table IV expressed/unit of protein and liver. Each assay contained the same amount of protein. The difference in activity of microsomes prepared from CO and SFO rats was 17% ($p < 0.10$) and 47% ($p < 0.05$) when activity was expressed on the basis of protein and liver, respectively. The protein content of the SFO microsomal preparation was only 60% ($p < 0.05$) of the control as was the case in the mitochondrial preparations.

TABLE III

Mitochondrial Swelling

Swelling medium	Δ OD/5 min[a]	
	CO	SFO
0.05 Tris HCl, pH 7.4 0.25 sucrose buffer	17.3 ± 4.3 (24)[b]	15.8 ± 4.2 (18)
Tris-sucrose buffer 0.005 GSH[c]	22.7 ± 6.4[d] (17)	13.6 ± 4.4 (12)
Mitochondrial protein (mg/ml)	12.1 ± 2.7[d] (24)	7.1 ± 1.6 (18)

[a]ΔOD (520 nm)/5 min at 30 C x 100. Added mitochondria to give initial OD of ca. 0.7. Mean ± standard deviation. p-Values refer to significance of differences between corn oil (CO) and *Sterculia foetida* oil rats for the same treatment using the *t* test.

[b]Number of rats assayed.

[c]GSH = reduced glutathione.

[d]$p < 0.05$.

Lipid Analysis

The fatty acid composition of adipose tissue and the dietary oil for rats fed CO, SFO, and HSFO is tabulated in Table V. The sum of the saturated fatty acids 14:0, 16:0, and 18:0 for SFO rat adipose lipid is 2.4 times that of the CO controls. This is not a reflection of the saturation of the dietary oil but the result of inhibition of fatty acid desaturation by CPFA. The saturated acids in dietary SFO are 24% of the total fatty acids compared to 67% for SFO rat adipose tissue. Hydrogenation lowered the CPFA content from 49.2 to 1.5% in the HSFO and resulted in a 17% decrease in the percent saturation and a 25% increase in 16:1 and 18:1 adipose fatty acids. Note that 16% adipose lipid in SFO fed rats was CPFA.

Table VI shows that differences in percent saturation of lipid in liver subcellular fractions

TABLE IV

Microsomal Codeine Demethylase Activity

Demethylase activity	CO[a]	SFO[a]
μg HCHO/mg Protein/HR	1.8 ± 0.6[b]	1.5 ± 0.5
μg HCHO/g LIver/HR	25.1 ± 7.6[c]	13.3 ± 4.9
Microsomal protein mg/ml	14.5 ± 2.6[c]	8.9 ± 1.6

[a]Control is the mean of 24 rats; Cyclopropenoid fatty acids is the mean of 18 rats. Values are mean ± standard deviation. p-Values refer to differences between corn oil (CO) and *Sterculia foetida* oil (SFO) rats for the same treatment using the *t* test.

[b]$p < 0.10$.

[c]$p < 0.05$.

TABLE V

Percentage Fatty Acid Composition[a]

Fatty acid	Rat adipose tissue			Dietary oil		
	CO	HSFO	SFO	CO	HSFO	SFO
14:0	1.6	2.4	2.0	—	—	—
16:0	24.4	34.7	40.7	11.2	23.5	21.3
16:1	11.5	5.3	2.0	0.7	0.4	1.9
18:0	1.6	12.5	24.2	2.1	6.5	2.2
18:1	45.2	34.4	12.7	27.3	13.5	8.2
18:2	16.2		2.1	58.8	11.9	9.7
Cyclopropane		7.4[b]			33.1	
CPFA	—	1.5	16.2		1.5	55.6[c]
Sum of saturated fatty acids[d]	27.6	50.0	66.9	13.3	30.0	23.5

[a]CO = corn oil, HSFO = hydrogenated *Sterculia foetida* oil, SFO = *Sterculia foetida* oil, and CPFA = cyclopropenoid fatty acids.

[b]Sum of 18:2 and cyclopropane fatty acids which could not be separated by gas liquid chromatography.

[c]SFO was assayed by Schneider procedure (21). Halphen assay gave a value of 49.2% CPFA.

[d]Sum of 14:0, 16:0 and 18:0 fatty acids.

for CO and SFO fed rats were not as dramatic as in adipose lipid. Percent saturation did increase by at least 8% in all fractions.

Histopathology

CPFA had a marked effect upon the pathology of liver and kidney tissues. The liver parenchymal cell nuclei of a majority of the rats fed SFO were shriveled (Fig. 2A). In some livers there were areas of necrosis (Fig. 2B). Focal degeneration of the kidney tubules was common in the SFO group (Fig. 2C), and droplets of an olive-brown substance were present in the epithelial cells of many tubules (Fig. 20). By microscopic visualization, it was estimated that the tubules of the SFO fed rats contained more than three times the quantity of these droplets found in the controls. The average age of the SFO fed rats was less than the controls, and the sexes were ca. evenly divided. The degeneration of the tubule epithelium and its replacement by droplets is not due to aging or sex.

DISCUSSION

The effect of subacute toxicity levels of dietary CPFA on several physiological parameters was determined in the rat. Diets containing 2% CO, 2% SFO (1% CPFA), or 2% HSFO were compared.

Growth was retarded sharply in SFO fed rats, and male rats were affected more than females. Rats fed SFO with the cyclopropene hydrogenated to cyclopropane grew much better than rats fed untreated oil and grew at a rate comparable to CO fed rats after they recovered from an initial lag in growth. Although feed consumption by the SFO rats was initially 25% below the controls, inefficient feed conversion was responsible for much of the slow growth. SFO rats required 10.6 g feed/g wt gain compared to 5.7 for CO fed rats. Feed consumption for the 2 groups was ca. equal during

TABLE VI

Percentage Saturated Fatty Acids[a] in Liver Subcellular Fractions

Diet[b]	Nuclei	Mitochondria	Microsomes	Soluble supernatant
CO	41.4	41.6	50.4	38.4
SFO	58.0	53.6	58.8	61.9
Percent increase	16.6	12.0	8.4	23.5

[a]Sum of 14:0, 16:0, and 18:0 fatty acids.

[b]CO = corn oil and SFO = *Sterculia foetida* oil.

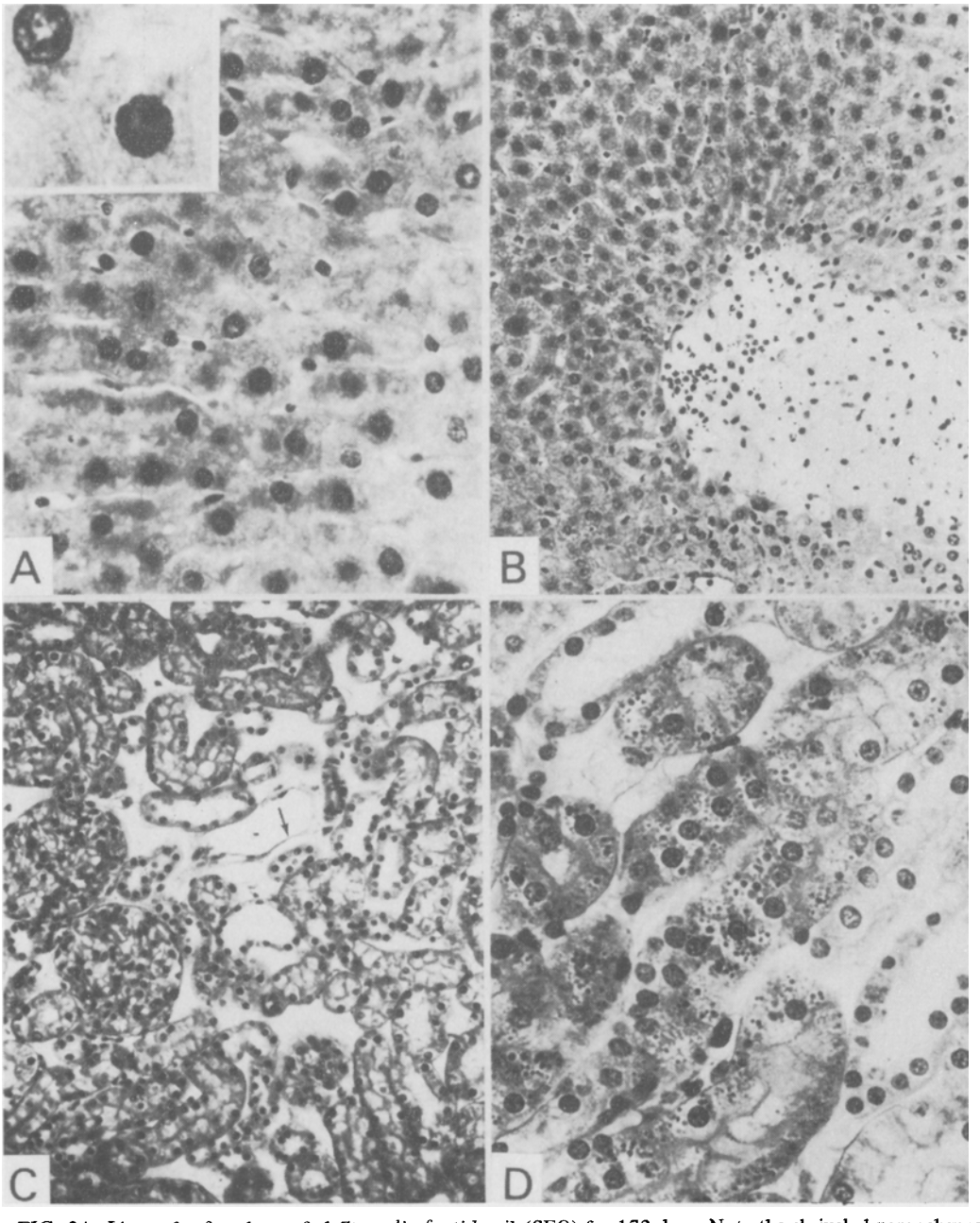

FIG. 2A. Liver of a female rat fed *Sterculia foetida* oil (SFO) for 172 days. Note the shriveled parenchymal cell nuclei. H. & E. stain. X560. Inset: detail of nuclei. X1400. 2B. Liver of a male rat fed SFO for 189 days, showing a necrotic focus. H. & E. stain. X250. 2C. Kidney of a female rat fed SFO for 188 days. Arrow indicates the connective tissue surrounding a degenerate tubule. H. & E. stain. X250. 2D. Kidney tubules of a female rat fed SFO for 190 days. Note the degeneration of the epithelial cells and their replacement by droplets of an olive-brown substance. H. & E. stain. X560.

the last 12 weeks of the trial, but SFO rats never recovered the wt difference and always appeared emaciated. SFO rats developed fragile bones and several broke bones during handling in the hemolysis assays. Until a special holding apparatus was built to allow the rear legs to kick freely, nearly every SFO rat broke one or both tibia bones.

The essential fatty acid (EFA) content of CO and SFO diets was ca. 3.0 and 0.5% dietary calories, respectively. Intake of EFA by SFO rats may have been inadequate and could have contributed to the observed CPFA effects. However, SFO and HSFO rats received ca. the same level of EFA in the diet; and HSFO rats did not exhibit a marked CPFA effect. Although SFO rats did consume less total EFA than HSFO rats, because of differences in feed consumption, the SFO rats did not develop symptoms of EFA deficiency.

Organ wt relative to body wt for all measured tissue was elevated in SFO fed rats. The value for liver was more than double the control. The enlarged organs in SFO fed rats is attributed to the toxic effect of CPFA and not to retarded growth. Feron, et al., (23) demonstrated that simple growth retardation does not affect organ to body wt ratios in the organs compared in this experiment. HSFO fed rats did not exhibit the extensive elevation of relative organ wt, demonstrating that lowering the CPFA content lowers the effects observed in SFO fed rats.

Histological examination revealed that the two most enlarged organs in SFO rats, liver and kidney, also had extensive tissue degeneration. This work confirms that CPFA do cause degenerative changes as reported by Miller, et al. (5). However, capillary fragility and hemorrhage in liver, kidney, and lungs was not as pronounced in these rats as in prenatal and postnatal rats, as reported by Miller, et al., (5) and previously observed in this laboratory.

The saturation of tissue lipid was increased dramatically in SFO fed rats. Other workers (7-9, 24) have reported that dietary CPFA increased lipid saturation by inhibiting fatty acid desaturation. The observed change from 28 to 70% saturation in adipose tissue was much more pronounced than previous reports. However, the conditions in this experiment were more severe, since no unsaturated fatty acids, other than those present in SFO, were provided. Mild hydrogenation of the SFO reduced the CPFA effect, indicating that inhibition of desaturation was reduced. The ratio of 16:0 to 16:1 and 18:0 to 18:1 was lowered from 20.3 to 6.5 and from 1.9 to 0.4, respectively, when HSFO replaced SFO in the diet.

The change in lipid saturation of liver subcellular fractions was less than in adipose tissue. Presumably, the animal would attempt to maintain the lipid composition of essential tissue in a functional state. When CPFA elevates the lipid saturate to unsaturate ratio, the less plentiful unsaturated fatty acids would be used to maintain lipid structural integrity and the excess saturated fatty acids would be metabolized or stored in adipose tissue. Changes of 8 and 12% observed in liver microsomal and mitochondrial lipid, respectively, may be severe enough to impair liver function.

Stimulation of swelling in liver mitochondria from CPFA fed rats by GSH was impaired sharply. The main action of GSH in swelling induction is stimulation of peroxide formation in unsaturated lipids of the mitochondrial membranes (13). The 12% reduction in unsaturated fatty acids in SFO mitochondria might have contributed to the failure of GSH to induce swelling by reducing conditions for peroxide formation in the membranes. Another function of GSH in swelling is stimulation of electron transport dependent swelling by serving as a redox agent. CPFA have been shown to react with thiol compounds (25) and conceivably could reduce mitochondrial swelling by interfering with thiol or disulfide groups involved in electron transport or by interfering with the redox activity of GSH.

In the SFO microsomes, the lipid saturation was 8% more, and the protein concentration was 40% less than in control microsomes. Ernster (26) reported that the activity of tightly bound microsomal enzymes varied according to the structural state of the microsomes. Siekevitz (27) further surmised that anything which primarily affects the lipid portion of the membranes may secondarily affect activity of enzymes residing in the membranes. Thus, structural changes in microsomal membranes caused by changes in lipid composition and lipid to protein ratio may explain the lower activity of membrane-bound codeine demethylase in microsomes from CPFA fed rats.

The faster hemolysis rate of erythrocytes in glycerol from SFO fed rats also may be explained by the ability of CPFA to inhibit fatty acid desaturation and alter lipid composition in the membranes. Kogle, et al., (10) postulated that fatty acid composition in erythrocyte membranes determines permeability to glycerol. They found that hemolysis time in isotonic glycerol of erythrocytes from several species of mammals increased as the proportion of unsaturated fatty acids increased in the cell ghosts. Walker and Kummerow (11) reported that erythrocytes from rats fed hydrogenated coconut oil hemolyzed faster in glycerol than cells from CO fed rats. As the dietary linoleic acid increased, the erythrocyte content of linoleic and arachidonic acids increased; and rate of hemolysis decreased. A decrease in the unsaturated fatty acid content of erythrocyte membranes caused by dietary CPFA may be responsible for higher rate of hemolysis in

glycerol. An increase in the saturation of the cell wall lipid also would make the membrane more rigid and reduce its ability to flex under osmotic pressure. The control of permeability to nonelectrolytes by the erythrocyte membrane is complex (28), so it is difficult to explain why thiorea and diethylene glycol hemolysis did not exhibit a CPFA effect similar to glycerol.

Rats fed SFO exhibited retarded growth, elevated organ to body wt ratios, increased saturation of tissue lipid, degeneration of cellular structure, and altered performance of three membrane systems when compared to CO fed controls. Destruction of the CPFA in SFO eliminated much of the differences between CO and SFO fed rats. Most of the physiological effects observed in CPFA fed rats may be associated with disruption of normal lipid metabolism via inhibition of fatty acid desaturation. In all three membrane systems, erythrocyte hemolysis, mitochondrial swelling, and microsomal enzyme activity, the data may be explained in terms of changes in lipid composition of membranes resulting from dietary CPFA. Speculation in this discussion assumes that lipid composition of a tissue fraction is representative of membranes residing in the tissue. The membranes in question should be isolated in pure form and assayed to determine whether CPFA do alter the lipid composition of essential membranes. A malfunction in these essential membranes would be followed by deterioration of other processes and the general health of the animal.

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Effects of Different Culture Media and Oxygen upon Lipids of *Escherichia coli* K-12

WILLIAM F. NACCARATO, Department of Biochemistry, Faculty of Arts and Sciences, and **JOHN R. GILBERTSON** and **ROSE A. GELMAN,** Department of Pharmacology and Physiology, School of Dental Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

ABSTRACT

The effects of altering the chemical composition of the culture media and the oxygen content of the environment upon the lipid metabolism of *Escherichia coli* K-12 were investigated. When *E. coli* cells were grown on the same culture medium but under aerobic and anaerobic conditions, an increase in the free fatty acids of anaerobically grown cells was observed with a disproportionate increase in the unsaturated fatty acids. When glucose was the sole carbon source, both fatty alcohols and hydrocarbons were detected as component lipids of these cells, whether growth occurred under aerobic or anaerobic conditions. Based upon this observation, acetate is considered the initial precursor for fatty alcohol and hydrocarbon biosynthesis. A possible metabolic pathway involving fatty alcohols in hydrocarbon synthesis has been postulated.

INTRODUCTION

Fatty alcohols of *Escherichia coli* K-12 have been characterized as 1- and 2- alkanols. A preliminary investigation into the metabolism of these alcohols implicated oxygen as a regulatory factor. When *E. coli* were grown anaerobically, there was a quantitative decrease in the total alcohol content. Gas chromatographic analysis showed this decrease resulted mainly from a selective loss of 2-alkanols (1).

Taking into consideration the effect of oxygen upon the 2-alkanols, it was hypothesized that they were synthesized by hydrocarbon oxidation (1), a process known to require molecular oxygen (2). The hydrocarbon substrates were thought to be derived from the media (analysis of the trypticase soy broth [TSB] media showed compounds that had properties of hydrocarbons on thin layer chromatography [TLC] and gas liquid chromatography [GLC]. No further characterization was carried out). If this were true, 2-alkanols should disappear when *E. coli* are grown anaerobically in media with glucose as the only carbon source. Alternatively, alcohol formation would suggest acetate as the ultimate precursor and fatty acids as a more direct precursor to the 1-alkanols. The first part of this study involved characterizing the free fatty alcohols and free fatty acids (FFA) from *E. coli* K-12 grown aerobically and anaerobically on a chemically defined media. The second part involved isolating and characterizing the hydrocarbons from these cells. This was undertaken to evaluate the possibility of alcohol reduction to hydrocarbons, a logical step in hydrocarbon biosynthesis.

EXPERIMENTAL PROCEDURES

Bacteria

The *E. coli* were grown as described (1), only a chemically defined medium was substituted for TSB. This medium was 0.06 M in potassium phosphate buffer (pH = 7.1), 7.5 mM $(NH_4)_2SO_4$, 0.4 mM Mg SO_4, and 0.027 M glucose. Cells grown on this chemically defined media will be referred to as glucose-grown and those grown on TSB as TSB-grown.

TLC

Preparation of the chromatoplates and the techniques used for isolation and recovery of lipid have been described (1). The plates used for isolation of hydrocarbons were prewashed with ethyl ether, air-dried, and activated (1). The hydrocarbons were purified using hexane as the developing solvent.

Isolation of Lipids

Lipids were extracted (3), concentrated under vacuum, and diluted to a known volume with nitrogen-equilibrated *n*-heptane. Ca. 400 mg lipid were applied to 18 g *n*-heptane-equilibrated silicic acid columns (4) and the column developed (1). Hydrocarbons eluted in the 1% ethyl ether/*n*-heptane fraction were purified by TLC. The free fatty alcohols, FFA, and phospholipids were isolated as before (1). Esterified fatty acids were liberated from the phospholipids by transesterification with anhydrous 2% HCl/methanol at 70 C for 1 hr, and the methyl esters extracted and isolated by TLC (1). In each instance, a blank containing the same volume of solvents as the samples was concentrated to dryness and carried through the entire lipid isolation procedure. The residue in the

fractions corresponding to the individual lipid types was anlyzed to prevent artifactual assignments.

GLC

Conditons for GLC of free fatty alcohosl and acids are published (1). Hydrocarbons were analyzed qualitatively and quantitatively under the same conditions, only the column temperature was programed from 100 C-250 C at 2 C/min. Peak areas were related to wt using a calibration curve derived from hexadecanol.

RESULTS

Quantitative Changes under Growth Conditions

Table I shows the quantitative changes that occurred in cell yield and lipid when *E. coli* K-12 were cultured aerobically and anaerobically on TSB and on the chemically defined medium. Significant changes in the cell yield, FFA, and free fatty alcohols but no change in percent lipids or hydrocarbons were observed. Note that there was less fatty alcohol and more FFA in cells grown anaerobically than in those cultured aerobically on the same media and that cell yield always decreased under anaerobiosis. Also observe that a smaller cell yield and lower fatty alcohol content occurred in glucose-grown cells as compared to the TSB-grown cells cultured under the same oxygen environment. A decrease in FFA occurred in aerobic glucose-grown *E. coli* as compared to those cultured on TSB, but an increase in FFA was noted in glucsoe-grown *E. coli* cultured anaerobically.

Qualitative Lipid Changes under Different Nutritional Conditions

In Table II, the chain length distribution of the alcohols isolated from aerobic glucose-grown *E. coli* is compared with the distribution of alochols isolated from cells cultured on TSB. While slight differences exist in the lower percentage components, the major alcohols are 1-tetradecanol, 1-hexadecanol, and 2-pentadecanol in both cases.

The FFA were isolated from aerobic glucose-grown *E. coli* and analyzed via GLC as described previously (1). No significant differences were noted between these values and those obtained from cells cultured on TSB (1). Methyl esters of fatty acids haying carbon numbers greater than those noted were not detected.

The identification of hydrocarbons in this study was based upon three criteria. (A) A natural lipid component was eluted from a silicic acid column in a fraction known to elute

TABLE I

Quantitative Lipid Changes in *E. coli* K-12 Grown in Different Media and under Different Oxygen Environments[a]

Growth media	Experiment no.	Wet wt (g/L)		Total lipid %		Free fatty acids μmoles/100 mg TL		Fatty alcohols μmoles/100 mg TL		Hydrocarbons μgrams/100 mg TL	
		aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic
Trypticase soy broth	1	6.00	3.2	1.01	1.1	0.820	2.9	0.15	.080	–	–
	2	6.00	2.9	1.01	1.1	0.640	2.6	0.14	.070	–	–
Glucose salts	1	2.30	1.40	1.50	1.40	0.400	4.00	0.04	nd[b]	200	220
	2	2.30	1.40	1.60	1.50	0.450	4.05	0.05	nd	200	220

[a] Lipids were isolated and quantitated as described in "Methods." TL = total lipid.
[b] – = not determined and nd = not detected.

TABLE II

Qualitative Distribution Fatty Alcohols from Aerobically Grown *E. Coli* Cultured in Two Media

Chain length[a]	Wt %, Glucose grown[b]	Trypticase soy broth grown[c] (i)
12:0	3.7	2.8
13:0 sec	2.7	8.4
13:0	2.7	
14:0 sec		1.5
14:0	14.1	18.2
15:0 sec	30.0	26.7
15:0	7.4	
16:1		2.7
16:0	32.8	28.6
17:0	5.9	
18:0	1.1	13.6

[a]Carbon number: number of double bonds, sec = secondary.

[b]Average of two experiments.

[c]Average of three experiments.

hydrocarbons (4) that had the same mobility as hexadecane during TLC. (B) GLC of this component indicated there were a number of species having relative retention times identical to known saturated aliphatic hydrocarbons. (C) The mass spectra of natural compounds were identical with fragmentation patterns of known hydrocarbons (5) and were consistent with the structure assigned to the molecule by GLC.

A gas chromatogram of the hydrocarbon fraction isolated from aerobic glucose-grown *E. coli* is shown in Figure 1. The components that were identified are all members of the normal saturated series. Other unidentified components also are present and account for 15% total peak area. At present, the identity of these components is being investigated. Identification of hydrocarbons in TSB-grown cells was not attempted because hydrocarbons from TSB enter the cellular pool of hydrocarbons and make it impossible to differentiate those synthesized by the cell from those obtained from the medium. However, when the chain length distribution of hydrocarbons from aerobically, glucose-grown *E. coli* is compared with previously published results (6), differences in the hydrocarbon types are immediately obvious (Table III). Our analysis shows that hexadecane is the major hydrocarbon of *E. coli* K-12, as opposed to octadecane in the previously reported data. Also, nonadecane and eicosonane were reported in significant amounts previously but our data indicate that these are not significant species in cells grown only on glucose. Tri-, tetra-, penta-, and hexacosane are the other significant hydrocarbons in the fraction we analyzed. A third difference is that the unidentifiable components found in our analysis were not reported in the previous publication.

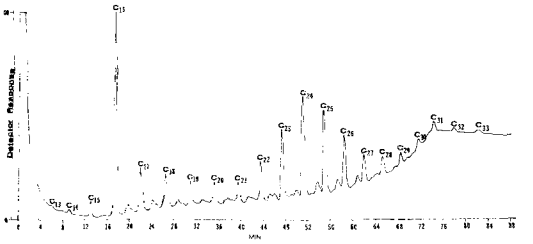


FIG. 1. Typical gas liquid chromatogram of the hydrocarbons isolated from *E. coli* grown on glucose as a sole carbon source in an aerobic environment. Carbon number of each component identified by gas liquid chromatography and mass spectrometry is noted in the figure.

Qualitative Lipid Changes under Anaerobiosis

The bound fatty acids (Table IV) and hydrocarbons of glucose-grown *E. coli* K-12 did not change qualitatively when these cells were grown anaerobically. This is consistent with published results (6-8).

Figure 2 compares the chain length distribution of the FFA in aerobic and anaerobic glucose-grown *E. coli*. The data show that, under anaerobic conditions, the amount of monoenoic fatty acids increased significantly. Hexadecenoic acid increased from 2.0% to 9.4% and octadecenoic acid from 13.3% to 40.0%.

Since fatty alcohols were not detectable in the anaerobically cultured glucose-grown *E. coli*, we were not able to determine the chain length distribution and no observation concerning the selective decrease of 2-alkanols could be made.

Analysis of the individual fractions corresponding to the FFA, fatty alcohols, and hydrocarbons from the solvent blank by TLC and GLC indicated that artifactual components were not present. This result is of particular significance with respect to the hydrocarbon fraction from glucose-grown cells, since, if artifacts were not detected and glucose was the sole carbon source, the hydrocarbons must have been formed biosynthetically.

DISCUSSION

The detection of 1- and 2-alkanols in *E. coli* grown on a chemically defined media proves that the free fatty alcohols are not derived from oxidation of hydrocarbons in the growth media. The ultimate source of the 1- and 2-alkanols must be glucose, since it is the only carbon source in the media. It is well known that the synthesis of fatty chains from glucose proceeds through acetate condensations (9).

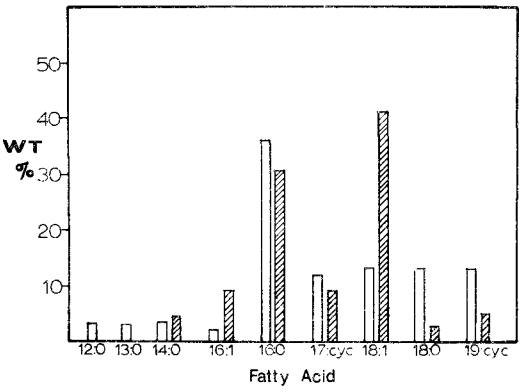


FIG. 2. Histogram depicting the chain length distribution of the free fatty acids isolated from *E. coli* grown on glucose as a sole carbon source in an aerobic and anaerobic environment. Ordinate-wt %; abscissa-number of carbon atoms: number of double bonds. Cyclopropane fatty acids are denoted by the number of double bonds: cyclic. □ aerobic ▨ anerobic

The fatty chains of the alcohols probably are derived from the fatty acid, but the mechanism for the formation of the alcohol function remains unknown.

Reduction of fatty acid or oxidation of endogenous hydrocarbons are possibilities. The change in alcohol content under anaerobiosis indicates that oxygen is a factor regulating alcohol metabolism in glucose-grown *E. coli*, as well as TSB-grown cells. However, it must be pointed out that the decrease in the fatty alcohols of the anaerobic glucose-grown cells must have involved the primary, as well as the secondary, alcohols. A selective loss of only the secondary alcohols would have resulted in ca. 30% decrease. Our methods would have been able to measure the remaining 70%; however, no alcohol was detected chemically or by GLC. This is not the case in the TSB-grown *E. coli* where there is a selective decrease of secondary alcohols (1).

The identification of hydrocarbons in *E. coli* and other microorganisms has been published (6). The qualitative differences in hydrocarbon composition reported here and elsewhere (6) can be the result of a number of factors. Bacterial lipids vary with the culture's age, temperature, pH, and growth media (10). Since the previous report did not give the growth conditions, we cannot evaluate the difference in the two sets of data.

Studies carried out with *Sarcina lutea* are the only extensive investigations of hydrocarbon biosynthesis in bacteria (11-13). Interestingly enough, the biosynthetic pathway in this bacteria is different from the elongation-decarboxylation pathway in plants proposed by

TABLE III

Hydrocarbons of *E. Coli* K-12 Grown on Glucose under Aerobic Conditions

Chain length	Wt % Glucose grown[a]	Wt % Previous data (6)
13:0	0.6	
14:0	1.5	
15:0	3.9	0.5
16:0	21.8	1.7
17:0	7.8	5.5
18:0	4.2	27.6
19:0	4.0	12.0
20:0	3.5	10.0
21:0	2.8	5.5
22:0	5.0	6.0
23:0	7.2	8.3
24:0	9.5	7.4
25:0	8.9	6.0
26:0	7.4	3.3
27:0	3.8	3.3
28:0	3.4	0.5
29:0	1.6	1.4
30:0	1.4	
31:0	1.1	
32:0	0.6	
33:0	0.7	
Unidentified	17.8	

[a]Average of two experiments.

Kolattukudy (14). Evidence indicates that *Sarcina lutea* synthesize hydrocarbons by condensation of a fatty acid and the aliphatic group of the neutral lipid 1-0-alk-1-enyl glycerols (13). The possibility that this mechanism operates in *E. coli* is unlikely, since this bacterium contains no alk-1-enyl glycerol ethers (1, 15). In addition, *E. coli* contain hydrocarbons that have chain lengths corresponding to the FFA. This suggests direct reduction of fatty acids to hydrocarbons as a biosynthetic mechanism along with a type of FFA elongation pathway for the very long chain hydrocarbons.

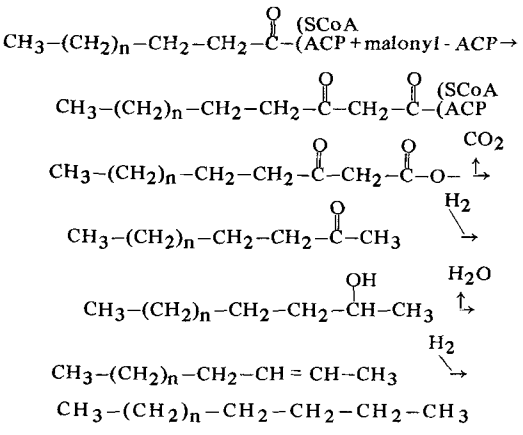
It is conceivable that the fatty alcohols are intermediates in the reduction of fatty acids to

TABLE IV

Esterified Fatty Acids of Glucose-Grown *E. Coli* K-12 Grown Aerobically and Anaerobically

Chain length	Wt % Aerobic	Wt % Anaerobic	Aerobic (8)
16:1	1.4	1.9	7.0
16:0	44.6	50.0	40.0
17:cyc	28.3	25.0	22.0
18:1	8.2	11.1	19.0
18:0			
19:cyc	12.2	7.7	7.4

hydrocarbons. An aldehyde would most likely also be involved in the reduction, and we have isolated these compounds from *E. coli* grown on TSB (1). Lipids having chromatographic properties of hexadecanal on Silica Gel G were isolated from the glucose-grown cells also, but the amount present made any further characterization impossible. 2-Alkanols also might be intermediates in hydrocarbon biosynthesis and may arise in the following pathway:



A common mechanism of decarboxylation in biological systems involves a β-keto intermediate (16), and this is what is postulated here.

Investigations into the metabolism of fatty acids in *E. coli* usually are concerned with only the esterified acids (8, 17-19). However, the data in this report indicate that changes in esterified fatty acids may not always reflect changes in FFA. The esterified fatty acid pool may remain quite constant under a given set of conditions, while the smaller pool of FFA changes quite rapidly. As a result, it must be kept in mind that measuring changes in esterified fatty acids will not reflect changes in de novo fatty acid synthesis.

It also is noted from the FFA analysis that cyclopropane fatty acids occur in the free state. Since they are biosynthesized while esterified to phospholipids (20, 21), those present as free acids must be arising from chemical or enzymatic hydrolysis. Chemical hydrolysis seems rather unlikely, because the ratio of 9, 10-methylene hexadecanoic acid:11, 12-methylene octadecanoic acid is 1.58 in the FFA and 3.5 in the bound. If chemical hydrolysis occurred during lipid extraction, similar ratios would be expected. Selective enzymatic hydrolysis might be releasing this acid to initiate their degradation.

A comparison of the chain length of the FFA from aerobically and anaerobically grown *E. coli* (Fig. 2) immediately suggests a role for oxygen in the control of fatty acid metabolism. The monoenoic acids in *E. coli* are known to be synthesized by branching of the saturated fatty acid pathway (22). α,β Dehydration of the β-hydroxyacyl derivative leads to saturated fatty acids, while β γ dehydration of this derivative yields the monenes. Our experiments suggest that lack of oxygen exerts a control at this point.

Saturated fatty acids also were observed to increase quantitatively under anaerobiosis. The amount of hexadecanoic acid measured by quantitative GLC in aerobically grown cells was 0.140 μmoles/100 mg total lipid. The content in anaerobically grown cells was 1.25 μmoles/100 mg total lipid, a ninefold increase. We have no evidence to indicate if the increased levels of FFA are due to increased synthesis or decreased degradation, thus a comment concerning their metabolism cannot be made. However, these changes do occur, and the causes and nature of these regulatory mechanism are of interest.

One other observation can be made from the data in Table I. When the FFA increase, the fatty alcohols decrease. It may be that the acyl CoA is the precursor to the 1-alkanols, as well as the FFA (23-25), and the product of one pathway may inhibit the other pathway at some point. It may be this type of mechanism that is decreasing fatty alcohols in the anaerobic *E. coli* and not the effect of oxygen directly. Experiments are now in progress to investigate this possibility.

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Fluorescent Products of Lipid Peroxidation: II. Methods for Analysis and Characterization

V.G. MALSHET, A.L. TAPPEL, and **V.M. BURNS,** Departments of Food Science and Technology and Physiological Sciences, University of California, Davis, California 95616

ABSTRACT

The effects of pH and of the metal chelator, europium (Tric[2,2,6,6-tetramethyl-3,5-heptanedionate]), upon fluorescence of lipid peroxidation products were tested, and fluorescence decay times and fluorescence polarization values were determined. Measurements of fluorescence intensity showed that the fluorescence of these compounds was quenched at basic pH and that it was restored by adjustment of pH to neutrality. Metal chelator decreased the fluorescence intensity 8-15%. pH Effects and metal coordination effects are useful for analysis and characterization of these fluorescent products. Fluorescence polarization and fluorescence decay times are also useful analytical techniques for characterization of fluorescent products.

INTRODUCTION

The peroxidation reactions of polyunsaturated fatty acids to form mainly hydroperoxides also produce numerous carbonyl compounds, such as malonaldehyde (1). These carbonyl compounds, in turn, react with amino groups of proteins, amino acids, and phosphatidyl ethanolamine to form fluorescent compounds in small yield (2-4). Measurement of these compounds via fluorescence spectroscopy may provide sensitive and selective methods to determine the occurrence of in vivo and in vitro lipid peroxidation (2,5,6).

This article reports on an investigation of the effect of pH and the effect of chelation upon fluorescence characteristics of in vivo and in vitro lipid peroxidation products and the possible use of fluorescence polarization and fluorescence decay time for the detection of these products.

EXPERIMENTAL PROCEDURE

Materials

Europium (Tric[2,2,6,6-tetramethyl-3,5-heptanedionate]), hereafter referred to as $Eu(thd)_3$, was obtained from Varian Instrument Division, Palo Alto, Calif.; synthetic dipalmityl phosphatidyl ethanolamine, from Schwartz/Mann, Orangeburg, N.Y., malonaldehyde bis-(dimethyl acetal), from Aldrich Chemical Co., Milwaukee, Wisc.; arachidonic acid and methyl docosahexaenoate, from The Hormel Institute, Austin, Minn.; and thixotropic gel (Cab-O-Sil) from Beckman Instruments, Palo Alto, Calif.

Methods

N,N-dileucinyl-1-amino-3-iminopropene (I) and N,N'-dihexanyl-1-amino-3-iminopropene (II) were prepared as described by Chio and Tappel (2). Fluorescent pigments were produced by reaction of oxidizing arachidonic acid (III) or methyl docosahexaenoate (IV) with phosphatidyl ethanolamine. The fatty acids (40 mM), emulsified in 0.1 M potassium phosphate buffer, pH 7, were oxidized for 24 hr in the presence of phosphatidyl ethanolamine (40 mM).

Microsomes and mitochondria were prepared as described by Ragab, et al., (7) from livers of rats fed a vitamin E-deficient diet for 1-1/2 months. Microsomes (V) and mitochondria (VI) were peroxidized as described by Dillard and Tappel (8), except that peroxidation was allowed to proceed for 48 hr in 0.1 M potassium phosphate buffer, pH 7.

Fluorescent pigments (VII) from the renal adipose tissue of rats fed a vitamin E-deficient diet for 1-1/2 months were extracted as described by Reddy et al. (9). Normal brain lipofuscin age pigments (VIII) were obtained from A.N. Siakotos, Indiana University Medical Center (10). Polymerized fluorescent products from peroxidized arachidonic acid (IX) were prepared in a similar manner as used for preparation of compounds III and IV, with the exclusion of dipalmityl phosphatidyl ethanolamine from the reaction mixture. For the preparation of polymerized malonaldehyde (X), 15 nmoles (2.46 g) malonaldehyde acetal in 1.5 ml 1 N HCl were allowed to stand at 40 C with occasional shaking until miscible, after which the solution was neutralized with 1 N NaOH and stirred for 4 hr. Browning pigments (XI) were prepared as described by Adhikari and Tappel (11).

For measurements of fluorescence, aliquots of the oxidizing reaction mixtures were diluted to 1 ml with water and then extracted at room temperature with 2 ml chloroform-methanol 2:1 by mixing for 2 min on a vortex mixer at

high speed. After centrifugation for 2-3 min, the water-methanol layer was separated from the chloroform layer. The chloroform layer was diluted with one-half volume of methanol for measurement of fluorescence.

In assessing the effect of pH upon the water-soluble fluorescent chromophore, N,N′-dileucinyl-1-amino-3-iminopropene, the buffers used to maintain pH were 0.1 M HCl-KCl, pH 1 and 2; 0.05 M citrate-phosphate, pH 2.5-7.0; and 0.05 M carbonate-bicarbonate, pH 9-10.5. The fluorescence intensity of 1.4 x 10^{-7} M N,N′-dileucinyl-1-amino-3-iminopropene was determined at different pH values with excitation at 375 nm and emission at 445 nm.

The effect of pH upon the fluorescence intensity of the chloroform-methanol soluble products was determined. The initial pH of the products varied from pH 5.8-7.8 pH. The pH was increased by the addition of 10 μliters 4 N sodium methoxide in methanol to 2 ml chloroform-methanol solution of fluorescent chromophore; the pH of the solution varied from 11.2-12.2 pH and was readjusted to neutrality by the addition of 10 μliters 4 N acetic acid in methanol. pH Was determined with a pH meter (Radiometer).

The effect of metal chelation upon the fluorescence intensity of N,N -dileucinyl-1-amino-3-iminopropene was determined in the presence of 10^{-6}-10^{-4} M $Eu(thd)_3$ in chloroform-methanol 2:1; with the remaining compounds, 5 x 10^{-5} M $Eu(thd)_3$ in chloroform-methanol 2:1 was used.

The fluorescence emission and excitation spectra were determined with an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, Md.) with an IP-21 photomultiplier tube. The instrument was calibrated with quinine sulfate, and measurements were made at ambient temperature. The slit arrangement for recording the fluorescence spectra was slits 3, 4, and 6 set at 3, 1, and 3 mm, respectively. The sensitivity setting was 50. Spectra were recorded on an X-Y recorder (Houston Instrument, Bellaire, Tex.). Under these instrument parameters, 1 μg standard quinine sulfate/ml 0.1 N H_2SO_4 had a fluorescence intensity of 70 at a 0.3 meter multiplier setting.

Fluorescence polarization studies were done with products II-V and VII in 3% thixotropic gel in chloroform. Fluorescence intensity measurements were made with an Aminco-Bowman spectrophotofluorometer fitted with glan prism polarizers; the emission wave length was kept constant at 460 nm and the excitation wave length was scanned from 300-430 nm. Polarization is expressed by the equation:

$$p = \frac{I_{VV} - I_{VH}}{I_{VV} + I_{VH}},$$

where I_{VV} and I_{VH} are the measured fluorescence intensities with the polarizers oriented vertically and horizontally, respectively. The polarization spectra were not corrected, thus the interpretation is limited to qualitative and semiquantitative comparisons.

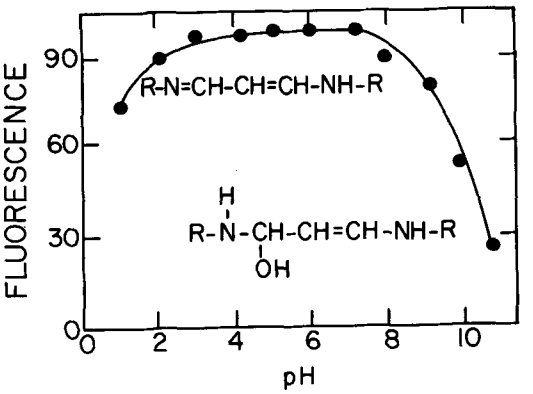

FIG. 1. Effect of pH upon fluorescence of N,N′-dileucinyl-1-amino-3-iminopropene.

Decay time measurements were made with the TRW nanosecond decay time fluorometer (12). A pulse lamp, either N_2 or D_2, excited the sample, and the fluorescence pulse was displayed on a double-beam oscilloscope. The fluorescence pulse trace was fitted with a simulated pulse from an on-line computer, which compensated for the finite light pulse and allowed direct read-out of decay time. Time periods greater than ca. 2 nsec can be determined within ± 0.5 nsec. Color filters were used to select excitation (Corning 7-54, 280-390 nm) and emission (Corning 3-75 cut off filter) wave lengths.

RESULTS

Figure 1 shows the effect of pH upon the fluorescence intensity of the water soluble Schiff base, N,N′-dileucinyl-1-amino-3-iminopropene. There was a gradual decrease in fluorescence intensity between pH 3-1 and an abrupt decrease in fluorescence intensity between pH 7.0-10.5 The initial fluorescence intensity was restored when the solution was adjusted from pH 10.5-pH 7.

The fluorescence intensity of chloroform-methanol soluble products II through VIII decreased 33-60% in presence of base (Table I). The initial pH ranged from pH 5.8-7.8 and in

TABLE I

Effect of pH and Chelation upon Fluorescence

Products	Fluorescence maxima		Addition		
	Excitation$_{nm}$	Emission$_{nm}$	Base[a]	Acid[b]	Chelator[c]
			Percent fluorescence		
Hexylamine + malonaldehyde (II)	388	437	63	97	90
Peroxidized arachidonic acid + phosphatidyl ethanolamine (III)	358	434	33	60	85
Peroxidized docosahexaenoate + phosphatidyl ethanolamine (IV)	360	439	60	97	89
Peroxidized microsomes (V)[d]	358	434	63	83	89
Peroxidized mitochondria (VI)[d]	358	432	71	100	88
Adipose tissue (VII)[d]	355	432	40	80	85
Lipofuscin age pigment (VIII)	355	437	70	97	92
Peroxidized arachidonic acid (XI)	355	450	1,000	300	100
Polymerized malonaldehyde (x)	384	480	800	800	100

[a]Ten μliters 4 N sodium methoxide was added to 2 ml solution.

[b]Ten μliters 4 N acetic acid in methanol was added.

[c]The final concentration of $Eu(thd)_3$ was 5×10^{-5} M.

[d]Rat liver microsomes and mitochondria, and adipose tissue were obtained from rats fed a vitamin E-deficient diet for 1-1/2 months.

presence of base from pH 11.2-12.2. When an equal amount of acid was added to the alkaline solution, the pH ranged from pH 7.1-7.6. When pH of the solution was adjusted to neutrality, the fluorescence intensity was restored to 60-100% original value.

Peroxidized arachidonic acid (IX) and polymerized malonaldehyde (X) had very low fluorescence yields when compared with that of quinine sulfate. The fluorescence intensity of these products increased 800-1000% upon addition of sodium methoxide (Table I). When the pH was adjusted to neutrality, the fluorescence intensity remained at a level higher than that of the original solution.

The fluorescence intensity of N,N'-dileucinyl-1-amino-3-iminopropene decreased 9-10% in the presence of 10^{-5}-10^{-4} M $Eu(thd)_3$. There was no decrease in fluorescence intensity when the concentration of the metal chelator was 10^{-6}M. The fluorescence intensities of products II-VIII decreased 8-15% in the presence of 5×10^{-5}M $Eu(thd)_3$ (Table I). The fluorescence intensity of peroxidized arachidonic acid and polymerized malonaldehyde did not decrease in the presence of $Eu(thd)_3$.

Table II shows the fluorescence decay time for each lipid peroxidation product measured. For chloroform-methanol soluble products II-V and VII, the values ranged from 5.1-8.1 nsec, and for water soluble products I and XI the values were 8.6 nsec and 10.2 nsec, respectively. The values obtained were reproducible.

Table II also shows the fluorescence polarization p values for products II-V and VII at an excitation maximum of 400 nm. The values ranged from 0.20-0.36. Figures 2 and 3 show fluorescence polarization values for these compounds at different wave lengths. With the

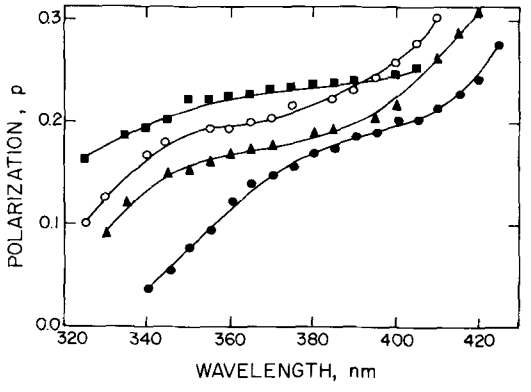

FIG. 2. Excitation polarization spectra of product II (●), III (▲), IV (■), and V (○).

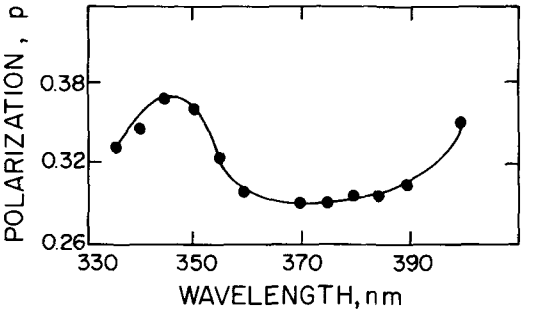

FIG. 3. Excitation polarization spectrum of product VII.

TABLE II

Fluorescence Decay Time and Fluorescence Polarization Value

Products	Nsec		Polarization
	Mean values	Individual values	
N,N'-dileucinyl-1-amino-3-iminopropene (I)	8.0	(8.0, 8.0)	---
N,N'-dihexanyl-1-amino-3-iminopropene (II)	5.2	(5.0, 5.3)	0.20
Ethyl arachidonate + phosphatidyl ethanolamine (III)	5.1	(4.7, 5.5)	0.21
Docosahexaenoate + phosphatidyl ethanolamine (IV)	7.0	(7.2, 6.8)	0.24
Peroxidized microsomes (V)[a]	7.0	(6.8, 7.2)	0.25
Renal adipose extract (VII)[b]	8.1	(8.2, 8.0)	0.36
Browning pigment (XI)	10.2	(10.1, 10.4)	---

[a]Microsomes were prepared from the livers of rats fed a vitamin E-deficient diet for 1-1/2 months.
[b]Renal adipose was obtained from rats fed a vitamin E-deficient diet for 1-1/2 months.

exception of compound VII, the products had similar polarization spectra.

DISCUSSION

The major aim of this study was to develop analytical methods to detect and monitor lipid peroxidation products formed in vivo and in vitro. At present, measurement of fluorescence is one of the most convenient and reliable methods to determined lipid peroxidation products. Therefore, we attempted to perturb chemically the fluorescence of conjugated Schiff-base compounds and to use these chemical modifications as analytical diagnostic methods for identification of these compounds formed in vivo and in vitro.

The effect of pH upon the fluorescence properties of some compounds is well known (13). Ortho- and metahydroxybenzoic acids can be determined fluorometrically in a mixture by varying the pH (14). The fluorescent products produced during lipid peroxidation derive mainly from malonaldehyde, and they have fluorochromic structures of the 1-amino-3-iminopropene type (4). The influence of pH upon the fluorescence intensity of the model compound, N,N'-dileucinyl-1-amino-3-iminopropene (I), indicates that the fluorescence is quenched at basic pH but that the fluorescence is restored by adjustment of the pH to neutrality. At alkaline pH, there may be an equilibrium between fluorescent and nonfluorescent structures as shown below:

$$\text{R-N=CH-CH=CH-NH-R} \rightleftharpoons \text{R-NH-}\underset{\text{OH}}{\overset{\text{H}}{\overset{|}{\text{C}}}}\text{-CH=CH-NH-R} \quad [1]$$

$$\text{H}^+$$

The effect upon fluorescence properties of some metal chelates is well known (15). Hinckley (16,17) used $Eu(thd)_3$ as a chelating reagent in nuclear magnetic resonance spectroscopy. The 9-10% decrease in fluorescence intensity of compounds, such as N,N'-dileucinyl-1-amino-3-iminopropene, is due to the bonding of the lone pair of electrons on the nitrogen atom by the metal ions as shown below:

$$\text{R-N=CH-CH=CH-NH-R} \quad [2]$$

$$\text{Eu}^{+++}$$

The bonding decreases the electron donating capacity of the nitrogen.

The in vivo and in vitro lipid peroxidation products (products II through VIII), which have characteristic fluorescence spectra, responded in a similar manner to pH and metal chelator as did the model compounds. Although the meaning of pH changes considerably in nonaqueous solvents and hence the units of pH cannot be compared directly, the same effect of decreased fluorescence was shown when base was added to the chloroform-methanol soluble and water soluble Schiff-base fluorochromes. Peroxidized arachidonic acid and polymerized malonaldehyde can be typical of interfering compounds in products characteristic of lipid peroxidation, although there is no direct evidence to this effect. These two products do not show this response to pH or chelation, and they do not have the 1-amino-3-iminopropene structure. This further supports the hypothesis that fluorescent products produced during lipid peroxidation have fluorochromic structures of the 1-amino-3-iminopropene type.

Udenfriend (18) indicates that tyrosine and tyrosine containing proteins have similar fluorescence polarization spectra. Products III, IV, and V had polarization spectra similar to that of model compound II. Agreeing with the results of studies on the effect of pH and metal chelation, the polarization spectra of products II-V indicate the presence of similar chromophores. Also, increasing values of p indicate

increasing molecular size of the products, as well as increase in viscosity due to the amount of other solutes. The use of thixotropic gel for obtaining polarization spectra is demonstrated by these experiments.

A search of the literature revealed no background information on the application of fluorescence decay time to such complex products. Proteins that contain tryptophan, compounds that contain pyridine nucleotide, and compounds that contain flavin have a range of fluorescence decay times (19). Fluorescent lipid peroxidation products have a wider range of decay times, which can be attributed to other solutes that may act as quenchers. Even in these complex mixtures, it appears that the fluorescence decay times are consistent with the identification of these lipid peroxidation products.

This study has shown that, in addition to fluorescence spectral characteristics, the effects of pH and $Eu(thd)_3$ chelation upon fluorescence intensity can be used to characterize in vivo and in vitro fluorescent lipid peroxidation products. Also, fluorescence polarization and fluorescence decay time can be used as additional analytical techniques to identify 1-amino-3-iminopropene fluorescent Schiff-base chromophores.

ACKNOWLEDGMENT

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Purification of Campesterol and Preparation of 7-Dehydrocampesterol, 7-Campestenol and Campestanol[1]

HENRY W. KIRCHER and **FUMIKO U. ROSENSTEIN**, Department of Agricultural Biochemistry, University of Arizona, Tucson, Arizona 85721

ABSTRACT

Campesterol ([24R]-24-methyl-5-cholesten-3β-ol) was isolated from a soybean sterol mixture and purified on a small scale by column chromatography and on a large scale by crystallization from acetone to 97% purity. From this, 7-dehydrocampesterol ([24R]-24-methyl-5,7-cholestadien-3β-ol) and campestanol ([24R]-24-methyl-cholestan-3β-ol) were prepared. The acetates and benzoates of all four compounds also were obtained and compared to the corresponding members of the ergostane series.

INTRODUCTION

Campesterol is one of the most ubiquitous sterols in nature. Its abundance in terrestrial plants and especially in oilseeds is exceeded only by sitosterol (1-4). Indeed, wherever campesterol occurs, sitosterol also is present and always to a greater extent. No plant or portion of a plant has yet been described in which campesterol is the major sterol.

Campesterol was first isolated from rapeseed oil (*Brassica campestris*) (5), then from rye germ oil (6), more recently from the *Oenothera* species (7), and the bark of a tree, *Eurycoma longifolia* Jack (8). Its preparation from 22-dehydrocampesterol derived from marine organisms was described by W. Bergmann and coworkers (9,10). Two other syntheses, using 3β-methoxybisnor-5-cholenoyl chloride and an optically active cadmium reagent (11) and pregnenolone acetate with an optically active Grignard reagent (12), have been described in the last few years.

We required campesterol in quantity for preparation of 7-dehydrocampesterol, 7-campestenol, and campestanol for our work on the utilization of these compounds by *Drosophila* species. The best source for campesterol at the present time is the commercially available sterol mixture obtained from the steam deodorizer distillates of soybean oil after removal of most of the stigmasterol. This source recently has been used for preparation of >98% pure campesterol in unspecified quantity by manifold recrystallizations from acetone (13) and in mg quantities by liquid partition chromatography on Sephadex LH-20 (14).

EXPERIMENTAL PROCEDURES

Methods and Materials

The column, thin layer (TLC), and gas liquid (GLC) chromatographic techniques and the hydrogenation methods employed have been described in earlier works (15-18). Except where noted, melting points (mp) were taken in vacuo and are corrected. Benzoates were prepared from the sterols in pyridine with a 10-fold excess of benzoyl chloride added in 3 aliquots followed by evaporation of the reaction mixture to dryness, extraction of the residue with methanol, and recrystallization of the derivative from benzene-acetone.

Purification of Campesterol

Generol 115 (General Mills Chemicals, Minneapolis, Minn., 91% total sterols, 53% sitosterol, 32% campesterol, 6% stigmasterol) (~5 kg) was crystallized and recrystallized from Skellysolve B until a number of fractions rich in campesterol were collected. These were combined and acetylated with acetic anhydride on the steam bath overnight (17) to yield 133 g campesteryl-sitosteryl acetate mixture, with a ratio of peak heights by GLC 8.5:1, respectively.

This material, and purer materials derived from it, were placed on silver nitrate-silica gel columns and eluted with 0.5 or 1% dry ether in Skellysolve F. There was never any complete separation of campesteryl from sitosteryl acetate. Best results were obtained with freshly prepared, activated (110 C) 20% silver nitrate on silica gel-Celite 2:1 (17) and elution with 1% ether in Skellysolve F. When 10 g 8.5:1 mixture of acetates was placed on a 1 kg column, 1.47 g 28:1 mixture was obtained. When 10 g 28:1 mixture was placed on the same type of column, 1.12 g 50:1 mixture of the two acetates was obtained. A single column took 2-3 weeks for a complete run. From these results, it was decided that fractional crystallization from

[1]Contribution 2189, Arizona Agricultural Experiment Station.

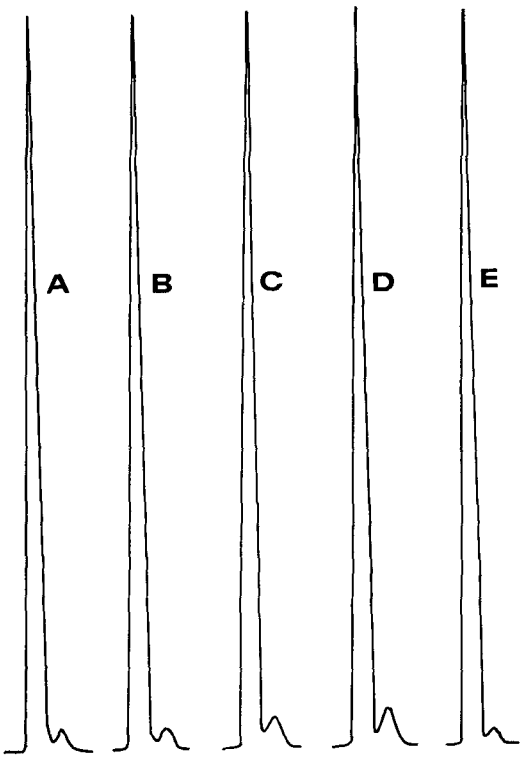

FIG. 1. Gas liquid chromatographic separation diagrams of campesteryl acetate fractions: A 97%, B 96.5%, C 95.5%, D 93.5%, E 98% (5% OV-101, 260 C, 80 psig Ar, McKee-Petersen recorder, chart speed 0.1 in./min).

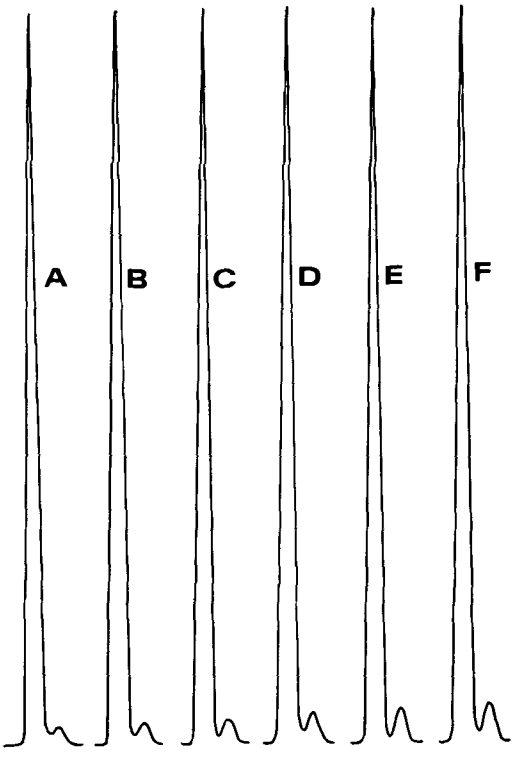

FIG. 2. Gas liquid chromatographic separation diagrams of 97% campesteryl acetate with: A 0%, B 0.5%, C 1%, D 2%, E 3%, F 4% sitosteryl acetate added (same conditions as in Figure 1).

acetone (7,13) would give a larger quantity of purified campesterol in a shorter time.

When the intensive crystallization process was started, 863 g impure campesterol was on hand divided over 11 fractions ranging from 1.2:1-9.5:1 in campesterol-sitosterol GLC peak height ratios. These fractions were crystallized, like fractions combined, and recrystallized many times from acetone (25-40 ml acetone/g sterol) over a period of 7 months after which the process was arbitrarily stopped. During this period a number of other crystallization solvents was tried; none was better than acetone. At the end of the process, the following fractions were available: (A) 52 g, ratio 50:1; dissolved in 400 ml chloroform, filtered, 500 ml methanol added, cooled to yield 40.5 g best campesterol, mp 161.5-162.3 C. A portion (20 g) was acetylated and recrystallized from ethanol to yield 19 g campesteryl acetate, mp 140-141 C, estimated purity 97% (Fig. 1A). (B) 49.5 g Campesteryl acetate, mp 140-141 C, estimated purity 96.5% (Fig. 1B). (C) 77.6 g Campesteryl acetate, estimated purity 95.5% (Fig. 1C). (D) 52 g Campesteryl acetate, estimated purity 93.5% (Fig. 1D). (E) 1.8 g Campesteryl acetate from silver nitrate column chromatography, mp 140.5-141.5 C, estimated purity 98% (Fig. 1E).

The purities of the various samples were estimated by addition of 0, 0.5, 1, 2, 3, and 4% of 99.5% pure sitosteryl acetate (19) to the acetate from fraction A and comparing the sitosterol peak sizes by GLC (Fig. 2). These artificial mixtures were evaporated to dryness and their mp taken (C, in air, corrected): 0: 140-140.1, 0.5: 139.7-140, 1: 139.1-140, 2: 139.1-139.8, 3: 139.1-140, and 4: 139-139.7.

7-Dehydrocampesterol

Campesteryl acetate (127 g, fractions B and C) was brominated in 5 batches in Skellysolve B with 30% excess N-bromosuccinimide and dehydrobrominated with collidine in refluxing mesitylene as described for the preparation of 7-dehydrositosteryl acetate (20). The final reaction products were crystallized from 130 ml hot acetone under N_2; cooling to room temperature gave a total of 46.6 g crude 7-dehydrocampesteryl acetate; further cooling in an ice

TABLE I

Melting Points of Campestane and Ergostane Derivatives

Sterol	Source	Melting point, C		
		Sterol	Acetate	Benzoate
Campesterol	Rapeseed oil (5)	157-8	137-8	158-60
	Rye germ oil (6)	157-8	139-40	158-60
	Oenothera spp. (7)	158-9	144-5	157-8
	Eurycoma longifolia (8)	157	139	
	22-Dehydrocampesterol (9)	157-8.5	137.5-8	156-7
	22-Dehydrocampesterol (10)	152	138-8.5	162-3
	Synthetic (11)	156-8	139-41	
	Synthetic (12)		138	
	Soybean sterols (13)[a]	160-1		
	Soybean sterols (14)[b]	158		
	This work[c]	161.5-2.3	140.5-1.5	164-4.5
5-Ergostenol	Brassicasterol (17)[c]	159.5-60.5	147.5-8.5	164-5
7-Dehydrocampesterol	Campesterol (21)	164-5		156-7[d]
	Campesterol (22)			156
	This work[c]	166-7	156.5-7	155.5-6.5[e]
22-Dihydroergosterol	Ergosterol (23)	152-3	157-8	
	Wood rotting fungus (24)	150-1	165-6	156-7
	Ergosterol[c,f]	161.5-2.5	161.5-2.5	162.5-3
7-Campestenol	This work[c]	147.7-8.8	158.7-9.3	179.5-9.8
7-Ergostenol	Ergosterol[c,f]	152-2.5	164-5.5	182.5-3
Campestanol	Campesterol (6)		143-4	
	22-Dehydrocampesterol (9)	145	143.5	
	Campesterol (25)	146-7	143-4	
	24-Methylene campesterol (26)	146	144	
	This work[c]	147-8	145.5-6	154-4.5
Ergostanol	Ergosterol (18)[c]	145.5-6	146-6.5	164.5-5

[a]>98% pure.

[b]Chromatographically pure.

[c]Melting point taken in vacuo, corrected.

[d]Clears at 164 (sharp).

[e]Clears at 165-6 (sharp).

[f]H.W. Kircher and F.U. Rosenstein, unpublished work.

chest gave an additional 17.7 g less pure (TLC) products. Subsequent recrystallizations of these materials from acetone under N_2 gave 40.0 g 7-dehydrocampesteryl acetate, mp 156.5-157 C, and two further products: 8.2 g less pure 7-dehydrocampesterol, mp 155.5-156.3 C, and 5.7 g less pure 7-dehydrocampesterol, mp 155-155.8 C. TLC of all three products showed no $\Delta^{4,6}$ contamination; GLC showed a small amount (3-4%) of contamination by the sitosteryl derivative.

The second two products were combined and hydrolyzed with 15 g KOH in 500 ml 95% ethanol on the steam bath for 3 hr under N_2. After cooling and addition of acetic acid, the free sterol was removed and recrystallized from 350 ml acetone under N_2 to give 8.6 g 7-dehydrocampesterol, mp 166-166.7 C and an additional 1.5 g product, mp 164-165 C.

7-Campestenol

The reduction of 30 g 7-dehydrocampesteryl acetate in 1200 ml freshly distilled 3:1 benzene-absolute ethanol over 2 g Tris(triphenylphosphine)-chlororhodium followed the procedure used for the reduction of 7-dehydrostigmasteryl acetate (16). After 5 days, a small amount of starting material was still evident (TLC, UV). An additional 1.5 g catalyst was added and the reaction continued for another day, after which no more $\Delta^{5,7}$ was present. The solution was evaporated to dryness, the product extracted from the catalyst with Skellysolve F and recrystallized twice from acetone to yield 18.0 g 7-campestenyl acetate, mp 158.7-159.3 C, and 4 g less pure product by workup of the mother liquors.

The acetate (12 g) was hydrolyzed with alcoholic KOH, acetic acid and water added and the product recrystallized from alcohol to give 8.1 g 7-campestenol, mp 147.7-148.8 C, and 1.4 g, mp 147.5-148 C.

Campestanol

Campesteryl acetate (15 g) in 400 ml ethyl acetate and 80 ml acetic acid was hydrogenated over 2 g 10% Pd/C at 150 C and 28 atmosphere for 5 hr. After cooling, catalyst removal, and solvent evaporation, the residue was crystallized

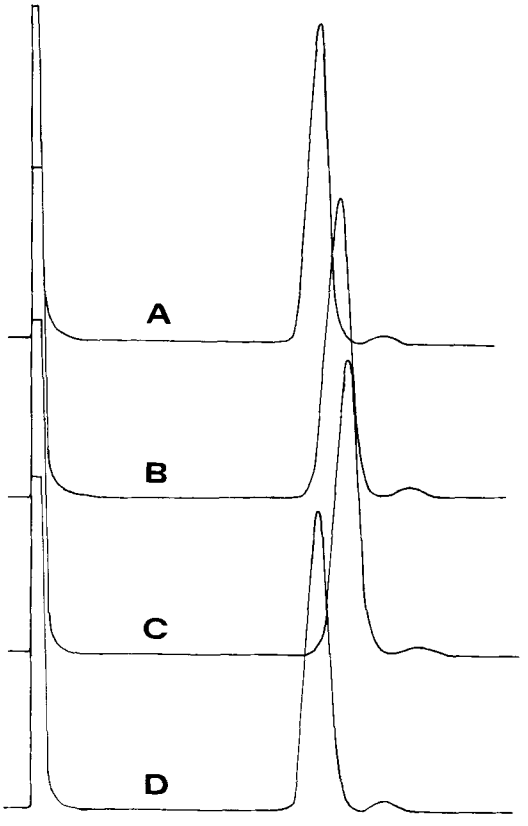

FIG. 3. Gas liquid chromatographic separation diagrams: A campesterol, B 7-dehydrocampesterol, C 7-campestenol, D campestanol (5% OV-101, 260 C, 40 psig Ar, Dynatronics recorder, chart speed 0.25 in./min).

twice from ethanol to yield 9.5 g campestanyl acetate, mp 145.5-146 C, negative Liebermann-Burchard test on a 10 mg sample.

The reaction was repeated and the product hydrolyzed with alcoholic KOH. Campestanol (10 g), mp 147-148 C, negative Liebermann-Burchard test, was obtained after crystallization from benzene-methanol.

DISCUSSION

The mp of the compounds prepared in this work are shown in Table I together with those from the literature and those of the comparable ergostane derivatives. The latter differ only in the configuration of the methyl group at C_{24} (R in campestanes, S in ergostanes). In all cases, a significant difference in mp between the corresponding campestane and ergostane derivatives occurs in only one of the three forms: for Δ^5, the acetates differ; for $\Delta^{5,7}$, the sterols differ; for Δ^7, the acetates differ; and for Δ^0, the benzoates differ.

The purity of campesterol and its acetate obtained since the advent of GLC of sterols (7,8,11-14) was in no case substantiated by GLC data, except perhaps where campesterol isolated from soybean sterols was reported to be >98% pure (13). Our efforts to get macroquantities of campesterol were rewarded by a number of fractions (A, B, C in "Experimental Procedures") that ranged in purity from 95.5-97% as estimated by quantitative GLC analysis. These materials then were used to prepare the other campestane derivatives (Fig. 3).

The presence of ca. 30% 22,23-dihydrobrassicasterol (5-ergostenol) in the campesterol isolated from soybean sterols by preparative GLC (L.J. Mulheirn, private communication) recently has been detected by 220 MHz NMR spectroscopy (27). Samples of our campesterol and of 22,23-dihydrobrassicasterol obtained previously (17) were analyzed by this method and each was shown to be virtually free of the other isomer (L.J. Mulheirn, private communication). Any 22,23-dihydrobrassicasterol present in our soybean sterol mixture must have been eliminated during the extensive crystallization processes.

7-Dehydrocampesterol first was prepared in very low yield (<2%) from campesterol by elimination of benzoic acid from its 7-benzoxy derivative (21), and, more recently, 7-dehydrocampesteryl benzoate was obtained by the photobromination of campesteryl benzoate followed by column chromatography of the reaction mixture (22). Our preparation from campesteryl acetate in good yield (43%, recrystallized product) followed the recently described method for preparation of 5,7-sterol dienes (16,20).

7-Campestenol heretofore has not been prepared or isolated from natural sources. It readily was obtained by the hydrogenation of 7-dehydrocampesteryl acetate with a soluble rhodium catalyst (16,28). Its IR spectrum was superimposable on that of 7-ergostenol (29) prepared by the Raney nickel catalyzed hydrogenation of ergosterol at 100 atmosphere (H.W. Kircher, unpublished work).

Campestanol has been prepared from the sources listed in Table I. The reduction of campesteryl acetate over Pd/C in ethyl acetate-acetic acid at 150 C and 28 atmosphere was complete in 5 hr. A macrosample (20 mg) taken after this time no longer gave even a weakly positive Liebermann-Burchard test.

The purities of campesterol, 7-dehydrocampesterol, 7-campestenol, and campestanol can be seen in Figure 3. The campesterol was derived from 97% pure campesteryl acetate; the

purities of the other compounds can be estimated by comparison. These sterols will be used to test their effect upon maturation and reproduction in Sonoran Desert species of *Drosophila.*

ACKNOWLEDGMENTS

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Lipolytic Activities of Intact Walker 256 Ascites Tumor Cells

RICHARD J. CENEDELLA and **BERNARD J. IMRICH**, School of Medicine, Department of Pharmacology, West Virginia University Medical Center, Morgantown, West Virginia 26506

ABSTRACT

The present studies confirm with Walker carcinosarcoma 256 in ascites form that ascites tumor cells produce and release large quantities of free fatty acid when incubated in vitro under the appropriate conditions. Two distinct lipolytic acitivities were recognized; a cold sensitive acidic lipolytic activity and a heat sensitive neutral-to-weakly-alkaline activity. Agents known to influence lipolysis in adipose tissue did not affect these activities. Glucose did inhibit the free fatty acid production at neutral pH, but this effect was apparently secondary to medium pH changes as a consequence of lactic acid production from glucose. These observations, together with finding a marked similarity between the composition of free fatty acid present in ascites fluid and those produced in vitro at both acid and neutral pH, are taken to support the concept that ascites tumor cells rapidly incorporate exogenous (medium) free fatty acid into a small and rapidly turning-over fatty acid-ester pool, probably a phospholipid pool.

INTRODUCTION

Free fatty acids (FFA) may be of special importance in tumor nutrition. Ehrlich ascites tumor cells readily assimilate and incorporate medium (exogenous) FFA into cellular lipids both in vitro and in vivo (1-4). A large fraction of their total energy requirement also may be supplied by oxidation of fatty acid (2). Calculations presented by Spector (4) and recently by Baker, et al., (5) show that essentially all of the fatty acid required by this tumor for growth can be supplied by the FFA present in ascites fluid. Baker and coworkers (5) further estimated that the rate of assimilation of medium FFA by the Ehrlich ascites tumor is some 25-30 times greater than the net rate of ester fatty acid accumulation seen during growth. This suggests a rapid turnover rate of tumor ester fatty acid and necessitates the presence of active lipolytic mechanisms in this cell. Indeed, apparently unlike other mammalian cells, except for the adipocyte, the ascites tumor cell can produce and release large quantities of FFA from endogenous lipid (3). This FFA production and release appears to be a continual process which proceeds concomitant with FFA uptake and esterification (3). However, net release of FFA by Ehrlich ascites cells is only seen if the cells are incubated in a medium containing albumin which is essentially depleted of FFA.

Spector (4) reported that soon after exposure of Ehrlich ascites cells in vivo to ^{14}C labeled fatty acids 70-80% cellular lipid radioactivity was present as phospholipid and 20-30% as neutral lipid. Less than 1% of the lipid radioactivity was present as FFA. When the labeled cells were reincubated in vitro for 2-3 hr, 20-30% initial cellular lipid radioactivity disappeared (3), but only 2-3% total cellular lipid ester was depleted during a similar incubation period (6). Thus, as FFA is taken-up by this tumor, it appears to be incorporated into a small and very rapidly turning-over ester fatty acid pool, probably a phospholipid pool. The FFA released from this pool then would be oxidized, reesterified, or released from the cell.

Although these earlier studies indicate an important role for lipolysis in ascites tumor cell metabolism, essentially no information is available on the nature of this lipolytic activity. The objectives of the present study are to examine the ability of Walker carcinosarcoma 256 in ascites form to produce FFA under conditions similar to those described by Spector and Steinberg (3) for the Ehrlich ascites tumor and to gather additional information on the nature of the lipolytic mechanisms operative in ascites tumor cells.

MATERIALS AND METHODS

Maintenance and Handling of Tumor Cells

Walker carcinosarcoma 256 (obtained from Microbiological Associates, Washington, D.C.) in ascites form was grown in male, Sprague-Dawley rats (300 g) by intraperitoneal injection of ca. 4×10^6 tumor cells in 0.2 ml saline. The cells were harvested 7 days after transplantation. At this time between 2 and 3×10^9 cells were recovered/rat. The ascites fluid-cell mixture from 2-4 rats was withdrawn from the peritoneal cavity by use of a 20 cc syringe (no anticoagulant) and the cells immediately centrifuged at 500 x g at 5 C for 10 min. The supernatant was drawn-off and the cells resuspended in 10 volumes of cold-hypotonic saline

TABLE I

Selected Substances in Cell-Free Ascites Fluid[a]

Substances	N[b]	Content (average ± standard error)
pH	13	7.40 ± 0.05
Free fatty acids	5	417 ± 44 μeq/l
Triglycerides	3	68 ± 19 mg/100 ml
Glucose	6	<1 mg/100 ml
Lactic acid	3	213 ± 20 mg/100 ml

[a]Supernatant remaining after centrifugation of freshly drawn ascites fluid-Walker 256 cell suspensions was analyzed for free fatty acid, triglyceride, glucose, and lactic acid content and for pH.

[b]The number of different ascites fluid samples analyzed (from 7 day infections).

(0.3 % NaCl) to hemolyze contaminating erythrocytes. The ascites cells then were recentrifuged, and the washing procedure was repeated. Prior to the last centrifugation, the total volume of the cell-hypotonic saline suspension was recorded, and an aliquot of the suspension was removed for counting of cells on a hemocytometer.

Incubation In Vitro of Ascites Cells

The packed cells recovered from the last wash were resuspended generally to a cell concentration of 0.4-0.5 x 10^8 cells/ml in ice-cold Krebs phosphate buffer (no Ca^{++}) containing 4% (w/v) fatty acid-poor bovine serum albumin (Calbiochem Corp., La Jolla, Calif.) The buffer was adjusted to desired pHs in the range of 3.7-9.3. The cells usually occupied less than 5% total volume of the suspensions. Calcium was eliminated from the buffer, since it forms insoluble salts with fatty acids and, thus, could influence the kinetics of FFA production and release. In fact, ca. 1 mM $CaCl_2$ in the buffer did reduce FFA production by ca. 10%. Also, Michaelis's barbital-sodium acetate buffer and McIlvaine's citric acid-phosphate buffer (7), containing the 4% albumin, were tested in place of the Krebs buffer and found to offer no advantage over this modified Krebs phosphate buffer in supporting FFA production by the Walker 256 ascites cells.

After adding medium to the recovered washed cells, 3 ml aliquots of the cell suspensions were transferred to 25 ml erlenmeyer flasks containing either 0.3 ml isotonic saline or 0.3 ml test substance in saline. The test substance was either glucose, sodium nicotinate, epinephrine hydrochloride (Parke, Davis & Co., Detroit, Mich.), norepinephrine bitartrate (Winthrop Labs., New York, N.Y.), crystalline glucagon (lot 258-234B-167-1, Eli Lilly & Co.), or prostaglandin E_1 (Upjohn Co., Kalamazoo, Mich.). All test substances were dissolved in isotonic saline at 10 times the final concentration desired in the cell suspensions. The cell suspensions were incubated with shaking (Dubnoff metabolic shaker) for up to 2 hr at 37 C in air. At the start of incubation and at various times during the incubation, aliquots of the cell suspensions were removed and centrifuged (1000 x g for 10 min), and 1.0 ml supernatant (medium) was taken and immediately added to 10 ml Dole's solution (8) containing 1.0 ml saline. The FFA was extracted according to Trout, et al. (9). In some cases, FFA was extracted from aliquots of the whole cell system (cells plus medium) taken before and after incubation. The FFA was measured via the automated colorimetric method of Lorch and Gey (10). The results of these measurements were expressed as the microequivalents (μeq) of FFA produced or released/10^8 ascites cells. When glucose, latic acid, and triglyceride levels were measured, the methods of Nelson (11), Barker and Summerson (12), and Lofland (13) were used, respectively.

Gas Chromatographic Identification of Individual FFA

To establish the identity of the FFA produced by the cells under various conditions, aliquots of the whole cell systems from before and after a 2 hr incubation were added to 20 volumes of 2:1 $CHCl_3$-CH_3OH for extraction of total lipid. The extracted lipids were recovered and separated into individual lipids by thin layer chromatography (TLC) as described earlier (14). The recovered FFA then were converted to the methyl ester derivatives and identified by gas liquid chromatography (GLC) as previously described (15). The relative composition of the FFA produced by the cells was determined from a knowledge of the total quantity of FFA in the medium before and after incubation and from identification of the

TABLE II

Comparison of Cellular Release vs. Net Production of Free Fatty Acids by Ascites Tumor Cells[a]

Experiment	Cell content of suspension	(A) Increase in μeq of free fatty acid/10 ml of medium (supernatant)	(B) Increase in μeq of free fatty acid/10 ml of whole cell system[b]	(A)/(B)
1	0.80 x 10^8/ml	3.74	3.96	0.944
2	1.17 x 10^8/ml	6.79	6.71	1.012
			Average	0.978

[a]Walker 256 ascites tumor cells were incubated at pH 7.4 for 2 hr at 37 C as described in "Material and Methods." The free fatty acid contents of the medium (supernatant) and of the whole cell system (medium + cells) were measured before and after incubation.

[b]Cells occupied ca. 8% total volume of the whole cell system.

relative composition of the FFA in the medium at these two times. The relative composition of the FFA present in fresh, cell-free ascites fluid also was determined by these methods.

RESULTS

Selected Substances in Ascites Fluid

Some of the chemical constituents of Walker 256 cell-free fresh ascites fluid are shown in Table I. Ascites fluid contains appreciable levels of FFA, ca. 400 μeq/liter. The virtual absence of glucose in this medium strengthens the possibility that the cells rely heavily upon available FFA as fuel for metabolic energy. However, the significant amount of lactic acid in the medium indicates that the cells had, at some time, metabolized appreciable glucose.

FFA Production by Ascites Cells

Walker 256 ascites tumor cells release comparatively large amounts of FFA (ca. 1.0 μeq/10^8 cells) to the medium in the course of a 2 hr incubation in vitro at pH 7.4 (Fig. 1). This FFA release proceeds at an essentially linear rate over the 2 hr incubation. Comparison of

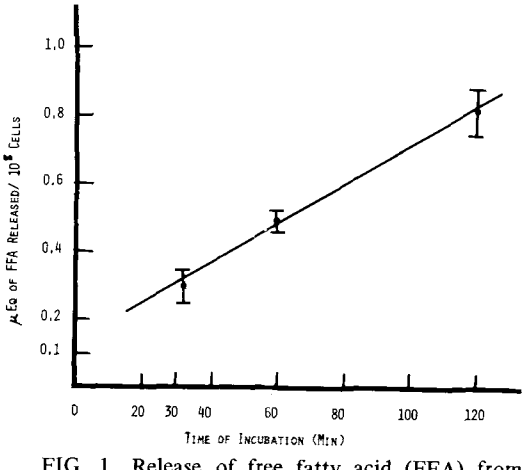

FIG. 1. Release of free fatty acid (FFA) from Walker 256 ascites cells vs. time of incubation. Washed, intact ascites tumor cells (average 0.95 ± 0.15 x 10^8 cells/ml) were incubated in vitro at 37 C for 120 min at pH 7.4. The FFA content of the medium was measured at 30, 60, and 120 min of incubation as described in "Material and Methods." The data represent the average of five experiments. Verticle bars are ± one standard error.

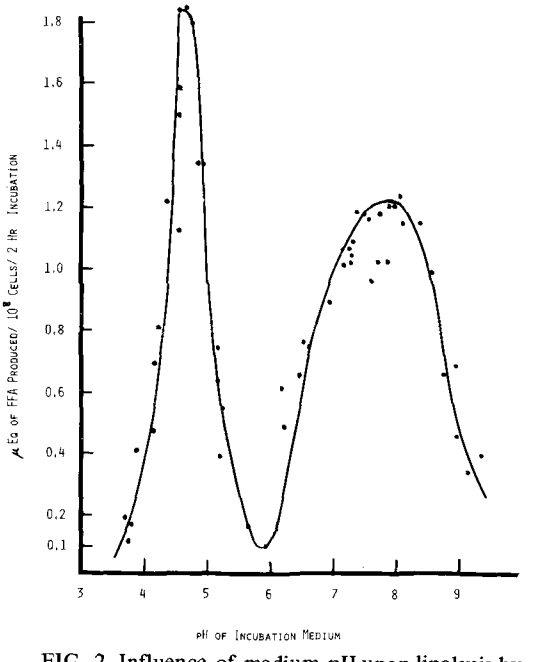

FIG. 2. Influence of medium pH upon lipolysis by intact ascites tumor cells. Ascites tumor cells (0.5 x 10^8/ml) were incubated for 2 hr in Kreb's phosphate buffer (pH 3.7-9.3) containing 4% fatty acid-poor bovine serum albumin. The data were collected from the incubation of eight separate cell pools.

TABLE III

Relative Composition of Free Fatty Acids

Fatty acid	Free fatty acids produced by lipolysis[a] At pH 7.4 %	At pH 4.7 %	Free fatty acids of cell-free ascites fluid (%)
12:0	0.6	0.3	0.6
14:0	2.1	1.6	4.2
14:1	2.5	1.5	0.7
16:0	18.9	18.7	24.0
16:1	5.3	4.7	7.3
18:0	15.1	11.2	10.5
18:1	23.4	26.4	22.8
18:2	14.5	13.4	12.6
18:3	<0.1	3.8	0.8

[a]Relative composition of the free fatty acids produced by intact Walker 256 ascites tumor cells upon incubation in vitro for 2 hr at 37 C at either pH 7.4 or 4.7. The presented values are averages of duplicate analyses.

the net increase in the FFA content of the incubation medium with the net increase of FFA of the whole system (cells plus medium) shows a ratio of ca. one (Table II). Thus, release of FFA by the cells is equivalent to net FFA production. Obviously, almost all of the FFA produced by the cells is released to the medium under the conditions used, i.e. an incubation medium containing albumin which is essentially depleted of FFA.

Effect of pH and Temperature Upon Ascites Cell FFA Production

When the cells were incubated in medium of varying pH, two distinct lipolytic acitivities were seen (Fig. 2), one with an acidic and narrow pH optimum of between ca. 4.6 and 4.8 and another with an apparent neutral-to-slightly-alkaline pH optimum of 7.4-8.2. The temperature sensitivites of the two lipolytic activities are clearly different (Fig. 3). The neutral-alkaline lipase activity (at pH 7.4) was relatively resistant to change at temperatures below 37 C, but the activity decreased sharply at temperatures greater than 37 C. The temperature optimum of the neutral-alkaline lipase activity actually seemed to be ca. 34-35 C. In contrast, the acid lipase activity (at pH 4.7) was lost rapidly when the cells were incubated below 37 C but was rather resistant to change at temperatures above 37 C. Free fatty acid production at pH 4.7, like that at pH 7.4, proceeds linearly over a 2 hr incubation.

Relative Composition of FFA

The compositions of the FFA produced by the intact cells at pH 4.7 and 7.4 and that of the ascites fluid are shown in Table III. The relative compositions of the FFA produced at acid and alkaline pH are very similar. In both cases, oleic acid was the principal FFA produced (ca. 25% total) with slightly less palmitic acid (ca. 19% total) and essentially equivalent percentages of stearic and linoleic acids (each 11-15% total). The only apparent difference in the composition of the FFA produced at the two pHs was the virtual absence of linolenic acid (18:3) in the FFA produced at the alkaline pH. The relative fatty acid composition of the cell-free ascites fluid FFA was similar to that of

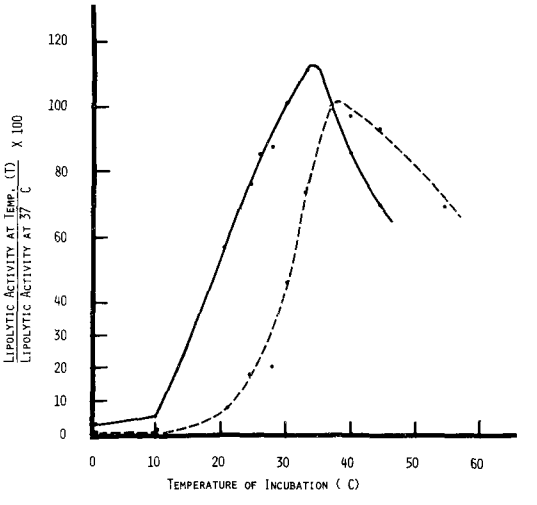

FIG. 3. Influence of temperature upon lipolytic activities of intact ascites tumor cells. Ascites tumor cells (0.5 x 10^8/ml) were incubated as described before for 2 hr at either pH 4.7 or 7.4 and at temperatures ranging from 0-55 C. The results are expressed as the free fatty acid production observed at experimental temperature (T) relative to the production observed at 37 C; the production at 37 C is, therefore, 100% activity. The data were collected from incubation of 10 different cell pools. —— alkaline lipase (pH 7.4) --- acidic lipase (pH 4.7).

TABLE IV

Effect of Various Agents upon Lipolysis by Intact Walker 256 Ascites Tumor Cells Incubated at Neutral and Acidic pH[a]

Agent	Concentrate	Percent inhibition of lipolysis (free fatty acid production)				
		pH 7.4			pH 4.7	
		Experiment 1	Experiment 2	Experiment 3	Experiment 1	Experiment 2
Glucose	20 mg%	3	-9[b]	-3	---	---
	40 mg%	-2	9	4	---	---
	60 mg%	---	6	9	---	---
	100 mg%	28	35	31	---	---
	200 mg%	53	55	53	8	3
	400 mg%	54	57	---	0	7
Epinephrine	1 μg/ml	6	5		10	-3
	10 μg/ml	5	4		3	1
	91 μg/ml	8	8		13	6
Norepinephrine	1 μg/ml	8	6		9	0
	10 μg/ml	8	-2		0	3
	91 μg/ml	12	2		12	-1
Glucagon	1 μg/ml	0	1		-2	1
	5 μg/ml	-9	4		5	-1
	50 μg/ml	-7	-1		0	2
Prostaglandin E_1	0.1 μg/ml	-7	-2		-3	-1
	1.0 μg/ml	1	3		-2	1
	10.0 μg/ml	-1	8		2	0
Na Nicotinate	10 mg%	-3	-5		4	1
	100 mg%	3	8		4	17
	200 mg%	14	13		18	29

[a]Ca. 0.5 x 10^8 cells/ml were incubated for 2 hr at 37 C in medium containing 4% serum albumin of pH 7.4 or 4.7. Free fatty acid production was measured in the presence and absence of test agent. In the absence of drug, ca. 1.0 μeq free fatty acid was produced/10^8 cells at both 7.4 and 4.7. Net release of free fatty acids from ascites tumor cells can be demonstrated if the molar ratio of free fatty acids to albumin ($\bar{v}$) in the incubation medium is low (3). This ratio was less than 0.15 for the buffer used.

[b]The negative sign denotes stimulation.

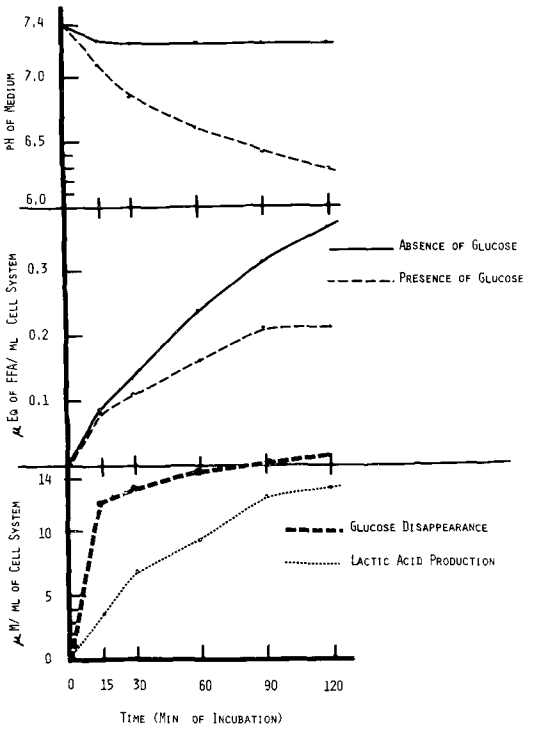

FIG. 4. Relationship of glucose metabolism to free fatty acid (FFA) production by intact ascites tumor cells and to change in medium pH. Changes in medium pH and FFA content were followed over a 2 hr period when cells (0.35 x 10^8/ml) were incubated in the absence and presence of glucose (400 mg%) at an initial medium pH of 7.4. The disappearance of glucose and appearance of lactic in a glucose containing cell system also were measured.

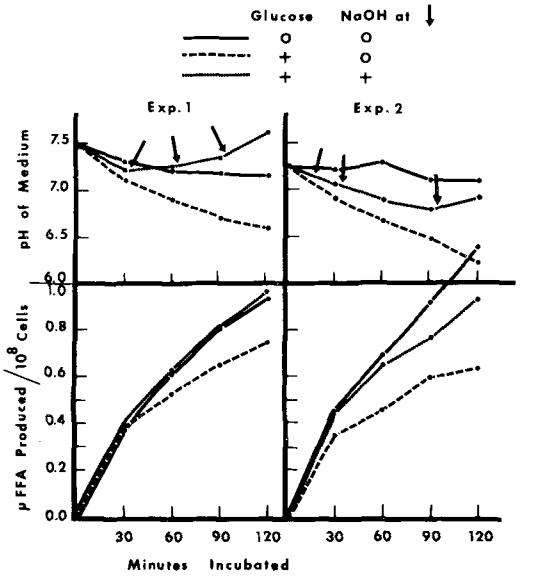

FIG. 5. Reversal of glucose inhibition of free fatty acid (FFA) production by intact ascites tumor cells. Medium pH and FFA production were measured over a 2 hr incubation of cells (0.33 x 10^8/ml in experiment 1 and 0.37 x 10^8/ml in experiment 2) at 37 C in either the presence or absence of glucose. The initial medium pH was 7.4. In both experiments, one cell system contained no added glucose and two others contained glucose at 400 mg%. To one of the glucose containing systems, 10-20 μliter aliquots of 1N NaOH were added at various times during the incubation to maintain the medium pH in the neutral range.

the FFA formed by cells incubated at either pH 4.7 or 7.4. Due to oversight, we did not measure for the presence of arachidonic acid. However, Spector (4) found no arachidonic acid in the ascites fluid FFA of the Ehrlich tumor.

Regulation of Ascites Tumor Cell Lipolysis

Substances known to influence lipolysis in normal mammalian cells were examined for ability to alter the lipase activities recognized in the Walker 256 ascites tumor (Table IV). As seen by Spector and Steinberg (3) with Ehrlich ascites cells, glucose in the incubation medium significantly inhibited FFA production at pH 7.4. Surprisingly, little or no inhibition of lipolysis occurred at an initial medium glucose level of less than ca. 100 mg%. Glucose did not influence lipolysis at the acid pH.

At the concentrations examined, epinephrine, norepinephrine, and glucagon, agents known to stimulate lipolysis in adipose tissue (16, 17), had no significant effect upon either the alkaline or acid lipolytic activity. Both prostaglandin E_1 and nicotinic acid can inhibit lipolysis in adipose tissue (16, 17). Prostaglandin E_1 did not affect lipolysis by the ascites cells. The combination of prostaglandin E_1 with epinephrine, norepinephrine, or glucagon also had no effect upon FFA production (data not shown). Nicotinate seemed to inhibit lipolysis at both the alkaline and acid pH but only at a very high concentration (200 mg%)

Experiments were conducted to determine the cause of the glucose related inhibition of FFA production at pH 7.4. When Walker 256 ascites tumor cells were incubated for 2 hr at 37 C in glucose-free medium (pH 7.4), the pH of the medium remained essentially constant, and FFA production proceeded at a linear rate over the incubation period (Fig. 4). However, in the presence of glucose (400 mg%), large amounts of lactic acid appeared in the medium, medium pH fell abruptly, and FFA production was inhibited. Virtually identical results were obtained in a second experiment (results not shown). Thus, the necessity for having high medium levels of glucose prior to seeing inhibition of lipolysis may be explained by decreases

in medium pH as a consequence of glucose conversion to lactic acid. Further support for this possibility is presented in Figure 5. If the pH of a glucose containing ascites cell suspension is maintained above neutral during the incubation by addition of a few μliter 1 N NaOH at various times, FFA production is not inhibited by glucose (Fig. 5, experiment 1). Similar results were obtained in a second experiment (Fig. 5, experiment 2), although, in this instance, insufficient base was added to keep the pH of the glucose containing system above neutral during the entire incubation period and, thus, there was some inhibition of FFA production.

DISCUSSION

The results of the present study with the Walker 256 ascites tumor confirm and extend earlier observations with the Ehrlich ascites tumor on tumor lipid metabolism. Like the Ehrlich tumor, the Walker tumor in ascites form produces and releases large quantities of FFA when incubated in vitro at neutral pH in a medium containing serum albumin depleted of FFA. However, the rate of FFA release by the Walker tumor appears to be 4-5 times greater than that reported for the Ehrlich ascites tumor (3). Spector and Steinberg (3) observed a net production of ca. 0.1 μeq FFA/10^8 Ehrlich ascites cells after 1 hr incubation at 37 C at neutral pH. The Walker tumor consistently produced 0.4-0.5 μeq FFA/10^8 cells during a first hr of incubation under similar conditions. This apparent difference between the tumors could reflect a more rapid turnover rate of ester fatty acid in the Walker tumor. The rapid production and release of FFA by the Walker ascites cell is consistent with the concept of a rapidly turning-over ester fatty acid pool in ascites tumor cells.

The Walker tumor possesses at least two lipolytic activities; one with a narrow and acidic pH optimum and another with a rather broad neutral-to-weakly-alkaline pH optimum. The lipolytic activity observed in the neutral-to-alkaline pH range could certainly represent a mixture of lipase activities. Phospholipases, triglyceride lipases, and lipoprotein lipase enzymes all can possess pH optima between ca. 7-8.5 (16, 18). The inability of Ehrlich ascites tumor to incorporate ^{14}C-labeled fatty acids from rat chylomicrons (19) suggests the absence of an active lipoprotein lipase in this tumor. Since the neutral-alkaline lipolytic activity of the Walker cells appeared to have a temperature optimum of ca. 34-35 C and was resistant to inactiviation at lower temperatures, it is tempting to speculate that this activity constitutes a triglyceride lipase involved with providing fatty acids for generating oxidative energy. However, Spector's observation (4) that Ehrlich ascites cells incorporate FFA principally into phospholipids and the indication that the ester fatty acids of this tumor turnover very rapidly, rather suggests that the neutral-alkaline lipolytic activity is one or a combination of phospholipases. The specific identity of the neutral-alkaline lipase(s) and also of the acid lipase requires additional investigation.

The recognition of acid lipases in normal body cells is a rather recent observation. Mahadevan and Tappel (20) and Guder, et al., (21) identified hepatic lysosomal lipases with pH optima of 4-5. Acid lipase also has been found in adipose tissue associated with particles which sediment upon centrifugation with the mitochondrial fraction (16). The acid lipolytic activity in the Walker cells could, thus, represent a lysosomal enzyme. It seems unlikely that this acid lipase contributes significantly to the rapid ester fatty acid turnover occurring in vivo, since the pH of ascites fluid is ca. 7.4.

Studies on the regulation of lipolysis in Ehrlich ascites cells have been reported by Spector and Steinberg (3, 6). They found that neither cyanide nor fluoride significantly influenced lipolysis. However, the presence of rather high concentrations of glucose in the incubation medium stimulated the uptake of ^{14}C-palmitic acid into cellular esterified lipid and inhibited both the depletion of cellular total lipid ester (6) and the release of ^{14}C-labeled FFA to the incubation medium (3). Spector and Steinberg concluded that glucose decreases FFA release by stimulating FFA reesterification. We confirm that glucose in the incubation medium (pH 7.4) at a concentration of 100 mg% or more markedly depresses FFA production by Walker ascites tumor cells in vitro. However, in contrast to the conclusion of Spector and Steinberg, this inhibition is most likely due to changes in medium pH as a consequence of glucose metabolism to lactic acid. This possibility also may explain the observations made by Spector and Steinberg on glucose inhibition of FFA release by the Ehrlich tumor, since the buffering capacity of the medium used by them (3) and that used in the present study were essentially identical and also since the concentration of ascites cells incubated in the two studies were similar. Irrespective of the mechanism by which glucose influences ascites cell lipolysis in vitro, it is unlikely that glucose plays any significant direct role in the physiological regulation of lipolysis in vivo. Ascites fluid, at least for the

Walker tumor, contains no detectable free glucose.

The activity of hormone sensitive lipolysis, as recognized in mammalian adipose tissue, is stimulated by epinephrine, norepinephrine, and glucagon and inhibited by nicotinic acid and prostaglandin E_1 (16, 17). Also, acid triglyceride lipase of fat cells is stimulated by epinephrine and inhibited by nicotinic acid (16). Neither the neutral-alkaline nor the acid lipolytic activity of the Walker ascites cells was influenced significantly by any of these agents. Thus, it seems that neither of the lipolytic activities in this tumor is comparable to the hormone sensitive lipase or to the acidic triglyceride lipase of adipose tissue.

What functions do these lipolytic activities serve in ascites tumor cell metabolism? As discussed earlier, both the findings of Spector and Steinberg (4) and Baker, et al., (5) indicate that the fatty acid requirements of the Ehrlich tumor can be supplied by FFA present in the ascites fluid. In fact, the flux of FFA into Ehrlich ascites tumor cells was found to be 25-30 times greater than the net rate of ester fatty acid accumulation seen during growth (5). Further, since essentially all of the FFA taken-up by ascites cells is esterified (4), this newly esterified fatty acid must be rapidly hydrolyzed to prevent a gross accumulation of lipid in these cells. Thus, the function of the recognized lipolytic activities may be to maintain an equilibrium between fatty acid assimilation and storage. If this concept is correct, selective inhibition of these lipolytic activities could have profound effects upon the growth of ascites tumor cells.

The observed similarities between the composition of FFA present in the ascites fluid and that of the FFA produced at both acid and neutral pH can be interpreted to suggest that the ascites fluid pool of FFA also turns over rapidly. Spector (4) reported substantial differences between the relative composition of the fatty acid present in ascites fluid as FFA and that of the total lipid of the Ehrlich ascites tumor. As ascites fluid FFA is taken-up by the cells in vivo, certain fatty acids could be incorporated into more slowly turning-over lipid-esters, perhaps those seen to accumulate with growth, whereas FFA incorporated into the more rapidly turning-over ester pools would soon be released to the ascites fluid. Thus, after some period of time, the composition of the ascites fluid FFA and that released by the cells via lipolysis would be similar.

ACKNOWLEDGMENT

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UV-Autoxidized Linolenic Acid in High Yield for Cancer Study

N. BAKER, and **L. WILSON,** Radioisotope Research Veterans Administration Wadsworth Hospital Center, Los Angeles, California 90073 and Department of Medicine, University of California School of Medicine, Los Angeles, California 90024

ABSTRACT

Aqueous emulsions of linolenic acid were exposed repeatedly to UV light in an oxygen atmosphere. After each exposure, a dense, yellow liquid formed which was insoluble in petroleum ether, poorly soluble in water, but highly soluble in ethyl ether. After 5 cyclic 90 min exposures, linolenic acid was almost quantitatively converted to autoxidation products which had the following properties: average mol wt, 510; 2-thiobarbituric acid-reactive (pink derivative); peroxidase-reactive; peroxide no., 1100 meq/kg; hydrogen no., 404 mg/mmole H_2; C, 61%; H, 9.0%; and O, 30%. Only trace amounts of free aldehydes were present. After treatment with BF_3-methanol, the major, volatile products found were methyl acetal-esters of azelaic semialdehyde and azelaic acid. These products did not vary qualitatively from one UV exposure to another as shown by gas liquid chromatographic analyses. Ca. 90% of the autoxidized material did not appear on gas liquid chromatography even after methylation. Our technique allows the rapid preparation of high yields of autoxidized linolenic acid sufficient to study chemical properties and anticancer activities of the autoxidation products.

INTRODUCTION

Shuster (1) has reported that, upon 90 min exposure of methyl linolenate to UV light, a water-soluble substance formed that was thiobarbituric acid (TBA) reactive and that inhibited both aerobic and anaerobic respiration of Ehrlich ascites carcinoma cells. As yet, the major water-soluble substances responsible for the anticancer activity of Shuster's preparation have not been identified. However, a major TBA-reactive product of autoxidized linolenic acid, free malonaldehyde, was identified and shown not to inhibit respiration of these cancer cells (2,3). Under conditions similar to those of Shuster, hydrogen peroxide also has been shown to be an autoxidation product of linolenic acid (2,3). However, the hydrogen peroxide formed could only account for a part of the oxidation product's inhibitory effect upon the tumor cells (3).

One of the major problems associated with this area of investigation has been the low yield of autoxidation products which one obtains by the method of Shuster. We have explored the possibility of using continuous oxygen flow and recycling of unused linolenic acid to obtain large yields of water-soluble, TBA-reactive products which would inhibit glycolysis and respiration of Ehrlich ascites carcinoma cells.

We have found conditions for converting linolenic acid almost quantitatively to highly oxidized products, including small amounts of TBA-reactive material. In this article, which is the first of four articles dealing with the crude autoxidized material, we describe how it may be prepared in high yield. Evidence is presented that the autoxidized material has an average of only 1 of the original 3 double bonds of the starting 18 C fatty acids; that ca. 4 g atoms of oxygen, on the average, have been added/18 C unit; and that only ca. one-sixth of the oxygen added is at the oxidation state of a peroxide.

Based upon mol wt measurements, elemental analysis, and acid number, the product appears to contain ca. 1 dimer (mol wt, ca. 700) for every monomer (mol wt, ca. 350) of autoxidized product. Most of the autoxidation product(s) is involatile; however, it decomposes partially during methylation to products which have been identified and quantified by gas liquid chromatography (GLC). Nevertheless, 90% of the crude product remains unidentified, despite extensive attempts to form volatile derivatives. In the second and third papers of this series (in preparation), we present evidence that as much as 120 mg/kg of the crude autoxidation product can be injected intraperitoneally into mice without any apparent signs of toxicity; moreover, under certain conditions, the autoxidized linolenic acid prevents the growth of Ehrlich ascites tumor cells in the peritoneal cavity of mice. In the fourth paper (in preparation), we show that the prevention of Ehrlich ascites carcinoma growth in mice by injection of the autoxidation product is associated with the development of a marked immune response against the same tumor.

MATERIALS AND METHODS

Preparation of Autoxidation Product

Linolenic acid and water (1:4) were emulsified by vigorous mechanical shaking for 5 min. A 4 ml aliquot of emulsion was transferred to a Hanovia quartz actinometer cell (7.5 mm diameter x 10 mm thick) and exposed to UV light, 100 watts for 90 min. This process was repeated until 20 ml emulsion was irradiated (first exposure). Throughout the exposure, the cell and sample were maintained at 8.6 cm from the lamp source, and oxygen was permitted to flow through the cell at 1600 ml/min. The UV light source was an Hanovia quartz mercury vapor arc tube, Hanovia Chemical and Manufacturing Co., Newark, N.J., catalog no. 16A-13, 100 watts, high pressure, U-shaped 1.7 in. arc length with the following filters: catalog no. 6541, 9863 nickel oxide, which transmits light between 2500-3700A° and catalog no. 6540, 7910 Vycor, which transmits light between 2100-4000A°.

Upon completion of the exposure, the sample was transferred to a 50 ml glass-stoppered tube and extracted two times with 4 volumes petroleum ether (PE), boiling range, 30-60 C. The removed PE was evaporated under nitrogen to reclaim the linolenic acid for recycling, i.e. for repeated exposure to UV light in the above manner, a total of five times (Fig. 1). The aqueous phase and the PE- and water-insoluble product of autoxidized 18:3 were extracted in two volumes of ethyl ether (EE). (The insoluble material dissolved.) The ether was evaporated; the residual dimer was dissolved in ethanol and again freed of solvent by vacuum distillation. The latter step usually was repeated twice to remove volatile material (2).

Chromatography

GLC was carried out using 6 ft columns packed with either 15% ethylene glycol succinate (EGS) on Chromosorb P, 100-120 mesh, or with 20% silicone fluid (SF) 96 on Chromosorb W, 60-80 mesh, column temperature, 175-195 C; flow rate, ca. 40 ml/min. Reverse phase thin layer chromatography (TLC) was carried out on either Eastman Chromagram sheets or on commercial glass plates coated with Silica Gel G. The nonpolar liquid phases were either undecane or dodecane, and the solvent systems were either hexane-ether (3:2) or acetonitrile, 90% saturated with nonpolar phase, e.g. dodecane (4,5). TLC using $AgNO_3$-impregnated plates and hexane-EE (3:2) as solvent also was carried out (6).

Chemical Properties

The methods for assaying TBA- and ROOH-reactivities have been described previously. ROOH refers to involatile substances which substitute for hydrogen peroxide in a peroxidase assay (2,3,7). Mol wt was measured by isothermal distillation in methyl acetate vs. methyl myristate (8). Methylation was carried out using BF_3-methanol (9). Oxidation of aldehydes to acids was carried out with Ag_2O (10). The following treatment, which can be used for methylation of carboxylic acids (11), also was found to convert aldehydes quantitatively to methyl esters of the corresponding fatty acids: 5% H_2SO_4 in methanol, 100 C for 60 min. We used this method in our attempts to identify suspected aldehydes. Reduction in H_2-Pt was carried out (according to R. Stein, personal communication) as follows. Chloroplatinic acid catalyst was heated in alcohol until the catalyst developed a black color, after which unsaturated compound was added and heated at 70 C for 60 min with H_2 passing through the system. Water was added and the reduced product extracted with petroleum ether (30-60 C, boiling point [BP]). Hydrogen number was determined according to the method of Brown (12). Peroxide number was determined iodometrically (13). Carbon and hydrogen analyses were carried out by Elek Microanalytical Laboratories, Harbor City, Calif.

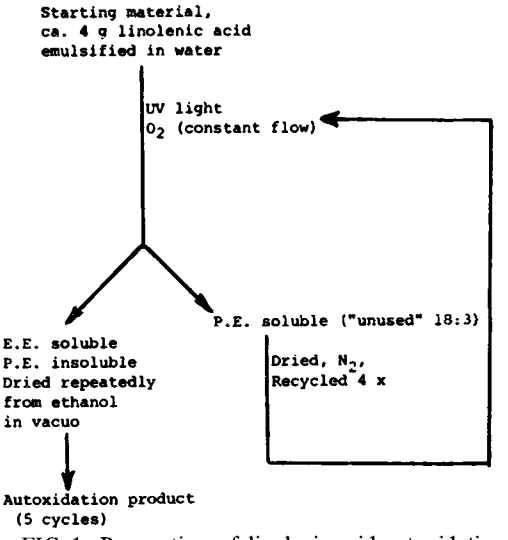

FIG. 1. Preparation of linolenic acid autoxidation product. PE = petroleum ether and EE = ethyl ether.

RESULTS

Physical Properties

Linolenic acid is less dense than water (Fig. 2). After emulsification in water, cyclic exposure to UV light and oxygen, extraction with PE, and centrifugation, no layer which is lighter

TABLE I

Yield of Autoxidized Linolenic Acid, Malonaldehyde, and ROOH-Like Substances after Cyclic Exposures of Emulsified Linolenic Acid to UV Light and O_2

Preparation	Cycle	Autoxidation product[a] mmoles/initial mmole 18:3	Malonaldehyde mmoles/mmole autoxidation product[a]	ROOH[b] mmoles/mmole autoxidation product[a]
1	(1+2)	0.16	0.026	0.029
	(3+4+5)	0.10	0.054	0.043
	(1-5)	0.26	0.036	0.034
2[c]	1	0.011	0.017	0.031
	2	0.071	0.032	0.049
	3	0.101	0.031	0.049
	4	0.063	0.034	0.047
	5	0.073	0.031	0.048
	(1-5)	0.32	0.032	0.049
3	1	0.113	0.022	0.023
	2	0.091	0.023	0.024
	3	0.121	0.030	0.028
	4	0.058	0.024	0.023
	5	0.061	0.028	0.028
	(1-5)	0.44	0.026	0.026
Mean	(1-5)	0.34	0.031	0.036

[a]Calculated on the basis of average MW = 510.

[b]"ROOH" here refers to substances which substitute for hydrogen peroxide in a peroxidase assay.

[c]The linolenic acid used for preparation 2 was freshly opened, whereas that used for preparations 1 and 3 had been opened, resealed under N_2, and stored for several months at -16 C. Preparations 2 and 3 were made from a common vial of linolenic acid.

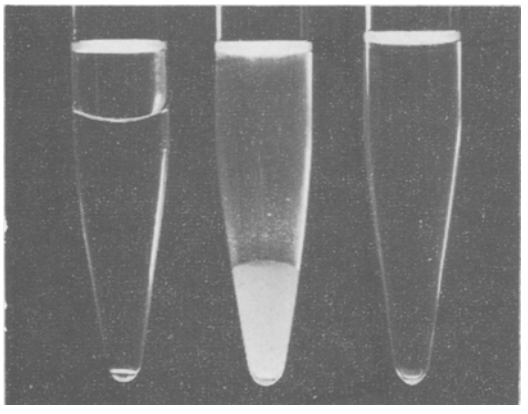

FIG. 2. Solubility properties of the autoxidation product produced by cyclic exposure of linolenic acid to UV light, oxygen, and water. The tube in the left contains ca. 1 ml starting material (linolenic acid) layered on 4 ml water (lower phase) prior to emulsification and cyclic exposure to UV light and O_2. The middle tube shows the cumulative product formed following five cyclic exposures to UV light and O_2 and following repeated extraction with petroleum ether. The petroleum ether phase has been removed. The dense autoxidation product, after centrifugation, has settled to the bottom of the tube. The tube at the right shows the appearance of a typical preparation of autoxidized linolenic acid similar to that shown in the middle tube after extraction of the autoxidation product with ethyl ether. Removal of the autoxidation product is almost quantitative. (Volumes of the 2 tubes on the right have been arbitrarily adjusted to correspond to ca. 5 ml total volume.)

than water remains. However, a large quantity of dense, PE-insoluble material is found beneath the water (Fig. 2, middle tube). The dense autoxidation product can be quantitatively extracted by a single shake-out with two volumes of EE (Fig. 2, right tube). The PE-insoluble fraction of autoxidized linolenic acid is poorly soluble in $HCCl_3$ or CCl_4, highly soluble in EE, methyl acetate, methanol, ethanol, and slightly soluble in water. Since it is denser than water, extensive addition of oxygen to the starting polyunsaturated fatty acid is indicated. The autoxidized material is a viscous yellow liquid which does not solidify at -76 C. It contains substances which have a characteristic odor unlike any of the aldehydes, alcohols, or acids with which we have worked.

Chemical Characterization

The average mol wt of the autoxidation product is 510 ± 40 (mean, ± standard deviation, n = 6). It has 1.3 meq titratable acid groups/average mmole; a hydrogen number of 406 mg/mmole H_2 at standard temperature and pressure (theoretical for 18:3, 93 mg/mmole H_2); and a peroxide no. of 1100 meq/kg (ca. 1 meq/average 2 mmoles). Elemental analysis (C, 61.1%; H, 9.0%; oxygen, by difference) indicated that 30% mass was oxygen, in contrast to linolenic acid which only has 11.5% oxygen. The autoxidized material contains substances

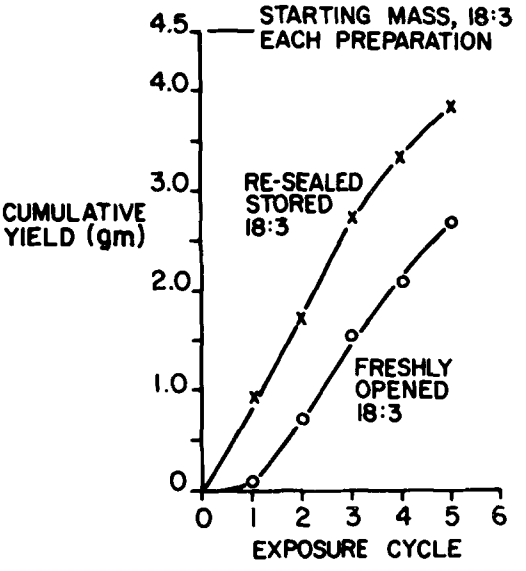

FIG. 3. Cumulative formation of the autoxidation products of linolenic acid during cyclic exposure of fresh and stored linolenic acid to UV light, O_2, and water.

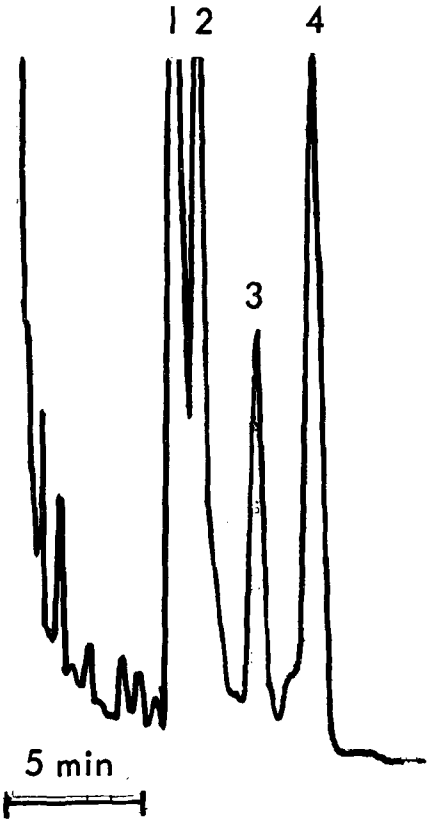

FIG. 4. Gas liquid chromatography (GLC) of methylated autoxidation products. The methylated material was passed through an SF96 GLC column and the eluate trapped. The eluate was then rechromatographed and the record shown here. Liquid phase is 20% silicone fluid 96 on Chromosorb W. Four major peaks have been identified as: peaks 1 and 2, *cis,trans*-isomers of 9-methoxy-8-nonenoic acid methyl ester; peak 3, azelaic acid dimethyl ester; and peak 4, azelaic semialdehyde methyl ester dimethyl acetal. A fifth major peak, corresponding to linolenic acid methyl ester t_r = 75 min is not shown; see Figure 6.

which form a pink derivative with 2-TBA (0.031 mmoles equivalent malonaldehyde/average mmole of autoxidation product) and substitutes for hydrogen peroxide in a peroxidase assay (0.036 mmoles equivalent ROOH/average mmole of autoxidation product). Most of the free malonaldehyde and H_2O_2 should have been removed by the repeated vacuum distillations to dryness from ethanolic solutions prior to the above determinations (2,3); however, if, after drying, malonaldehyde and H_2O_2 remain associated with the major product(s), they could only be trace contaminants of the major linolenic acid autoxidation products.

Recycling of Linolenic Acid— Constancy of Product

The yield of autoxidized linolenic acid dimer is relatively low when highly purified linolenic acid is first exposed to UV light and oxygen. However, if the linolenic acid is separated from the oxidation product by extraction with PE and the solvent removed under nitrogen, the previously exposed linolenic acid serves as a much more efficient source of oxidation product than does previously unexposed 18:3 (Fig. 3). Moreover, as shown in Figure 3 and Table I (preparations 2 and 3), the product's yield/cycle and properties remain almost constant through at least 4 cycles (the second through the fifth). If a vial of highly purified linolenic acid is opened then again sealed under nitrogen (without using special precautions to ensure the complete absence of trace oxygen), the linolenic acid appears to become altered during storage (several months at -16 C), so that it will give rise to a nearly maximal yield of autoxidized dimer during the first exposure to UV light and oxygen (Fig. 2 and Table I, preparation 3). Thus, the yield from the first cycle in preparations 2 and 3 shown in Table I differ by an order of magnitude even though both products were made from one vial of commercial 18:3. Preparation 2 was made from freshly opened 18:3, whereas preparation 3 was made from 18:3 which had been stored for several months under N_2 at -16 C in a sealed ampoule. This phenomenon also was observed in other preparations.

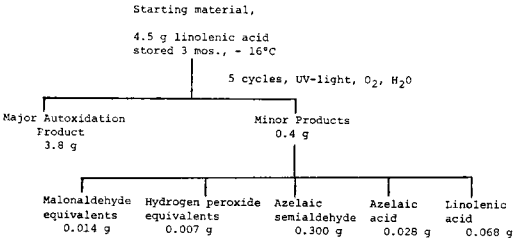

FIG. 5. Summary of products of UV light initiated, cyclic autoxidation of aqueous linolenic acid.

GLC

Only four major components appeared on GLC after methylation of the autoxidation product with BF_3-methanol. The products have been identified by retention times, mass spectroscopy, reductive ozonolysis, Ag_2O oxidation of aldehydes to acids, and IR spectroscopy (compound 2 only) as follows: compound 1, azelaic semialdehyde dimethyl acetal methyl ester; compound 2, azelaic acid dimethyl ester; compound 3, 9-methoxy-8-nonenoic acid methyl ester, the vinyl ether of which is formed as an on-column artifact (14) from compound 1; and compound 4, linolenic acid methyl ester (position and *cis,trans*-isomerization of double bonds not investigated). The approximate proportions of each component which appear on GLC (ignoring minor peaks) are as follows: 9C semialdehyde, 76%; 9C-dicarboxylic acid, 7%; and linolenic acid, 17%.

The azelaic semialdehyde (methylated in BF_3-methanol) sometimes was represented on GLC as 3 peaks, depending upon the column used for separation (Fig. 4). Thus, on a 20% SF 96 column, the vinyl ether methyl ester separated into ca. equal *cis-trans*-isomers, either of which, when collected and reinjected, gave rise to both of the original peaks. The vinyl ether methyl ester was characterized by mass spectral analysis and conversion on reductive ozonolysis to a substance having the retention time of the corresponding 8-C semialdehyde methyl ester. The GLC peaks derived from azelaic semialdehyde were reduced greatly when the autoxidation product first was oxidized with Ag_2O or H_2SO_4 and methylated. The peak corresponding to azelaic acid dimethyl ester was augmented under these conditions to the degree expected from oxidation of azelaic semialdehyde to azelaic acid. A small peak appeared on GLC where malonaldehyde tetramethyl acetal would be expected. This peak, when collected during gas chromatography gave a pink TBA derivative. The peak height on GLC was of the order of magnitude necessary to account for the TBA-reactivity of the autoxidation product.

The total mass represented by the sum of all peaks was ca. 10% that of the starting material. Thus, 90% mass was not seen on GLC. It also follows that malonaldehyde, azelaic acid, and linolenic acid were not formed on the GLC column from the autoxidized material by thermal decomposition, since they were methylated totally. Therefore, these substances were either formed during autoxidation process or during methylation or both. Evidence that free malonaldehyde forms during the autoxidation has been presented previously (2,3). Reduction (Pt, H_2) of the autoxidation product, followed by methylation of the reduced substance(s), resulted in the virtually complete conversion of all of the major GLC-resolvable peaks to one substance, methyl stearate. Again, only ca. 10% starting mass was represented by the methyl stearate peak. Thus, we may conclude that most of the major product visible on GLC, azelaic semialdehyde, was not present in free form prior to methylation but, rather, was formed during methylation from a substance which could be reduced to methyl stearate. However, to account for the azelaic acid in the product, and because free malonaldehyde is known to be formed in small amounts (2,3), we assume that some free azelaic semialdehyde probably was formed during the autoxidation period; this semialdehyde would be expected to be oxidized further, at least partially, to azelaic acid in the presence of O_2. Several minor GLC peaks which were collected behaved as methyl acetals of 2,4-dienals (2,4-dinitrophenylhydrazine derivatives) of intermediate chain lengths. However, appreciable quantities of free dienals or free medium chain mono- or dicarboxylic acids could not be separated by TLC prior to methylation and GLC analysis, and the relative absence of free aldehydes in the autoxidation product was confirmed by both NMR and IR spectral analyses. Therefore, the dienals are either bound in a polymeric form or are formed from another product and released during methylation and GLC.

Only ca. 15% methylated autoxidation product could be distilled at reduced pressure (4 mm Hg), in a 240 C oil bath. The distillate appeared on GLC as peaks, some of which had the same retention times as the undistilled material. However, much of the azelaic acid semialdehyde dimethyl acetal methyl ester appeared to be converted to vinyl ethers during the distillation.

The mass of the products, which appeared on GLC both before and after reduction of the autoxidation product followed by methylation, was only ca. 10% starting mass, despite the use of a variety of columns, including 1% XE-60 on

Gas Chrom P, 100-120 mesh (Applied Science Laboratory, State College, Pa.) which separates cholesterol from other high boiling sterols. Therefore, GLC analysis failed to yield information regarding 90% mass of the autoxidation product. A summary of the approximate yields of the various major products is given in Figure 5.

The GLC records of the autoxidation products after each of five cycles of linolenic acid exposure to UV light and oxygen are shown in Figure 6. Two series of cyclic exposures (A and B) are represented in Figure 6; they correspond, respectively, to preparations 2 and 3 of Table I and to the freshly opened and resealed, stored materials shown in Figure 3. The major peaks correspond to methyl esters, acetals, and vinyl ethers derived from azelaic semialdehyde, azelaic acid, and linolenic acid. The relatively high yield of linolenic acid in the first cycle of preparation 2 (Fig. 4A) is probably due to contamination by starting material; however, this mass of 18:3 in the autoxidation product represents less than 1% of the starting 18:3. The GLC patterns remained virtually constant during each exposure in confirmation of data shown in Table I. The small peak, labeled 2a shown in Figure 6B, was probably also present but unresolved from peak 2 in the preparation shown in Figure 6A. Peak 2a tends to be augmented during storage or after high temperature distillation and may be 9-methoxy-8-nonenoic acid methyl ester formed from the dimethyl acetal methyl ester of azelaic semialdehyde (14). Thus, we found no major qualitative changes in the products formed during repetitive exposures of linolenic acid to UV light and oxygen.

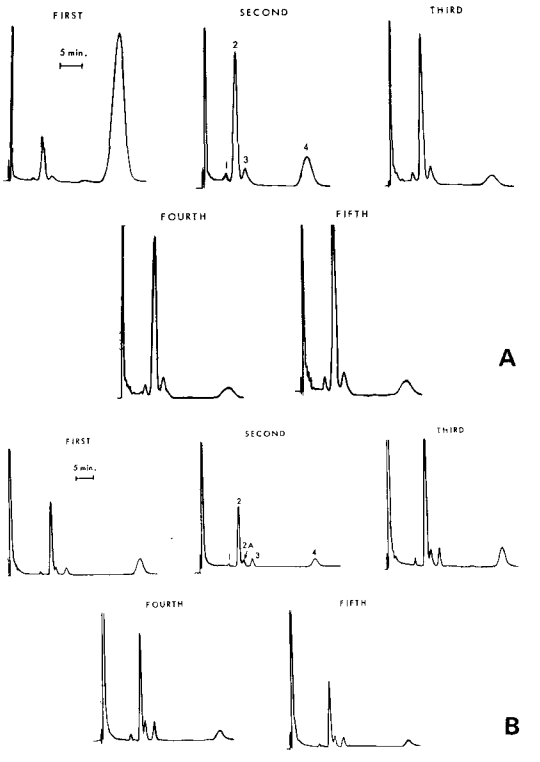

FIG. 6. Degree of constancy of gas liquid chromatographic pattern after cyclic exposure of linolenic acid to UV irradiation, water, and oxygen. (A) Methylated autoxidation products formed from fresh 18:2; (B) from stored 18:3. 15% ethylene glycol succinate on Chromosorb P. Peak 1, unidentified; peak 2, azelaic semiladehyde methyl ester dimethyl acetal; peak 2a, unidentified; peak 3, azelaic acid dimethyl ester; and peak 4, methyl linolenate. The number of exposures of linolenic acid to UV light is indicated (first, second, etc.; Fig. 1). Note that peak 3 in Figure 6B has been resolved from peak 2 by decreasing the flow rate. All peaks are delayed correspondingly in Figure 6B, see for example, 6A.

Stability

The autoxidation product is relatively stable when stored at -16 C under N_2 as evaluated by the properties described above (TBA-reactivity, peroxidase-reactivity, GLC pattern, solubility, volatility, TLC behavior) and based upon anticancer activity in vitro and in vivo (unpublished observations).

DISCUSSION

We have been interested in the isolation and characterization of a water-soluble, potential anticancer agent which forms during the UV initiated autoxidation of linolenic acid (2,3). Despite the great potentiality for producing myriad water-soluble compounds from autoxidized linolenic acid (15,16), we have found that, by cyclic exposure of aqueous linolenic acid emulsions to UV irradiation and oxygen, this polyunsaturated fatty acid can be converted rapidly and almost quantitatively to a stable material which differs in its chemical properties from previously described products. The resulting material is so highly polar that it is poorly soluble in PE; yet, it can be quantitatively and easily extracted from an aqueous emulsion with ethyl ether. Moreover, although a part of the material is somewhat soluble in water (2,3), it forms in such a large yield and is so dense that it can be centrifuged readily from an aqueous emulsion as a heavy (highly oxidized), yellow, viscous liquid (Fig. 2). Based upon acid number, elemental analysis and mol wt considerations, we estimate that the autoxidized material contains ca. 1 mole of dimer (mol wt ca. 700)/mole monomer (mol wt ca. 350). The material is considerably more satu-

rated than linolenic acid and is extensively oxidized (30% oxygen). In fact, we estimate that ca. 4 atoms of oxygen have been added and 2 double bonds have disappeared/18 C of the starting material. Only one sixth of the added oxygen is peroxidic. Some lower mol wt fragments also are present in, or can be formed from, the autoxidized material, but these fragments account for only ca. 10% total mass. They seem to be derived to a large extent from 18 C-compounds, some of which can be reduced to methyl stearate but which also appear to break down, in part, to azelaic semialdehyde (methyl ester, dimethyl acetal) and malonaldehyde (tetramethyl acetal) during treatment with BF_3-methanol. Since both malonaldehyde (17) and azelaaldehyde (18) are known oxidation products of polyunsaturated lipids, it is possible that these compounds (as well as azelaic acid) also were present prior to treatment with BF_3-methanol. However, no free azelaaldehyde could be detected by combined TLC and GLC analyses of the nonmethylated oxidation product (N. Baker, V. Slawson, and L. Wilson, unpublished observations).

In view of the very striking inhibition of Ehrlich ascites tumor growth in mice which can be brought about with the autoxidized material and the relative lack of toxicity after intraperitoneal injection into mice, further attempts to fractionate and to characterize the crude oxidation product seem warranted.

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Esterification of Cholesterol and Hydrolysis of Cholesteryl Ester in Alcohol Induced Fatty Liver of Rats

NOZOMU TAKEUCHI, MASAMI ITO, and **YUICHI YAMAMURA,** Third Department of Internal Medicine, Osaka University Hospital, Fukushima, Osaka, Japan

ABSTRACT

Repeated oral administrations of ethanol to rats induced accumulation of cholesteryl ester, as well as triglyceride in the livers. The contents of free cholesterol and phospholipid in the livers were not changed significantly in the present experiment in which ethanol ingestions were repeated four times. Although in vitro esterification of cholesterol by particle fractions of the alcoholic fatty liver was not affected, hydrolysis of cholesteryl palmitate by the supernatant fraction of the liver homogenate was reduced when compared with those of the control group which was given water or isocaloric glucose. The results of in vitro esterification of cholesterol and hydrolysis of cholesteryl palmitate in the liver of the rats which were ingested with glucose were larger than those of the control rats which were given water.

INTRODUCTION

It is well known that chronic alcoholics often have fatty livers. Experimentally, an oral administration of large amount of ethanol to animals is able to produce fatty infiltration of the liver easily, and profound alterations in lipid metabolism have been suggested in the alcohol induced fatty liver.

The accumulation of hepatic triglyceride is characteristic in fatty liver, meanwhile increase of hepatic cholesteryl ester is observed with it (1-4). Ridout and his colleagues (5) reported that the increase of hepatic cholesteryl ester was proportional to the amount of hepatic triglyceride in fatty liver which was induced by feeding a hypolipotropic diet.

Intrahepatic cholesterol metabolism in fatty liver has not been well known. In this paper, esterification of cholesterol and hydrolysis of cholesteryl ester were investigated in vitro using rat liver which was ingested with large amounts of ethanol. The effect of glucose administration upon cholesterol metabolism in rats also was examined in vitro at the same time.

MATERIALS AND METHODS

4-^{14}C Cholesterol (55.7 mCi/mM) was purchased from Radiochemical Center, Amersham, England, and 4-^{14}C cholesteryl palmitate (36.2 mCi/mM) was obtained from Daiichi Kagaku Ltd., Tokyo, Japan. Labeled cholesterol and cholesteryl palmitate were purifed by thin layer chromatography (TLC) before use.

Female Sprague-Dawley rats weighing 200 g had been fed a balanced stock diet (Oriental Kobo Ltd., Osaka, Japan) ad libitum. The rats were fasted for 13-15 hr before the experiment and 2 ml/100 g body wt of 40% ethanol was ingested by means of a stomach tube in the rats. The same volume of water was given to one control group and an isocaloric glucose solution to another group. Four hr after the ingestions, some of the rats were sacrificed for the experiment.

The ingestions of ethanol or glucose were repeated every 4 consecutive days to other animals of 3 groups fed ad libitum. They were fasted overnight before the experiment day and sacrificed 4 hr after the last treatments. Some of the rats whose body wt decreased more than 5% by the treatments were excluded from the experiment.

In another experimental series, rats were fed for the previous 3 days with a high cholesterol diet containing 1.5% cholesterol and 0.5% sodium cholate. After overnight fasting, they were ingested with ethanol or water as described above and used for the experiment.

The liver (3 g) was homogenized with 3 volume cold potassium phosphate buffer (0.1 M, pH 7.4) and centrifuged at 2000 G for 20 min to remove the cell debris, nuclei, and floating fluffy layer of lipids. Thereafter, the supernatant was centrifuged at 105,000 G for 60 min. The precipitate consisting of particle fractions was suspended in the potassium phosphate buffer which corresponded to equal volume of the original supernatant before the centrifugation at 105,000 G. The suspension (1 ml) was used for the determination of cholesterol esterification, and 0.05 μCi 4-^{14}C cholesterol in 0.05 ml 10% Tween 80 solution was added to the medium. For hydrolysis of cholesteryl ester in vitro, 1 ml 105,000 G supernatant was put into a flask, and 0.05 μCi cholesteryl palmitate was added. In the experiment with the low cholesterol diet, 50 μg cold substrate in 0.05 ml acetone:diethyl ether (2:1 v/v) was added to it, because cholesteryl ester was

TABLE I

Effect of Ethanol or Glucose Ingestion upon Hepatic Lipid of Rats

Frequencies of ingestions	Animal groups	Hepatic lipid (mg/g wet tissue)									
		Total lipid		Triglyceride		Free fatty acid		Phospholipid	Cholesterol		
									Free	Esterified	
1	Control 11[a]	40.57 ± 1.17[b]	C	7.02 ± 0.36	A	2.30 ± 0.19	B	27.49 ± 0.57	1.85 ± 0.08	0.30 ± 0.05	
	Alcohol 11	47.12 ± 1.89	B	13.30 ± 0.95	A	1.99 ± 0.25		28.09 ± 0.61	1.80 ± 0.12	0.43 ± 0.06	
	Glucose 8	37.36 ± 1.20	B	6.26 ± 0.61	A	1.39 ± 0.19		27.62 ± 1.80	1.87 ± 0.13	0.35 ± 0.08	
4	Control 12	38.03 ± 0.74	A	5.82 ± 0.33	A	1.80 ± 0.16	B	26.97 ± 0.58	1.82 ± 0.07	0.29 ± 0.02	B
	Alcohol 16	61.90 ± 5.11	A	29.58 ± 5.33	B	1.44 ± 0.15		28.68 ± 0.61	1.80 ± 0.05	0.56 ± 0.06	C
	Glucose 10	38.95 ± 1.08	B	8.40 ± 0.86	B	1.22 ± 0.06		25.64 ± 0.48	1.88 ± 0.11	0.28 ± 0.04	

[a]Numbers of rats.
[b]Mean ± standard error. A = $p<0.001$, B = $p<0.01$, and C = $p<0.02$.

negligible in the supernatant fraction. Methods of incubation and lipid analysis were carried out by the same methods reported previously (6).

Esterification of cholesterol was active in microsomal fraction. Mitochondrial fraction had some activity, but supernatant fraction at 105,000 G had no activity at all. Therefore, the combined fraction of mitochondria and microsome of liver was used to estimate the hepatic esterification activity of cholesterol in the present experiment.

The remaining portion of the liver (1 g) was homogenized with 40 ml chloroform:methanol (2:1 v/v), and the extracted lipid fraction was washed with Folch's procedure (7). After the dried lipid was weighed, a portion of the total lipid was applied on a TLC plate coated with activated silica gel (20 x 20 cm, 0.25 mm in thickness), which was developed with solvent mixture of petroleum ether:diethyl ether:acetic acid (90:10:1 v/v/v) for 40-45 min. Lipid bands on the plate were detected by iodine vapor and scrapped off from the plate after the brown colors were fainted. Silica gel was extracted 3 times with chloroform:methanol (1:1 v/v) or methanol containing 1% acetic acid (for the extraction of phospholipid). After evaporation of the solvents, free cholesterol, tri-, di-, and monoglyceride, free fatty acid and phospholipid were determined respectively by the methods described by Leffler (8), Fletcher (9), Dole (10), and Hoeflmayr (11) with slight modifications. The cholesteryl ester fraction at solvent front was hydrolyzed with 1 ml 5% ethanolic KOH solution at 65 C for 1 hr. Hydrolyzed cholesterol was extracted with 5 ml redistilled petroleum ether, and after washing the ether extract with 1 ml distilled water 2 times, cholesterol was determined with Leffler's method. Statistical significance was estimated with Student's t-test.

RESULTS

Table I demonstrates the results of the analysis of hepatic lipid of the rats which were treated with ethanol or glucose. As shown in Table I, total lipid of liver increased significantly even by a single administration of ethanol. Repeated ingestions of ethanol to the rats further promoted the increase of hepatic total lipid. Hepatic total lipid of the rat which recieved a single alcohol ingestion was 47.12 mg/g wet tissue and that treated 4 times with alcohol was 61.90 mg/g ($p<0.05$). Hepatic triglyceride increased also by an alcohol administration, and repeating it further elevated the triglyceride content from 13.3 mg/g to 29.58

mg/g ($p<0.02$). Glucose feeding did not affect the amount of hepatic total lipid, but triglyceride increased significantly with reciprocal decrease of free fatty acid by repeating the treatments.

Free cholesterol was not influenced either by alcohol or glucose. However, cholesteryl ester increased from 0.29 mg/g to 0.56 mg/g ($p<0.01$), when the ingestions of alcohol were repeated.

Figure 1 demonstrates the relationship of hepatic cholesteryl ester and hepatic triglyceride in control and alcohol treated rats. These two factors were well correlated as shown in the figure.

The results of in vitro esterification of hepatic cholesterol are shown in Table II. There was no significant difference between esterification of cholesterol by particle fractions of alcoholic fatty liver and those of the control group, while glucose administration stimulated esterification of cholesterol in vitro, as indicated in Table II.

From the results of in vitro experiments for the hydrolysis of cholesteryl palmitate, it was shown that the hydrolysis of cholesteryl palmi-

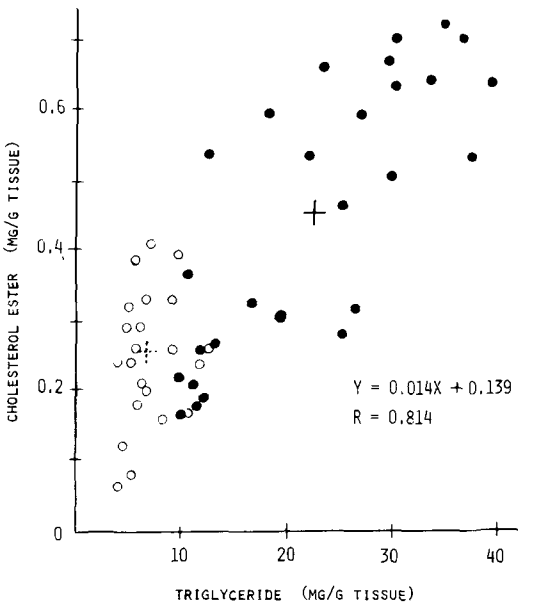

FIG. 1. Relationship between hepatic cholesteryl ester and triglyceride in alcohol ingested rats. ○ = control ┼ (mean) ● = alcohol treated ┼ (mean)

TABLE II

Effect of Ethanol or Glucose Administration upon Esterification of Cholesterol by Rat Liver Particle Fractions in Vitro

Frequencies of ingestions	Animal groups		Percentage esterification (percentage/2 hr/10 mg protein)		Esterified cholesterol (nMoles/2 hr/10 mg protein)	
1	Control	10	15.14 ± 0.87	A	38.6 ± 2.4	B
	Alcohol	10	15.10 ± 1.22		36.7 ± 3.9	
	Glucose	8	21.65 ± 1.10	B	53.6 ± 2.9	C
4	Control	7	13.41 ± 1.01	C	34.8 ± 2.3	C
	Alcohol	7	13.25 ± 0.62		34.3 ± 1.7	
	Glucose	6	18.51 ± 1.34	C	48.2 ± 2.4	B

[a]Numbers of rats.

[b]Mean ± standard error. A = $p<0.001$, B = $p<0.01$, and C = $p<0.02$.

TABLE III

Effect of Ethanol or Glucose Administration upon Hydrolysis of Cholesteryl Palmitate by 105,000 G Supernatant of Liver

Frequencies of ingestions	Animal groups		Percentage of hydrolysis (Percentage/2 hr/50 mg protein)		Hydrolyzed ester (nMoles/2 hr/50 mg protein)	
1	Control	7	10.69 ± 0.52[b]	D	8.9 ± 0.45	C
	Alcohol	5	8.29 ± 0.93	B	6.8 ± 0.80	D B
	Glucose	8	14.25 ± 1.10		11.9 ± 0.95	
4	Control	7	11.69 ± 0.45	B D	9.3 ± 0.42	C C
	Alcohol	7	8.15 ± 0.67	B	6.9 ± 0.57	A
	Glucose	6	14.97 ± 1.08		12.1 ± 0.84	

[a]Numbers of rats.

[b]Mean ± standard error. A = $p<0.001$, B = $p<0.01$, C = $p<0.02$, and D = $p<0.05$.

TABLE IV

Effect of Ethanol Ingestion upon in Vitro Esterification of Cholesterol and Hydrolysis of Cholesteryl Palmitate in Liver from Cholesterol Fed Rats

Esterification	Percentage esterification (Percentage/2 hr/10 mg protein)	Esterified cholesterol (nMoles/2 hr/10 mg protein)
Control 4	14.48 ± 0.62[b]	117.7 ± 9.3
Alcohol 4	13.95 ± 1.39	116.3 ± 12.6
Hydrolysis	Percentage hydrolysis (Percentage/2 hr/50 mg protein)	Hydrolyzed ester (nMoles/2 hr/50 mg protein)
Control 4	9.06 ± 1.02	24.8 ± 2.7
Alcohol 4	4.04 ± 0.93 $p<0.02$	10.9 ± 2.8 $p<0.02$

[a]Number of the rats.

[b]Mean ± standard error.

tate decreased in alcoholic fatty liver (Table III). In the glucose treated rats, hydrolysis of cholesteryl palmitate was significantly higher than that of the control group.

When the rats were fed for the previous 3 days of the experiment with a high cholesterol diet which suppressed the hepatic cholesterogenesis, ethanol administration also reduced the hydrolysis of cholesteryl palmitate in vitro, though it did not affect the esterification of cholesterol as shown in Table IV.

DISCUSSION

Hepatic triglyceide occupied ca. 15-16% of total lipid in control rats, whereas it became 48% after the repeated ingestions of alcohol as indicated in Table I. So, the increase of total lipid by alcohol was due mainly to the increase of triglyceride, because other lipids were not significantly changed except for cholesteryl ester which made only a small contribution to the wt of total lipid. Cholesteryl ester is usually ca. two-tenths the total cholesterol in normal rat liver. However, as fatty infiltration is developed in the liver, cholesteryl ester increases remarkably accompanied with an increase of hepatic triglyceried (1-4). In the present experiment in which 6.4 g ethanol/kg body wt was ingested 4 times to rats, cholesteryl ester in the liver increased ca. twofold compared to the control group and had good correlation with hepatic triglyceride content.

It is well known that serum cholesterol exclusively is esterified by transacylation of fatty acid on β-position of serum phosphatidyl choline by lecithin:cholesterol acyltransferase (12). The uptake of serum cholesteryl ester is rapid without hydrolysis by the liver (13-17), and the cholesteryl ester is hydrolyzed to free form by hydrolyrase in the liver (13-16). The free cholesterol is degraded to bile acid (18), reesterified in the liver (15,16,19,20), or secreted into the blood stream (15, 20-22). Esterification of cholesterol in the liver takes place with acyl CoA by acyl CoA:cholesterol acyltransferase which localizes in particle fractions of the liver (23-27). But reesterification of cholesterol usually is considered to be slow in the liver (15-16). So, hydrolysis of cholesteryl ester might be more important than the esterification of cholesterol for hepatic accumulation of ester form.

In the present experiment, it was shown that the esterification of cholesterol was not affected by ethanol as demonstrated in Tables II and IV. But the hydrolysis of cholesteryl palmitate was decreased by alcohol administration (Table III and IV). Lefèvre, et al., (4) reported that ethanol feeding to rats delayed the turnover rate of bile acid and decreased the daily amount of fecal bile acid when a high cholesterol diet was fed and proposed that ethanol affected cholesterol transformation into bile acid. Such impairment of the degradation to bile acid might have some relationship with decreased hydrolysis of cholesteryl ester. However, it is not clear that the decrease of hydrolysis of cholesteryl ester is either the cause or the effect of the impairment of cholesterol degradation.

Lieber, et al., (2) suggested the increased cholesterogenesis in alcoholic fatty liver. Scheig and Isselbacher (28) also demonstrated that direct addition of ethanol to rat liver slices enhanced in vitro lipogenesis from acetate 1-^{14}C. In the present experiment when cholesterogenesis was suppressed by feeding a high cholesterol diet, hydrolysis of cholesteryl palmitate was low in the rat liver treated with ethanol. Therefore, it may be independent of the activity of hepatic cholesterol synthesis.

The effects of ethanol are not derived from an excess of calories, because the effects of isocaloric glucose ingestion upon hepatic cholesterol metabolism are completely different from that of ethanol. Glucose administration causes increase of in vitro esterification of cholesterol and hydrolysis of cholesteryl palmitate.

From these results in the present experiments, it is suggested that the impairment of hydrolytic activity of cholesteryl ester by ethanol has some contribution to the accumulation of cholesteryl ester in the liver.

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SHORT COMMUNICATIONS

Preparation of Psychosines (1-O-Hexosyl Sphingosine) from Cerebrosides

ABSTRACT

A convenient method for large or small scale preparation of psychosine from cerebroside has been developed by adaptation of published procedures. Cerebroside is refluxed with butanol and aqueous KOH, then the KOH is removed with perchloric acid. The fatty acids are removed by extraction with hexane and the excess perchloric acid is removed by partitioning between chloroform, ethanol, and water.

INTRODUCTION

The first practical psychosine preparation from cerebroside was described by Klenk in 1926 (1) and modified by Carter and Fujino (2). (Cerebroside is galactosyl ceramide and glucosyl ceramide; psychosine, in analogy with this custom, is used for galactosyl sphingosine and glucosyl sphingosine.) The latter method involves refluxing with dioxan and aqueous barium hydroxide, but, in our hands, this gives rise to obstructive foaming and coalescence of the lipid-base complex into hard lumps. We found that only the cerebroside containing hydroxy acids appears to be hydrolyzed under these conditions. The yield is poor and the final product, psychosine sulfate, does not recrystallize as originally described (2). It is said to be hygroscopic (3).

The method of isolating the psychosine, which involves precipitating the base as the sulfuric acid salt $(Psy)_2H_2SO_4$, has led to an unfortunate terminology which has caused many individuals to assume the salt is actually a sulfate ester. The salt ought to be called psychosinium sulfate.

A marked improvement came from Taketomi and Yamakawa (4), who refluxed the cerebroside for 2 hr in 1 N KOH in butanol-water 90:10. The KOH was removed by washing with water, and the fatty acid was removed with a silica gel column. We found a serious problem with emulsions in the alkaline partition; this was particularly difficult with large samples. Attempts at removing the KOH with other partition systems gave unsatisfactory results, and we finally resorted to dialysis (5). This method is tedious with large samples, and the dialysis bags sometimes break, so we investigated the recent method of Cumar, et al. (6). These workers refluxed cerebroside with 1 N KOH in 2-methoxyethanol-water 70:30 for 6 hr, removed the KOH with perchloric acid, and removed the fatty acid with a Florisil column. This method has the disadvantage of calling for a peroxide-prone solvent. We found a foaming problem when refluxing, but this could be controlled by addition of a little octanol. However, there was an appreciable amount of sphingosine formation, and removal of the relatively nonvolatile solvent necessitated desiccation for some time over sulfuric acid.

It was decided to combine the advantages of these two methods, the use of butanol for hydrolysis and the use of perchlorate for removing the alkali. We found that we could remove the fatty acid by a simple solvent partition, leaving almost pure psychosine. The 2 hr recommended initially for the hydrolysis was found to give incomplete hydrolysis, so the period was extended. A final chromatographic purification step can be avoided for many

purposes.

METHODS AND RESULTS

Galactosyl ceramide was prepared from crude sphingolipids (7) with a Florisil column (8). By using a relatively tall column (4.7 x 210 cm), we were able to isolate rather pure material. The column was packed dry, using 2000 g Florisil that had been dried at 100 C overnight and hydrated with 160 ml water. The packing was wetted with chloroform until most of the air bubbles had been removed, then washed with 500 ml chloroform-methanol 88:12. A 50 g sample of mixed sphingolipids, prepared by solvent extractions (7), was added as a solution in 2500 ml of the same chloroform-methanol mixture and rinsed in with 500 ml more solvent. Elution was carried out with 6000 ml chloroform-methanol 82:18, then with 6000 ml of a 72:28 mixture. Air pressure (ca. 10 psi) was used to drive the solvents at ca. 1000 ml/hr and 800 ml fractions were collected with a modified Technicon fraction collector (9). (A good deal of sulfatide can be obtained by further elution with a 60:40 mixture.) Fractions found to contain cerebroside by thin layer chromatography (TLC) were pooled and lyophilized from benzene.

Glucosyl ceramide was prepared from a Gaucher patient's spleen, following a similar procedure. The sphingolipid concentrate was made from a total lipid extract by alkaline methanolysis (5).

Cerebroside hydrolysis was carried out in a 100 ml round-bottom flask containing 28 g KOH dissolved in 40 ml water. To this was added 10 g cerebroside and 360 ml n-butanol; the flask joint was wiped clean, and refluxing was carried out for 4 hr with the use of an oil bath held at ca. 125 C. A soda lime tube protected the alkali. The level of the oil was kept just below the level of the flask's liquid. One experiment in which the KOH was not first dissolved in the water led to a very dark mixture.

The hydrolysate was diluted with 400 ml methanol, and ca. 120 ml of 5 N $HClO_4$ was added to bring the pH to ca. 7 (overacidification was corrected with KOH). The potassium perchlorate was removed by filtration through a Celite-coated glass funnel (600 ml size) and rinsed with 400 ml methanol. The methanol rinse was used to complete the transfer of the filtrate to a 2 gal glass bottle. Some fatty acid precipitates when the methanol is added.

To remove the fatty acids, we acidified with 5 N perchloric acid (ca. 26 ml, to yield pH 3-4) and added 1350 ml water and 2700 ml hexane. The mixture was mixed well by shaking the bottle in a swirling motion then left until the upper layer cleared. The hexane was sucked off with an aspirator (it can be saved for preparation of hydroxy fatty acids if galactosyl ceramide was used). We removed residual fatty acids with a similar extraction with 1350 ml hexane; 200 ml methanol was added to prevent emulsification.

The perchloric acid was now removed by adding 2 N NaOH (ca. 42 ml to yield pH ca. 10) and 1350 ml chloroform to form the partition system. The mixture was swirled, and, when the lower layer cleared, the upper layer was sucked off and discarded. The lower layer was washed twice more with 1350 ml portions of methanol-water 1:1. The clear lower layer then was evaporated to dryness under vacuum with the aid of benzene. Since small amounts of butanol could be detected by odor, the psychosine was left in a vacuum desiccator over sulfuric acid for a while. The lipid then was transferred to a small flask with benzene and lyophilized again. The yield was ca. 4.3 g with either type of cerebroside.

TLC of the product with chloroform-methanol-water-ammonium hydroxide 70:30:4:1 shows only psychosine, with just a trace of sphingosine, when examined with bromothymol blue, ninhydrin, and a charring spray (10). (A very small spot, presumably dihydropsychosine, can be seen just below the primary spot.) The psychosine gives characteristic colors with the different sprays: blue or blue + white with the pH indicator and pink with ninhydrin. Acylation with fatty acids has yielded cerebrosides, which were identified by TLC on silica gel and silica gel-borate plates, and by their IR spectra. Elemental analysis of a sample by Spang Microanalytical Laboratory, Ann Arbor, Mich., yielded the values, 62.25% C and 10.07% H (theor.: 62.45% C and 10.25% H).

The time required for each partition step varied according to the degree of shaking but was generally 1-5 hr. We usually did not wait for both layers to clear before discarding the unwanted layer. In one trial, we found that several hundred mg of KCl was useful in speeding the clearing of the methanol-water washes. Of course, when one works on a smaller scale, it is possible to speed the washing steps by centrifugation. It is not recommended that any solvent volume ratios given in the above procedure be changed as emulsion or recovery problems might arise. Because the purification steps involve solvent partitioning primarily, the method is particularly suited to small scale preparations as well as to large scale work.

NORMAN S. RADIN
Mental Health Research Institute,
The University of Michigan,
Ann Arbor, Michigan 48104

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In Vitro Desaturation of 1-^{14}C Linoleic Acid in Novikoff Hepatoma

ABSTRACT

The lipid fatty acid pattern of normal liver, host liver, and Novikoff hepatoma was determined by gas liquid chromatography, and Δ6-desaturase activity for linoleic acid was measured in the microsomal fractions. The results showed that, in Novikoff hepatoma, there is a correlation between the low content of arachidonic acid and the low activity of Δ6-desaturase, a key enzyme in the biosynthetic pathway of this acid.

INTRODUCTION

Earlier studies carried out in our laboratories (unpublished results) have shown that the lipid fatty acid pattern of normal liver no longer occurs in Novikoff hepatoma. The main features of the fatty acid composition in this tumor are the relatively high oleic acid content, concomitant with a relatively low amount of arachidonic acid. Similar disturbances, especially with respect to the oleic acid content, also were described in other experimental hepatomas (1-3).

The present experiment was performed to find out if the low level of arachidonic acid content in the Novikoff hepatoma is related to changes in the activity of the Δ6-desaturase, a key enzyme in the biosynthetic pathway of this unsaturated fatty acid (4-5).

EXPERIMENTAL PROCEDURES

The Novikoff hepatoma was maintained by intraperitoneal implants into Holtzman rats. Normal liver was obtained from rats of the same breed. Fatty acid composition of total lipids from normal liver, host liver, and Novikoff hepatoma was determined by gas liquid chromatography (GLC) (6) in columns packed with 15% diethylene glycol succinate on Chromosorb WAW (100-120 mesh) at 180 C. The activity of the desaturating enzyme was measured in the microsomal fractions separated by differential centrifugation at 105,000 x g (7). The assay conditions for desaturating activity were as follows: 5 mg microsomal protein were incubated in an open test tube with 100 nmoles diluted labeled fatty acid (1-^{14}C linoleic acid, 57.0 mC/mmole, 99% radiochemically pure, Radiochemical Center, Amersham, England, diluted to a specific activity of ca. 1.7 mC/mmole with the corresponding unlabeled pure fatty acid). The incubation was performed in a Dubnoff Shaker at 37 C for 30 min in a total volume of 1.5 ml 0.15 M KCl, 0.25 M sucrose solution containing, in μmoles: adenosine 5′-triphosphate, 2; coenzyme A, 0.1; nicotinamide adenine dinucleotide, reduced form, 1.2; $MgCl_2$, 7.5; glutathione, 2.2; sodium fluoride (NaF), 62; nicotinamide, 0.5; and phosphate

NORMAN S. RADIN
Mental Health Research Institute,
The University of Michigan,
Ann Arbor, Michigan 48104

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TABLE I

Fatty Acid Composition of Total Lipids from Normal Liver, Host Liver, and Novikoff Hepatoma[a]

Fatty acid[b]	Mole%		
	Normal liver	Host liver	Novikoff hepatoma
14:0	2.6	2.7	3.8
14:1	2.7	1.9	3.0
16:0	5.6	4.2	6.9
18:0	21.0	14.6	10.7
18:1	14.5	16.4	29.3
18:2	19.4	19.0	17.7
20:4	10.6	10.5	2.4

[a]Each value in the table was determined by calculating the mean of the results of three separate analysis of tissue samples of different animals.

[b]Other components not tabulated account for 100%.

buffer (pH 7.0), 62. After incubation, the mixture was saponified and the extracted free fatty acid esterified (7). The distribution of radioactivity between linoleic and γ-linolenic methyl esters was determined by GLC in a Pye apparatus (7).

RESULTS

An impression of the variability in the fatty acid spectrum of the total lipids from normal liver, host liver, and Novikoff hepatoma is given in Table I. Specially intriguing is the relatively high amount of oleate in the tumors when compared with the values obtained from normal liver and host liver, concomitant with an inversion of the oleate-stearate proportion in the tumors. The low amount of arachidonic acid observed in the tumor tissue is also remarkable, although the content of its precursor, linoleic acid, does not differ from that of normal liver and host liver. These results resemble those from essential fatty acid deficient rats (8), specially with respect to the high content of oleic acid.

As can be seen in Table II, the conversion of linoleic acid to γ-linolenic acid is depressed significantly in the Novikoff hepatoma when compared to normal liver and host liver.

DISCUSSION

Although no definitive conclusion can be drawn from the present data, apparently the low amount of arachidonic acid observed in the hepatoma could be a consequence of the depressed fatty acid desaturating activity of the microsomes for linoleic acid. Moreover, in spite of the different possible pathways that have been shown in mammalian cells (9), Marcel, et al., (10) demonstrated that the dominant pathway for arachidonic acid biosynthesis involves aerobic desaturation of linoleic acid prior to the elongation step. According to these findings, the growth of the Novikoff hepatoma cells is not affected by the low content of arachidonic acid, as was demonstrated for two other rapidly dividing tumors (11). To achieve a further insight into the molecular basis of the altered metabolism of the cancer cells, studies on the arachidonic acid metabolism in Novikoff hepatoma are currently in progress.

LILIANA CHIAPPE
OSVALDO MERCURI
MARÍA ELENA DETOMÁS
Cátedra de Bioquímica
Instituto de Fisiología
Facultad de Ciencias Médicas
La Plata, Argentina

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TABLE II

In Vitro Oxidative Desaturation of Linoleic Acid to γ-Linolenic Acid by Microsomes from Normal Liver, Host Liver, and Novikoff Hepatoma

Tissue[a]	Percentage of conversion Linoleic 18:2 → γ-18:3
Normal liver	8.0 ± 2.0
Host liver	8.0 ± 1.0 NS[b]
Novikoff hepatoma	3.0 ± 0.6 P< 0.001

[a]Five animals were tested in each group. Data are the means ± standard deviations of the means. Probability (P) values are related to normal liver.

[b]NS = not significant.

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LETTER TO THE EDITOR

Occurrence of Triglycerides in Earthworms

Sir: Studies on the lipid composition of earthworms have been reported (Lovern, *Biochem. J.* 34:709 [1940] and Cerbulis and Taylor, *Lipids* 4:363 [1969]). These investigators made reference, *inter alia*, to the complexity of earthworm lipids and to the apparent absence of triglycerides. The inefficient lead salt method of analysis available to Lovern was not capable of isolating glycerides, but the high content of unsaponifiable matter led him to state that "it seems doubtful whether much ordinary triglyceride could have been present, most of the fat presumably being sterol, etc., esters."

Cerbulis and Taylor, who employed modern chromatographic techniques, reported that no tri-, di-, or monoglycerides were detected and that this feature, together with the brown-black color, were two of the most characteristic properties of earthworm lipids.

The purpose of this letter is to report that current investigations upon the lipids of earthworms have shown that triglycerides are present in substantial proportions in worms whose lipids are extracted freshly but that, upon storage of dead earthworms, the triglycerides are lipolyzed rapidly to free fatty acids and glycerol.

Earthworms, identified as a mixture of *Lumbricus rubellus* and *Allolobophora caliginosa*, were collected at different seasons from the same paddock of ryegrass-clover pasture land by digging and hand-sorting from soil ca. 3-6 in. below the surface. Within 1 hr of collection, the worms were washed several times with dilute saline solution, followed by distilled water, and the total lipids were extracted with a mixture of chloroform-methanol (2:1 v/v) in a Sorvall Omnimixer according to the method of Folch, Lees, and Sloane-Stanley (*J. Biol. Chem.* 226:497 [1957]). Column chromatography using acid treated Florisil (Carroll, *J. Lipid Res.* 2:135 [1961]), was employed to separate the total earthworm lipids into neutral lipids, glycolipids, phospholipids, and a small residue. By means of a combination of silicic acid column chromatography and preparative thin layer chromatography (TLC), the neutral lipids were resolved into their constituent categories. The identity of all glyc-

TABLE I

Triglyceride and Free Fatty Acid Contents of Neutral Lipids of Earthworms Collected at Different Seasons

Quantitative data	Spring (October 1971)	Summer (February 1973)	Autumn (March 1973)	Winter (August 1973)
Wt fresh washed worms (g)	1050.0	178.0	137.0	90.0
Wt total lipid extract (g)	17.70	2.14	3.38	1.61
Total lipid content (% dry matter basis)	10.3	11.1	11.1	9.1
Neutral lipids (% total lipids)	39.3	28.5	29.5	35.2
Triglycerides (% neutral lipids)	37.3	19.0	20.9	26.2
Free fatty acids (% neutral lipids)	10.3	8.4	6.7	6.7

eride and free fatty acid fractions was demonstrated by conventional TLC procedures (see Mangold, *JAOCS* 41:762 [1964]) using authentic triglycerides and fatty acids as standards. Fatty acids combined as glycerides were transesterified with BF_3-methanol (Van Wijngaarden, *Anal. Chem.* 39:848 [1967]) and analyzed by gas liquid chromatography (GLC). The most significant fact to emerge from these analyses was that, not only were triglycerides present in all 4 samples of earthworms collected at different seasons, but that their contents ranged from 19.0-37.3% wt of the respective neutral lipid fractions. In Table I are recorded the results of analyses.

To establish that even cold storage of dead earthworms before extraction of lipids resulted in substantial decreases in triglyceride content with corresponding increases in free fatty acid levels, a second sample (180.0 g) of earthworms collected in winter was divided into two equal portions; one half was washed and extracted with chloroform-methanol immediately after collection, and the other half was washed, exposed to air in the laboratory for 24 hr, and then stored in a refrigerator at -13 C for 4 weeks before extraction. Analyses of these two batches revealed that the neutral lipids of the worms which were promptly extracted contained 26.2% triglycerides, while those which were stored prior to extraction contained only 4.0%. This reduction in triglycerides was offset by an increase in free fatty acids, the proportions changing from 6.7% to 22.6%. In another experiment, a sample of earthworms (309.7 g) was collected in autumn from agricultural farmland in a neighboring district and stored in a refrigerator at -13 C for several weeks before being washed and extracted. It was found that the neutral lipids contained 1.4% triglycerides and 23.7% free fatty acids and that the aqueous extraction solutions, when analyzed by the Lambert and Neish method (*Can. J. Res.* B28:83 [1950]), contained appreciable amounts of glycerol.

Preliminary identification of the glycerides, extracted from the second sample (180.0 g) of earthworms collected during winter, was based upon conventional column and TLC referred to above. Supporting evidence was furnished by applying microsamples alongside authentic monopalmitin, dipalmitin, and tripalmitin on silica gel plates impregnated with 3% boric acid and developing with chloroform-acetone (96:4 v/v) (Thomas, Scharoun, and Ralston, *JAOCS* 42:789 [1965]). It was found that these glyceride fractions possessed R_f properties which corresponded with those of the standard triglycerides but differed markedly from those of the mono- and diglycerides. Further confirmation that these fractions were correctly identified as glycerides was provided by GLC analyses of their fatty acids produced on hydrolysis. These fatty acid constituents, which were very diverse in nature and ranged from C_{10}-C_{32}, had a characteristic composition similar to that determined earlier for the total and neutral lipids of earthworms.

The foregoing results suggest that, in earthworms, there is present an active enzyme which affects lipolysis of the triglycerides of dead worms to free fatty acids and glycerol. Further studies are required to establish whether this enzyme participates in triglyceride metabolism in live worms.

R.P. HANSEN
ZOFIA CZOCHANSKA
Applied Biochemistry Division
Department of Scientific and
Industrial Research
Palmerston North
New Zealand

Effects of Dietary Docosenoic Acid upon Rats in Cold

J.L. BEARE-ROGERS and **E.A. NERA**, Food Research Laboratories, Health Protection Branch, Department of National Health and Welfare, Ottawa, Canada

ABSTRACT

Six week old rats fed rapeseed oil and subjected to cold at 4 C had a higher mortality rate than those fed oils lacking or low in docosenoic acids. A high frequency of deaths coincided with the period of pronounced cardiac lipidosis. Rats which had passed this phase before being exposed to the cold survived as well as the control animals.

INTRODUCTION

It is well established that young rats fed docosenoic acid deposit excessive amounts of lipid in the myocardium (1-5). Early work also had shown that rats fed rapeseed oil succumbed to the cold stress more readily than those fed corn oil (6). It was, therefore, of interest to determine if any correlation existed between cardiac lipid accumulation and cold susceptibility.

In this article, data are presented on cardiac fatty acids and mortality rates of rats maintained in the cold and fed a source of docosenoic acid.

METHODS

Weanling or 6 week old Sprague-Dawley male rats, COBS, were obtained from Charles River Breeding Laboratories, Wilmington, Mass., for experiments I and II and from Canadian Breeding Farm and Laboratories, St. Constant, Quebec, for experiments III and IV. They were housed in individual cages in a randomized block design and fed ad libitum a semipurified diet containing 20% w/w fat (5).

In experiment I, 6 week old rats supplied with a mixture of lard and corn oil (3:1), canbra oil (low in erucic acid), or rapeseed oil were housed for 1 week in a room maintained at 4 C. Cardiac fatty acids were determined by gas chromatography, as previously described (5), for five animals of each group that had survived in the cold. In experiment II, test diets first were fed to weanling rats kept at room temperature until they were 6 weeks of age. They then were transferred to the cold environment for another week, after which 5 rats from each group were killed for the determination of cardiac fatty acids. Experiments III and IV were initiated with 6 week old rats in the cold and continued for 4 and 3 weeks, respectively. Olive oil was substituted for the control mixture of lard and corn oil in experiment III and partially hydrogenated herring oil was included in experiment IV. Hearts of rats that died in experiment III were analyzed for fatty acids but in experiment IV were assessed histopathologically (2).

RESULTS

The amount of cardiac fatty acids in rats exposed to 4 C depended upon the type of dietary fat and the duration of feeding (Table I). When 6 week old rats were fed rapeseed oil for 1 week, the level of the cardiac lipids 1

TABLE I

Cardiac Fatty Acids of Rats Fed Test Oils for 1 Week at 4 C

Experiment	Age of rat (week)		Dietary fat	Cardiac fatty acids		
	Fed diets	Placed at 4 C		mg/Heart	mg/g	Percent 22:1
I	6	6	Lard:CO[a]	11.5 ± 0.8[d]	15.4 ± 2.0	0
			CBO[b]	11.5 ± 1.7	16.3 ± 2.6	1.1 ± 0.3
			RSO[c]	22.7 ± 3.4	33.4 ± 5.4	23.1 ± 2.7
II	3	6	Lard:CO	15.1 ± 2.0	16.5 ± 1.1	0
			CBO	17.9 ± 1.8	19.9 ± 2.2	0.8 ± 0.4
			RSO[e]	20.2 ± 0.9	24.4 ± 1.3	9.4 ± 2.1

[a]CO = corn oil.

[b]CBO = canbra oil (2.7% 22:1).

[c]In experiment I, rapeseed oil (RSO) had 23:1% 22:1.

[d]Mean of 5 rats ± standard error of the mean.

[e]In experiment II, rapeseed oil had 38.1% 22:1.

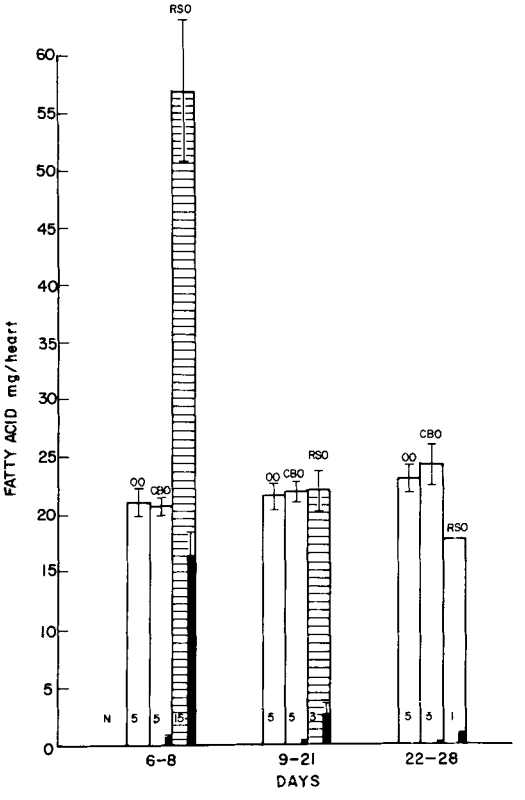

FIG. 1. Cardiac fatty acids in rats fed 20% olive oil (00), canbra oil (CBO), or rapeseed oil (RSO) for the time intervals shown of experiment III. Bars with horizontal lines represent rats which died on test and open bars, rats which were killed; narrow solid bars represent the amount of erucic acid (22:1). Number of rats indicated on bars.

week later was double that found with the low erucic canbra oil or the mixture of lard and corn oil. A high concentration of erucic acid characterized the fatty hearts. In the second experiment, rats that received the diets containing rapeseed oil for 3 weeks before cold exposure had a relatively low concentration of cardiac fatty acids, including erucic.

The food consumption in experiment II increased when rats were placed in the cold by ca. 70% for those fed lard and corn oil or canbra oil but only 47% for those fed rapeseed oil. During the week at 4 C, the food intake was 118±3 g/rat for the first two diets and 99±7 g/rat for the group fed rapeseed oil; the mortality rates were 1/16, 0/16, and 1/16 for rats fed lard and corn oil, canbra oil, and rapeseed oil, respectively. The differences in food consumption appeared not to influence mortality during the experiment.

Mortality rates in experiments I, III, and IV are shown in Table II. The greatest number of

TABLE II

Mortality of Rats Fed Test Diets and Placed in Cold at 6 Weeks of Age

Experiment	Dietary fat	Mortality, week[a] 1	2	3
I	Lard:CO[b]	4/16		
	CBO[c]	2/16		
	RSO[d]	10/16		
III	OO[e]	0/20		
	CBO	1/20		
	RSO[f]	13/20		
IV	Lard:CO	0/15	1/15	3/15
	CBO	2/15	5/15	6/15
	RSO[f]	8/15	12/15	13/15
	HHO[g]	4/15	10/15	10/15

[a]Cumulative mortality rates.

[b]CO = corn oil.

[c]CBO = canbra oil (2.7% 22:1).

[d]RSO = rapeseed oil (23.1% 22:1)

[e]OO = olive oil.

[f]Rapeseed oil (29.4% 22:1).

[g]HHO = hydrogenated herring oil, I.V. 76.0 (31.3% 22:1).

deaths in the first week occurred in rats receiving rapeseed oil. Subsequently, as seen in the last experiment, an increased mortality also occurred in rats fed a significant quantity of C22 in the form of partially hydrogenated herring oil.

The level of total fatty acids and of erucic acid in the hearts of rats that died or were killed are shown for different time intervals (Fig. 1). From 6-8 days, 15 rats fed rapeseed oil died and showed strikingly high levels of cardiac fatty acids, particularly erucic. Three more rats fed this oil died between 9-21 days, and 1 rat survived to 28 days. Olive oil or canbra oil did not increase the cardiac lipids nor mortality in the cold.

In experiment IV, the histopathological grading for intracellular fat globules on a 1-4 scale indicated that during the 6-8 day period, the highest degree of fat accumulation, 4+, occurred only with rapeseed oil, but 3+ was common with the partially hydrogenated herring oil. After 8 days, 2+ was the highest grade in either of these groups, and no fat accumulation was seen in the rats fed either the control fat or canbra oil. Ca. half of the animals which died during the early period showed myocardial necrosis.

DISCUSSION

The cause of death in the cold may have been related to a decreased ability of the adrenals to react to stress. In rats fed rapeseed

oil, Carroll (7) found large amounts of adrenal cholesterol esterified to erucic acid, and Walker and Carney (8) related this situation to a reduced secretion of corticosterone in the cold. Although, in this latter work, erucate added to corn oil was associated with a lower adrenal response than was olive oil, the cold stress still elicited a fourfold increase in plasma corticosterone in the rats fed erucate. A further adrenal change was noted by Carney, et al., (9) who found that the production of prostaglandin, as stimulated by adrenocorticotrophin, was decreased in rats fed rapeseed oil for 12 weeks. Cortical cell hypertrophy appeared to continue throughout an experimental period of up to 64 weeks (10).

The high incidence of deaths observed in the persent experiments occurred when cardiac lipidosis was most severe. After the cardiac fat deposits regressed, the rats could survive when placed in the cold environment. Impairment of adrenal function, therefore, appeared to be secondary to the condition of the heart and its relative inability to metabolize erucic acid.

In earlier experiments at room temperature (2,4,5), weanling rats received docosenoic acid and developed cardiac lipidosis within a week. The older the rat, the less susceptible was its heart to fatty accumulation (11). Rats subjected to cold were 6 weeks of age, a stage when control rats could survive the stress. The adverse fate of rats fed rapeseed oil coincided with a greatly reduced food intake before the onset of death, which did not occur at room temperature.

The docosenoic acid in rapeseed oil was erucic acid, but, in partially hydrogenated herring oil, it was a mixture of positional and geometric isomers (12). A *trans*-acid with its higher melting point would probably be less readily absorbed than its corresponding *cis*-acid. The fatty acids which accumulated in the heart of rats fed rapeseed oil reflected the composition of that oil (2). From their study of the effects of erucylcarnitene upon mitochondrial oxidation, Christophersen and Bremer (13) suggested that an erucyl metabolite inhibited the oxidation of other fatty acids.

Cardiac lipidosis in rats receiving docosenoic acid was greatest after ca. 1 week and then regressed, but during the acute phase the extra stress of cold was associated with a high mortality.

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Biochemical and Physiological Studies of Certain Ticks (Ixodoidea): Isolation and Partial Identification of a New Fatty Acid in Eggs of *Dermacentor andersoni* Stiles (Ixodidae)

IBRAHIM R. SHIMI,[1] NABILA A. MAROUN,[2] MAMDOUH Y. KAMEL,[3] HEMMAT H. RAGHEB, and **MOHGA M. BADRAN,[4]** Medical Zoology Department, U.S. Naval Medical Research Unit Three, c/o Fleet Post Office, New York, New York 09427

ABSTRACT

Gas liquid chromatographic analysis of the fatty acid methyl esters from eggs of *Dermacentor andersoni* Stiles (Ixodidae) revealed the presence of significant quantities (15% total fatty acids) of an unidentified component with a retention time between $C_{18:3}$-$C_{22:0}$ fatty acids. Smaller amounts of the unidentified component (ca. 5% total fatty acid) also were detected in host rabbit serum. Purified, the unidentified component's methyl ester collected from the tick eggs by preparative gas liquid chromatography was partially identified and characterized by chemical and spectroscopic analyses. The evidence suggests that the unidentified component is a methyl branched C_{15} tricarboxylic acid containing two vicinal carboxylic acid groups. Biosynthesis of the unidentified component by the tick is under investigation.

INTRODUCTION

Studies on eggs of *Dermacentor andersoni* Stiles in this laboratory (1) showed that 15% total fatty acids (FA) consist of an unknown (FAX) with a retention time in gas liquid chromatographic (GLC) separation of $C_{18:3}$-$C_{22:0}$. An unidentified compound with a similar retention time also occurs in the free and bound FA of the body fluids of two other ticks, *Hyalomma dromedarii* Koch and *H. anatolicum excavatum* Koch (2). We have attempted to purify and characterize the FAX of *D. andersoni* eggs and to determine its structure.

MATERIALS AND METHODS

Material

Adult *D. andersoni* from the U.S. Naval Medical Research Unit-3 Medical Zoology Department colony (originating in Montana), were fed on laboratory rabbits and held at 30 C and

[1]Present address: Biochemistry Department, Faculty of Science, Ain-Shams University, Cario, Egypt.

[2]Submitted in partial fulfillment of the requirements for the Ph.D. in Biochemistry, Ain-Shams University, Cairo, Egypt.

[3]Present address: Biochemistry Laboratory, National Research Center, Cairo, Egypt.

[4]Present address: Organic Chemistry Department, Faculty of Pharmacy, Cario University, Cairo, Egypt.

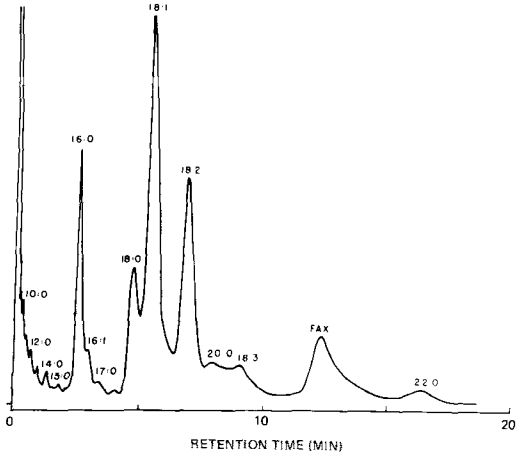


FIG. 1. Gas liquid chromatographic pattern of fatty acid methyl esters of tick egg.

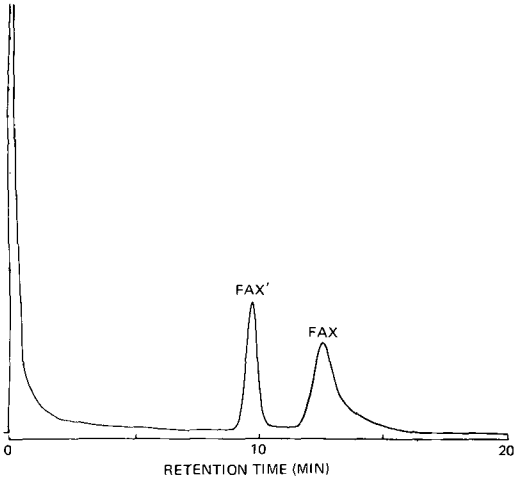


FIG. 2. Gas liquid chromatogram of the collected unidentified component (FAX) and (FAX[1]) methyl esters.

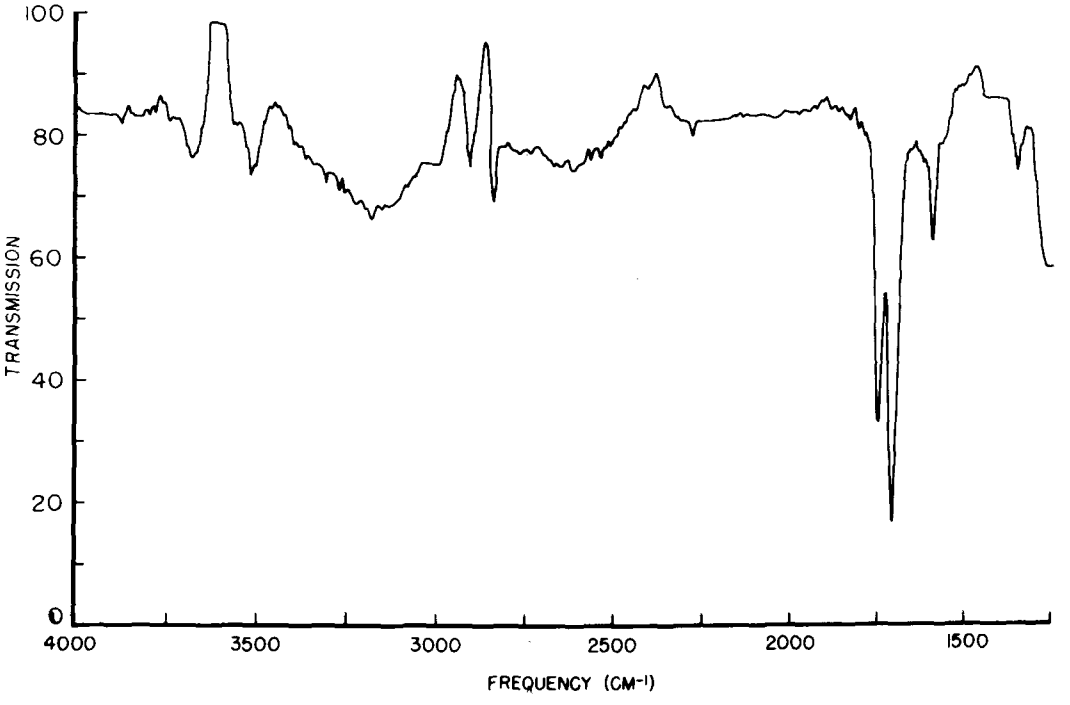


FIG. 3. IR spectrum of free fatty acid of tick egg in chloroform.

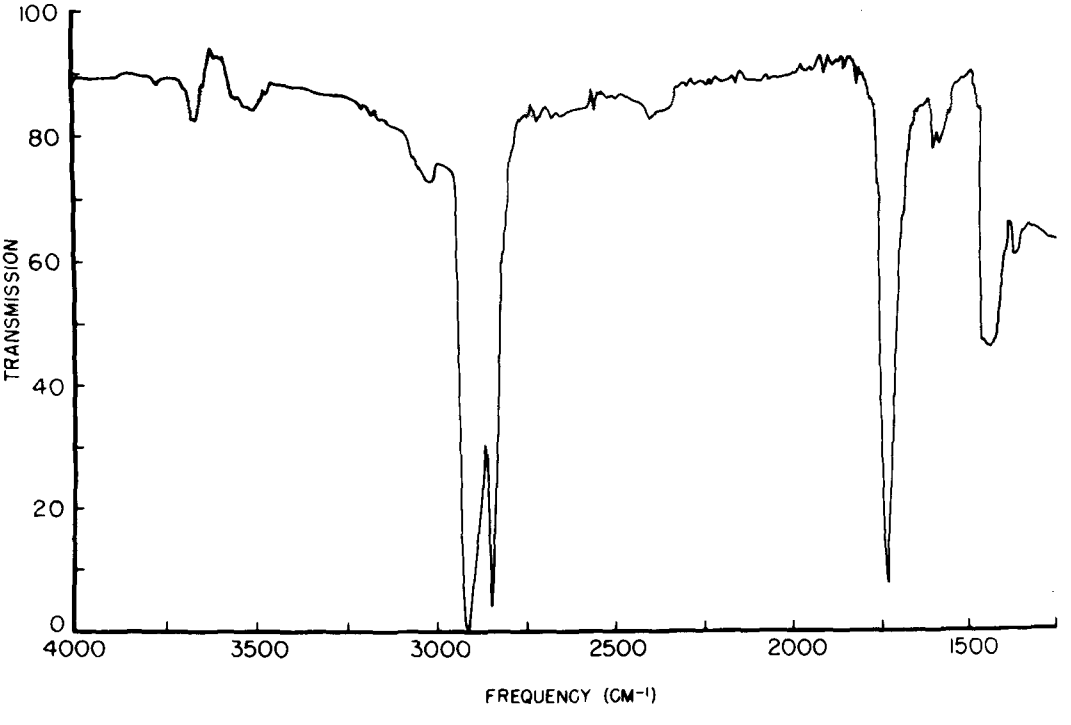


FIG. 4. IR spectrum of fatty acid methyl esters of tick egg in chloroform.

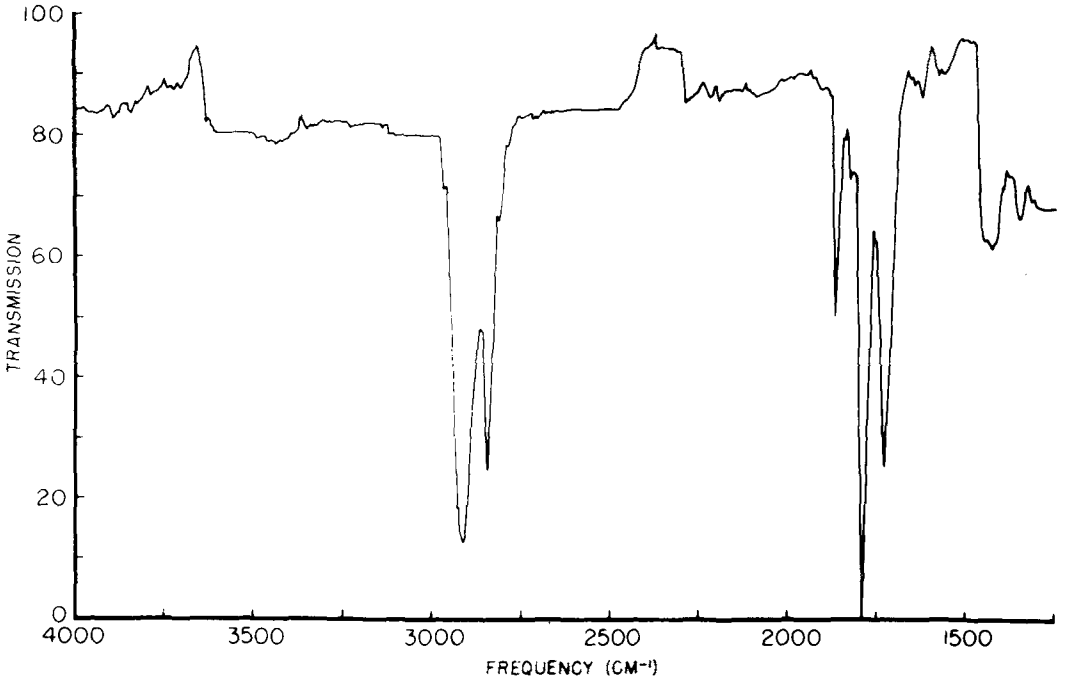


FIG. 5. IR spectrum of unidentified component (FAX[1]) methyl ester in chloroform.

75% relative humidity when not feeding. Eggs from engorged females were collected after the first day of deposition and stored at -50 C until used.

Extraction and Purification

Lipids were extracted from a known wt of homogenized eggs by the procedure of Bligh and Dyer (3), as modified by Kates (4). Total FA methyl esters were prepared by direct transesterification using the method described by Feldman and Rouser (5). The FAX methyl ester was separated and collected by preparative GLC using a Hewlett-Packard model 5750 with a 180 cm x 0.6 cm stainless steel column packed with 15% diethyleneglycolsuccinate on 100-120 mesh Gas Chrom P (Applied Science Laboratories, State College, Pa.) equipped with a thermal conductivity detector and a flame ionization detector. The temperatures of the injector, column, detector, and collection device were 200, 180, 250, and 300 C, respectively. Helium was used as a carrier gas at 50 ml/min.

Chemical Analysis

All experiments were made on the purified FA methyl ester collected by preparative GLC. Hydrogenation (6) and ozonolysis (7) were performed to determine the degree of unsaturation. Hydroxyl groups were estimated by formation of trifluoroacetyl or trimethylsilyl derivatives (8) and vicinal carboxylic acid groups by condensation with resorcinol (9). Aldehydes and ketones were determined by derivatization with dimedone (5,5-dimethylcyclohexane-1,3-dione) or 2,4-dinitrophenylhydrazine (10,11) and carboxylic acid anhydrides by reaction with hydroxylamine (9) to give hydroxamic acid. After each test, the reaction products were extracted with n-hexane or chloroform and analyzed by GLC.

Spectrometric Analysis

IR spectra were recorded on a Perkin-Elmer 337 grating IR spectrophotometer. Mass spectra were determined at the University of Montana and University of Kentucky Mass Spectrometry Center with identical results.

RESULTS AND DISCUSSIONS

In the tick egg GLC pattern (Fig. 1), $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, and $C_{18:2}$ compose ca. 80% (4.8 mg/100 mg egg dry wt) of the total FA. The remainder, ca. 15%, represents FAX, with a retention time of 12.3 min appearing between $C_{18:3}$-$C_{22:0}$.

When the FAX was collected by preparative GLC and rechromatographed, there was a new peak (FAX[1]) with a retention of 9.6 min (Fig. 2). The FAX[1] is probably a breakdown product

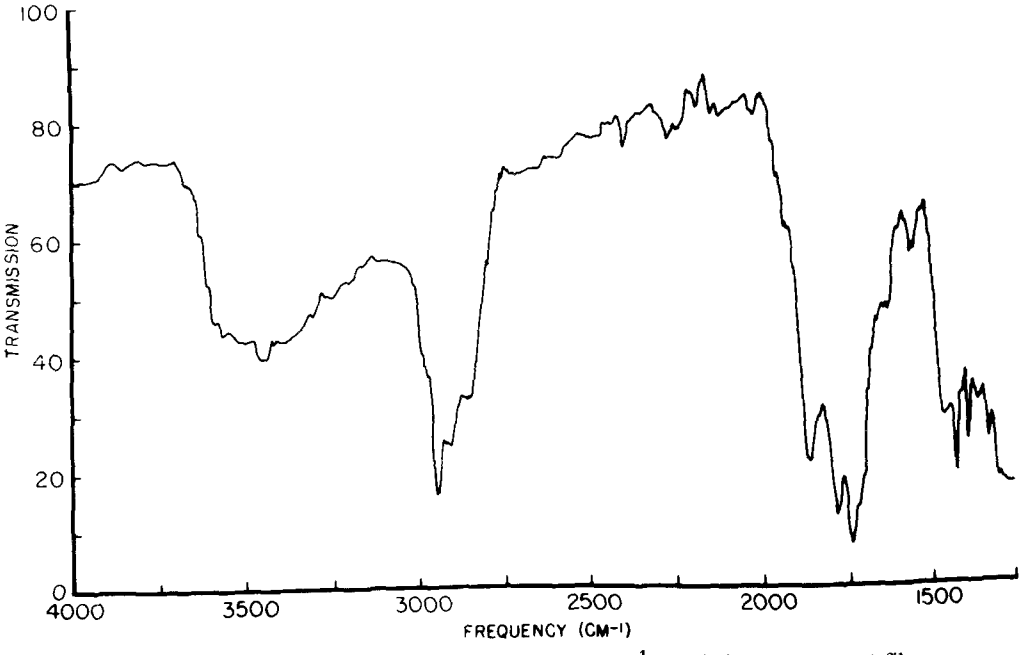


FIG. 6. IR spectrum of unidentified component (FAX[1]) methyl ester as a neat film.

or a chemically altered form of the original FAX.

When the total FA methyl esters of the egg homogenate were hydrogenated and the reaction products extracted and analyzed by GLC, the unsaturated FA peaks disappeared but the saturated FA peaks increased. Ozonolysis of the total FA methyl esters showed unaltered saturated acids, but unsaturated acids were broken down to shorter chain fatty aldehydes. Both reactions appear to have no effect upon the purified FAX or FAX[1] methyl esters, and their retention time remained unchanged. Isolated FAX and FAX[1] methyl esters also were unaltered after treatment with trifluoroacetic anhydride, trimethylchlorosilane, dimedone, and

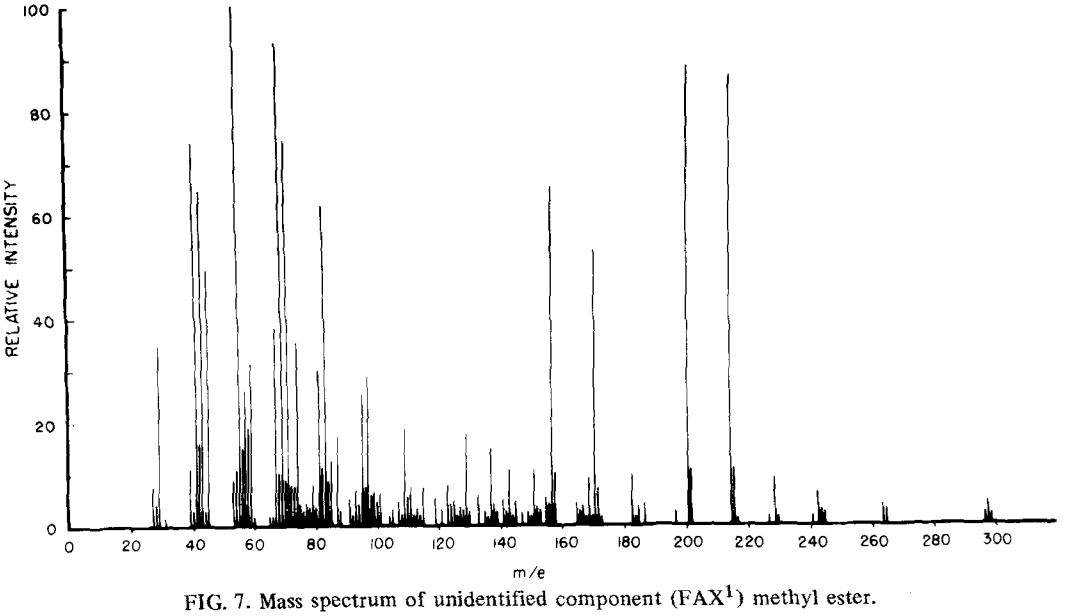


FIG. 7. Mass spectrum of unidentified component (FAX[1]) methyl ester.

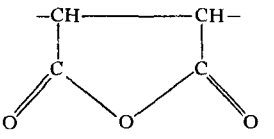
2,4-dinitrophenylhydrazine. Thus, FAX and FAX[1] methyl esters appear to be saturated FA containing no hydroxy, aldehydic, or ketonic groups.

An intense emerald green fluorescence developed in the reactions between resorcinol and total FA, FAX, and FAX[1] methyl esters. When the reaction products were extracted with n-hexane and analyzed by GLC, the peak corresponding to FAX disappeared from the total FA methyl ester. The n-hexane extracts of the reaction products of FAX and FAX[1] methyl esters with resorcinol showed no peaks corresponding to the original FAX and FAX[1] methyl ester peaks. These results indicate the presence of two vicinal carboxylic acid groups and carboxylic acid anhydride in the FAX and FAX[1] and also that FAX is the only acid with vicinal carboxylic acid groups in the total tick egg FA. In the GLC pattern, the peak corresponding to the FAX methyl ester remained unaltered after condensation with hydroxylamine, but the peak for the FAX[1] methyl ester disappeared. Thus, FAX[1] is probably the anhydride of the FAX methyl ester formed during the collection process due to the high temperature of the tungsten filaments in the thermal conductivity detector.

The IR spectra of free FA, total FA methyl esters, and FAX[1] methyl ester in chloroform show bands at 2915 cm^{-1} (νasym CH_2) and 2847 cm^{-1} (νsym CH_2), indicating the aliphatic nature of the compounds (Figs. 3-5). The spectrum of the free FA (Fig. 3) shows two carbonyl stretching bands at 1750 cm^{-1} and 1710 cm^{-1} due to the aliphatic FA mononer and dimer forms, respectively.

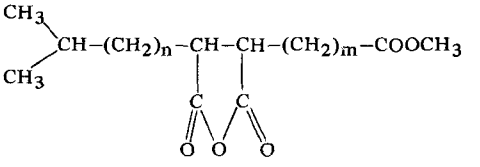
Methyl ester formation clearly is indicated in the IR spectra of total FA methyl esters and FAX[1] methyl ester by the carbonyl stretching vibration band at 1735 cm^{-1}. The IR spectra of the collected FAX[1] methyl ester in the chloroform solution and also as a neat film (Figs. 5,6), show two bands at 1860 cm^{-1} and 1790 cm^{-1}, characteristic to carbonyl stretching vibration of 5 membered cyclic anhydrides. The peaks at 1380 cm^{-1} and 1365 cm^{-1} were attributed to the presence of a gem-dimethyl group (Fig. 6).

The FAX[1] methyl ester fragmentation spectrum (Fig. 7) offers further confirmation. The peak at m/e 74 is a typical methyl ester rearrangement radical ion $[CH_2 = C(OH)\,OCH_3\cdot]^+$ and the peak at m/e 59 is due to $[CH_3OC{=}O]^+$. The prominent peak at m/e 43 in the mass spectrum may be attributed to $[CH_3CH_2CH_2]^+$ or to $[(CH_3)_2CH]^+$. The base peak at m/e 55 is probably due to the fragment $[CH_2{=}CHC{=}O]^+$ originating from the five membered cyclic anhydride group



by hydrogen rearrangement and loss of CO_2 as a neutral fragment. The peaks at m/e 81, 95, and 109 and the peaks at m/e 201, 215, 229, and 243 in the mass spectrum are considered to be due to loss of -CH_2 groups during fragmentation.

Since the parent peak in the mass spectrum suggests a mol wt of 298 for the anhydride, thus from the chemical tests and spectral data, the following structure is suggested for the collected FAX[1] methyl ester:



where n ± m = 7.

In conclusion, FAX is a saturated tricarboxylic fatty acid containing two vicinal carboxylic acid groups and a methyl branch and has a mol wt of 302 before esterification.

Additional studies are in progress to determine the exact position of the carboxylic acid groups on the chain and the process of FAX biosynthesis in the tick. The FAX is three times more concentrated in the tick egg than in the hos rabbit blood; therefore, it is obviously a physiologically important property of the tick organism.

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DDT Absorption and Chylomicron Transport in Rat

DOROTHY M. -E. POCOCK and **ALAN VOST**, McGill University Medical Clinic, Montreal General Hospital, Montreal, Quebec, Canada, H3G 1A4

ABSTRACT

Male rats were fed 100 nM dichlorodiphenyltrichloroethane-^{14}C in oil by gastric tube. Recovery of dichlorodiphenyltrichloroethane-^{14}C in thoracic duct lymph was 60% in 12 hr. Lymph dichlorodiphenyltrichloroethane-^{14}C (97%) occurred in lipoproteins of d <1.006, designated chylomicrons. Mechanical separation of chylomicron triglyceride core (labeled with triglyceride-^{3}H) from chylomicron membrane (labeled with phospholipid-^{32}P) showed that 97% dichlorodiphenyltrichloroethane-^{14}C was present in triglyceride core. To investigate possible association of plasma clearance of the two core lipids, rats were pulse injected with chylomicrons, doubly labeled with triglyceride-^{3}H and dichlorodiphenyltrichloroethane-^{14}C. The decay of dichlorodiphenyltrichloroethane-^{14}C in sequential serum samples was rapid (T½ = 2 min) and was independent of triglyceride-^{3}H decay. In tissues removed 14 min after injection of chylomicrons, 30% administered dichlorodiphenyltrichloroethane-^{14}C was found in liver but only 1% in adipose tissue. In hepatectomized (eviscerated) rats, the decay of serum dichlorodiphenyltrichloroethane-^{14}C (T½ = 10 min) was also independent of and more rapid than triglyceride-^{3}H decay. With sucrose density gradients, it was shown that chylomicron dichlorodiphenyltrichloroethane-^{14}C transferred to higher density serum proteins in vitro and in vivo and to bovine albumin in vitro. Thus, dichlorodiphenyltrichloroethane was transported from intestine largely in the triglyceride phase of chylomicrons; disappearance of chylomicron-dichlorodiphenyltrichloroethane from the systemic circulation was rapid and partly independent of the presence of the liver and of triglyceride hydrolysis; some dichlorodiphenyltrichloroethane was transported from serum chylomicrons to albumin or other plasma proteins before tissue uptake.

INTRODUCTION

The mechanisms by which hydrophobic lipids are absorbed from the intestinal tract and subsequently transported in intestinal lymph and in plasma are incompletely understood. Studies of intestinal absorption and subsequent transport of physiological hydrophobic lipids, such as long chain fatty acids and sterols, are complicated by metabolic transformations of these lipids during the absorptive process. As most absorbed lipid is transported as triglyceride in the central core of chylomicrons, triglyceride provides a hydrophobic phase of potential importance in transporting other hydrophobic lipids. We attempted, therefore, to find a nonphysiological hydrophobic lipid absorbed from the intestinal tract with high efficiency, without metabolic transformation and with transport in the triglyceride phase of chylomicrons. Dichlorodiphenyltrichloroethane (DDT) was selected because inspection of its structure indicated considerable hydrophobicity and because accumulation of dietary DDT in adipose tissue of rats (1), man, and other mammals (2), despite low concentrations of dietary DDT, suggested that DDT might be absorbed efficiently from mammalian gastrointestinal tract. Previous studies of DDT absorption have given contradictory results. Judah (3) found that only 19 ± 3% DDT was absorbed from the gut of rats within 3 hr, whereas Rothe, et al., (4) found that 85% labeled DDT was absorbed from gut and 47 ± 5% administered dose was absorbed via the thoracic lymph duct. In the latter study, after feeding unlabeled DDT, a variable fraction of absorbed DDT material in lymph was identified tentatively as dichlorodiphenyldichloroethylene (DDE).

This article describes in vivo absorption of DDT-^{14}C and the nature of its transport in chylomicrons in intestinal lymph and in plasma. Some aspects of distribution of DDT-^{14}C to liver and other tissues were examined.

MATERIALS AND METHODS

DDT-^{14}C [1, 1, 1-trichloro-2, 2-bis (p-chlorophenol) ethane, labeled on the ring] (specific activity 22.7 mC/mM) was obtained from Amersham-Searle Co., Toronto, Canada. Its radiochemical purity (>98%) was confirmed by thin layer chromatography (TLC), as described below. DDT (melting point [mp] = 109 C) from Aldrich Chemicals, Montreal, Canada, was used as a chromatographic standard. Glycerol-2-^{3}H (n) (specific activity 436 mC/mM, purity

>99%) was obtained from Amersham-Searle Co. and sodium phosphate-^{32}P from Charles Frosst and Co., Montreal, Canada. Sunflower seed oil (Safflo) was supplied by Co-Op Vegetable Oils Ltd., Altona, Manitoba Canada.

All chemicals and solvents were reagent grade. Isooctane (2, 2, 4-trimethyl pentane) was eluted through alumina prior to use. Glass fiber paper impregnated with silica gel or silicic acid was obtained from Gelman Instrument Co., Ann Arbor, Mich.

Animals

Male Wistar rats (250-350 g) were obtained from Canadian Breeding Farms (St. Constant, Canada). Rats were prepared surgically into three different groups as follows. (A) Animals with thoracic ducts cannulated below the diaphragm (5) with Silastic tubing were used to obtain lymph on the day after operation. (B) Fed rats with cannulated right and left jugular veins were used to inject labeled chylomicrons intravenously and subsequently to sample blood. (C) Rats with jugular catheters and evisceration of liver, pancreas, spleen, and the intestinal tract were used to study the effect of evisceration upon disappearance of radioisotope labeled chylomicron lipids from blood. The jugular polyethylene cannulas were kept filled with their void volume of acid-citrate dextrose (U.S. Pharmacopeia formula A, 10% in 0.9% saline) to reduce blood clotting. Animals with intravenous catheters were used for experiments 45 min after their recovery from ether anesthesia. All rats were kept in cages that permitted only longitudinal movement.

Chylomicrons

Chylomicrons labeled only with DDT-^{14}C were obtained by feeding rats 100 nM DDT-^{14}C, dissolved in 0.2 ml sunflower seed oil, by gastric tube and by collecting lymph in intervals up to 72 hr. Chylomicrons labeled with DDT-^{14}C and triglyceride-^{3}H were obtained from lymph after feeding glycerol-^{3}H (0.06-0.86 μM) and DDT-^{14}C (0.08-0.88 μM) in 0.2 ml ethanol via intraduodenal polyethylene catheters inserted previously via gastrostomies. Chylomicrons labeled with DDT-^{14}C and phospholipids-^{32}P were obtained by similar intraduodenal injection of 5 mCi sodium phosphate-^{32}P (aqueous) and 0.50 μM DDT-^{14}C in 0.2 ml sunflower seed oil, emulsified with 60 mg sodium taurocholate in 0.5 ml saline. Lymph was collected after each of these injections for 4 hr.

Radioisotope labeled chylomicrons were isolated from lymph immediately after collection (maximum collection time, 24 hr) by layering chyle below 0.9% saline in 30 ml polycarbonate ultracentrifuge tubes and centrifuging for 3.84 x 10^6 G min (A 211 head, B 60 International Centrifuge) at 14 C. The upper 2 cm layer of the 7 cm column was relayered below saline and the process repeated. The fraction obtained was termed chylomicrons, but it is recognized that this fraction also may contain lymph very low density lipoproteins (VLDL) which, in the oil-fed rat, form a continuous spectrum with lymph chylomicrons during ultracentrifugal flotation (6). Lymph chylomicrons of density <1.006 were isolated for analysis by layering chyle below 0.9% saline and ultracentrifuging for 13.86 x 10^6 G min (7).

To separate chylomicron surface membranes containing phospholipids and proteins from the inner triglyceride-rich chylomicron oil core, chylomicrons were disrupted physically by dehydration and rehydration in a rotary flash evaporator at 40 C, as described by Zilversmit (8).

Chylomicrons used for experiments on the plasma disappearance rates of chylomicron lipids were injected intravenously into the left jugular vein of the rats in a maximum volume of 1.5 ml over 30 sec. The chylomicron triglyceride loads were designed to exceed the maximum physiological lymph triglyceride influxes of 5 mg/kg/min in the rat (9). The pulse injection of chylomicron triglyceride gave loads over the usual 12 min observation time of 14.5 ± 3 mg/kg/min/rat. In these experiments, labeled metabolites of DDT, DDE, and dichlorodiphenyldichloroethane (DDD) formed <1% of ^{14}C lipid in two batches of chylomicrons and in serum taken 14 min after injection of a batch.

Chylomicrons from lymph collections and chylomicrons in serum samples from in vivo plasma disappearance experiments were separated from higher density proteins by flotation in linear sucrose gradients by a modification of the method of Pinter and Zilversmit (10). The density of the samples of chylomicrons and serum was adjusted by addition of sucrose (50%, W/W). The gradients consisted of 25 ml mixed 30-50% sucrose (W/W), so that the density at the bottom of the gradient was 1.1700 and at the top 1.1270. An additional 4 ml 60% sucrose was added at the bottom to fill the round bottom of the 40 ml centrifuge tubes. The samples (0.3-0.8 ml) were introduced into the gradients at the interface of the gradient and the 60% sucrose layer and were followed by sufficient 60% sucrose to total 1.0 ml consistently. The height of the gradients was 5.7 cm. All samples from the same experiment

were centrifuged simultaneously. Gradients were centrifuged for 17.28 x 10^6 G min in a SB-110 swinging bucket rotor (B 60 International Equipment Co. centrifuge). The centrifuge tubes then were punctured at the bottom, and aliquots of the gradients were drained successively into 16 or 17 tubes. Under these conditions >95% radioactivity of ^3H in the chylomicrons rose to the top of the gradients (tubes 12-17), whereas albumin-^{125}I remained at the origin of the gradients (tubes 1-4).

Lipids were extracted from homogenized tissues and feces with chloroform-methanol (11), from all aqueous samples by heptane-isopropanol (12), and from red cells with chloroform-isopropanol (13). The heptane-isopropanol system was 3-4% less efficient than the chloroform-methanol system in extraction of chylomicron DDT-^{14}C and triglyceride-^3H from lymph and serum samples; however, it was more convenient. Lipid extracts were stored under nitrogen at 8 C.

Lipids were separated by glass paper chromatography (GPC) using glass-fiber paper impregnated with silicic acid or with silica gel (Gelman Instrument Co., ITLC-SA or ITLC-SG, respectively). DDT and its metabolites, DDD and DDE, were separated from each other and isolated from other lipids on ITLC-SA chromatograms developed in 100% isooctane. R_f values in this system were: tetracosane, 0.98; DDE, 0.64; DDT, 0.48; and DDD, 0.26; cholesteryl palmitate, other neutral lipids, and polar lipids remained at the origin. Neutral lipids were separated from one another on ITLC-SG chromatograms developed in the solvent system isooctane-benzene-acetic acid-acetone, as previously described (14). Radioisotope labeled lipids separated by GPC were counted after direct addition of the glass-fiber paper to scintillation fluid (14).

Triglycerides were determined quantitatively by the enzymatic assay of glycerol released after saponification of lipid extracts (15).

Radioactivity of ^3H, ^{14}C, and ^{32}P was counted in a Nuclear Chicago Mark I scintillation system. Simultaneous dual radioisotope counting was achieved by channel ratio methods using a Ba-133 external standard (15). Lipid samples were counted in 10 ml toluene solution of butylphenylbiphenylyloxadiazole-1, 3, 4 (Nuclear Chicago Corp., Boston, Mass.) and aqueous samples were counted in Aquasol (New England Nuclear Corp., Boston, Mass.). Quenching of counting rates was calculated using appropriate quenched radioisotope standards. Radioactivity of ^{125}I was counted in a γ counting system (Model 4227, Nuclear Chicago Corp.).

RESULTS

Intestinal Absorption

DDT-^{14}C (100 nM), dissolved in sunflower seed oil, was given intragastrically to 3 rats 18 hr after cannulation of their thoracic ducts. Lymph was collected for 3 days, during which food and water were given ad libitum. Within 12 hrs 61.1 ± 1.6% administered radioactivity was recovered in lymph and, after 72 hr, 63.3 ± 1.9%. In one experiment, 4 hr collection intervals were used, and 55% administered radioactivity appeared in lymph within the first 4 hr. Lymph radioactivity was entirely lipid-soluble and GPC showed that >98% radioactivity ran with the R_f of DDT-^{14}C.

After 72 hr, 8-9% administered radioactivity was recovered in feces, but no significant radioactivity was found in urine. Total recoveries of the administered DDT-^{14}C in lymph and feces were >70% after 3 days.

In 2 of the rats, killed at 72 hr, certain tissues were analyzed, and 3.0% and 1.7% administered DDT-^{14}C was recovered in intra-abdominal white adipose tissue and 0.6% and 2.6% in liver. Gut contents, heart, brain and sera each contained <0.5%.

Ca. 25% administered radioactivity was not recovered, but analyses of all tissues, or of respiratory gases, were not made. In these experiments, DDT-^{14}C was absorbed with high efficiency after intragastric administration, and its major absorption pathway was the intestinal lymphatic system.

Lymph Transport

Chylomicrons plus VLDL (density <1.006) were isolated from lymph collections from 3 rats after intragastric administration of DDT-^{14}C and contained 97.4 ± 0.3% of the lymph ^{14}C radioactivity. Thus, all DDT-^{14}C was transported in lymph in the chylomicron-VLDL fraction.

To determine whether DDT-^{14}C was transported by the central triglyceride core of chylomicrons or by the outer protein and phospholipid-rich membrane of chylomicrons, the core of some chylomicrons was labeled with triglyceride-^3H, and the membrane of other chylomicrons was labeled with phospholipid-^{32}P. All chylomicrons were also labeled with DDT-^{14}C. The specificity of ^{14}C for DDT-^{14}C and of ^3H for triglyceride-^3H was demonstrated by GPC since 98% ^{14}C and 93% ^3H were recovered in the appropriate area of the chromatograms; similarly, ^{32}P was a specific label for phospholipid since 94% chylomicron ^{32}P was extracted in the chloroform phase of the chloroform-methanol extraction system.

The oil core and the membrane phases of each category of chylomicrons were separated by mechanical disruption (8) and analyzed for radioactivity. The results are shown in Table I. The ratios of triglyceride-^{3}H to DDT-^{14}C in intact chylomicrons and in their oil phase were the same, indicating that DDT was packaged in the core of the chylomicrons with triglyceride. The ratios of ^{32}P to ^{14}C in the membranes were 26-33 times greater than in the intact chylomicrons. Since the oil phases of these chylomicrons were uncontaminated with ^{32}P, it may be calculated that 3-4% chylomicron DDT occurred in the membranes. The possibility cannot be excluded that this membrane DDT-^{14}C resulted from contamination of membrane with core lipid.

Plasma Disappearance of Chylomicron DDT-^{14}C

As DDT-^{14}C was associated with the triglyceride oil phase of chylomicrons, a possible correlation between plasma disappearance of chylomicron triglyceride and chylomicron DDT was explored.

Chylomicrons containing DDT-^{14}C and triglyceride-^{3}H were injected into the left jugular vein of unanesthetized rats and plasma sequentially was sampled from the other jugular catheter. The results are shown in Figure 1. DDT-^{14}C plasma disappearance curves formed a single exponential in the first 6 min with a half-life of 2.75 ± 0.05 min. In two animals, earlier sampling was facilitated by continuous anesthesia and the DDT-^{14}C from their plasma disappeared exponentially for the first 4 min with a half-life of 1.4 ± 0.1 min (Fig. 2). The disappearance of the chylomicron triglyceride-^{3}H from plasma was much slower in all experiments, and it may be concluded that chylomicron DDT-^{14}C was cleared from the plasma compartment independently of chylomicron triglyceride-^{3}H clearance. Half-lives were not calculated for plasma triglyceride-^{3}H disappearance, since the results of multiple sampling showed that this disappearance was not a simple exponential function. In two nonanesthetized, eviscerated rats the clearance

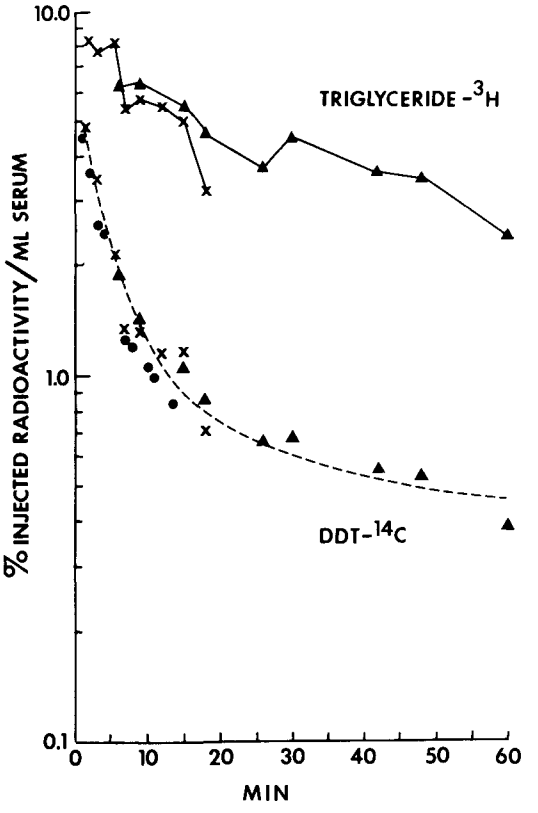

FIG. 1. Simultaneous decay curves of chylomicron dichlorodiphenyltrichloroethane-^{14}C (DDT-^{14}C) and triglyceride-^{3}H in the plasma of unanesthetized rats. x and ▲ each represent an experiment in which chylomicrons doubly labeled with DDT-^{14}C and triglyceride-^{3}H were injected intravenously. • represents one experiment in which chylomicrons labeled only with DDT-^{14}C were injected intravenously. — represents the trend of the DDT-^{14}C decay curves.

of chylomicron DDT-^{14}C from plasma was delayed (half-life 10.25 ± 1.75 min); however, chylomicron DDT clearance was still independent of chylomicron triglyceride clearance in both animals.

In all these experiments, chylomicron DDT-^{14}C clearance from plasma was very rapid and independent of triglyceride-^{3}H clearance;

TABLE I

Ratios of Triglyceride-^{3}H or Phospholipid-^{32}P to Dichlorodiphenyltrichloroethane-^{14}C in Intact Chylomicrons and in Their Oil Phase or Membrane Phase, Released after Mechanical Disruption

	Experiment 1	Experiment 2	Experiment 3
	$^{3}H/^{14}C$	$^{32}P/^{14}C$	$^{32}P/^{14}C$
Chylomicrons	1.0	1.0	1.0
Oil phase	0.97	→0	→0
Membrane phase	---	32.9	26.1

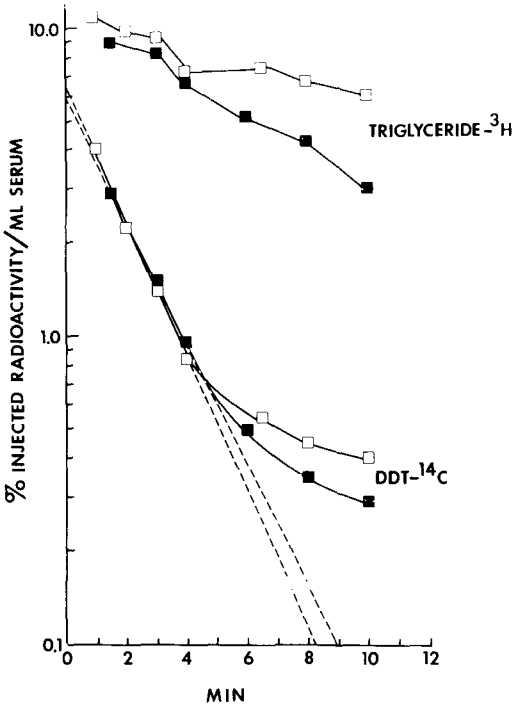

FIG. 2. Simultaneous decay curves of chylomicron dichlorodiphenyltrichloroethane-^{14}C (DDT-^{14}C) and triglyceride-^{3}H in the plasma of anesthetized rats. ■ and □ each represent an experiment in which chylomicrons doubly labeled with DDT-^{14}C and triglyceride-^{3}H were injected intravenously. — are the extrapolations of the exponential portions of each of the DDT-^{14}C decay curves.

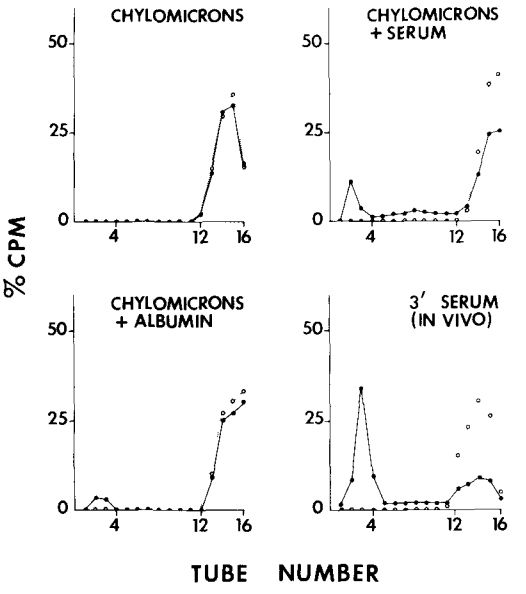

FIG. 3. Percent distribution of ^{14}C and ^{3}H in sucrose density gradients centrifuged simultaneously. Chylomicrons were doubly labeled with dichlorodiphenyltrichloroethane-^{14}C and triglyceride-^{3}H. Samples consisted of: chylomicrons alone; chylomicrons mixed with 5% bovine albumin or rat serum in vitro; and serum from blood withdrawn 3 min after chylomicrons were injected into a rat in vivo. Samples were introduced at the bottom of gradients. ●—●—● = ^{14}C. ○--○--○ = ^{3}H. CPM = total counts/min.

the presence of liver and gastrointestinal tract was not a prerequisite for the clearance of DDT from the plasma.

Sucrose Density Gradients

As chylomicron DDT-^{14}C was cleared from plasma rapidly and independently from triglyceride-^{3}H, it appeared possible that DDT was transported into tissues by plasma proteins other than chylomicrons. This possibility was examined by sucrose gradient centrifugation of chylomicrons (doubly-labeled with triglyceride-^{3}H and DDT-^{14}C) alone or with rat serum or bovine albumin, in vitro. Chylomicrons from the same collections also were injected intravenously into rats and serially timed serum samples were subjected to sucrose gradient centrifugation.

The results of a typical in vitro experiment are shown in Figure 3. When washed chylomicrons alone were added to gradients, both DDT-^{14}C and triglyceride-^{3}H rose to the top fraction. In the presence of bovine albumin and rat serum, a significant proportion of DDT-^{14}C remained at the origin. The transfer of DDT-^{14}C from chylomicrons to higher density proteins (or lipoproteins) was selective, although a small fraction of triglyceride-^{3}H also transferred from chylomicrons in the presence of serum. The mean results of in vitro experiments are shown in Table II. In unpurified lymph, 2.6 ± 0.25% (n=2) DDT-^{14}C was recovered at the origin of sucrose gradients, indicating that DDT-^{14}C transfer to other lymph proteins was minimal.

The results of sucrose gradient centrifugations of in vivo serum samples, after intravenous injection of doubly-labeled chylomicrons, are shown for a single typical experiment in Figure 4. In 2 min, 6 min, and 14 min serum samples, chylomicron DDT-^{14}C selectively transferred to higher density proteins in tubes 1-4 and always in excess of the proportion of triglyceride-^{3}H found in tubes 1-4.

In three in vivo experiments, the disappearance rate of DDT-^{14}C from both the top fractions, chylomicrons, and the bottom, higher density fractions, formed single exponential functions between 2-6 min, inclusively. The mean value for the half-life of DDT-^{14}C in the top fraction did not differ significantly from

that in the bottom fraction, and the mean of 6 values was 3.55 ± 0.7 min.

These results of in vitro and in vivo experiments show that DDT dissociated from chylomicron triglyceride and transferred selectively, in serum, both in vitro and in vivo, to proteins of a higher density than chylomicrons—possibly albumin or higher density lipoproteins. The possibility cannot be excluded that the observed net transfer of DDT-^{14}C from chylomicrons to higher density proteins observed in vivo may have represented an artifact of in vitro transfer during gradient preparation.

Tissue Distribution of DDT-^{14}C

At the end of six experiments in which DDT-^{14}C labeled chylomicrons were injected, livers contained 30 ± 3% administered ^{14}C (DDD = 65 ± 1.5%, DDT = 32 ± 1.3%, and DDE 1.5 ± .3% recovered radioactivity). In two experiments total lumbar and epididymal fat pads contained only 4% ^{14}C radioactivity recovered in livers. These results indicate that liver, not adipose tissue, is the major site of initial plasma clearance of chylomicron DDT.

DISCUSSION

The high efficiency of absorption of small quantities of DDT (<1 mg/kg) from gut into lymph in these experiments considerably exceeds that previously observed in the rat (4). It is possible that, in previous studies, the large quantities of DDT administered (75-110 mg [3] and 7-10 mg/kg [4]) became partly insoluble in the intestinal tract or saturated absorption pathways. The ready accumulation of DDT in mammalian systems in nature may be explained by this efficient absorption of small quantities. In this study, the efficiency of DDT absorption into intestinal lymph was comparable to that of oleic acid (16) and hexadecanoic and pentadecanoic acids (17). A variety of other hydrophobic lipids also are transported by intestinal lymph after absorption, but, for octadecanoic acid (18), cholesterol (19), hexadecane (20), and α- and γ- tocopherols (21), the absorption efficiency is reported at <50%.

The efficiency of DDT absorption by lymph in intact animals is underestimated from results in cannulated rats since anastomotic lymph channels may by-pass the cannula; it is probable that absorbed DDT-^{14}C was transported totally by the lymphatic, rather than the portal, system.

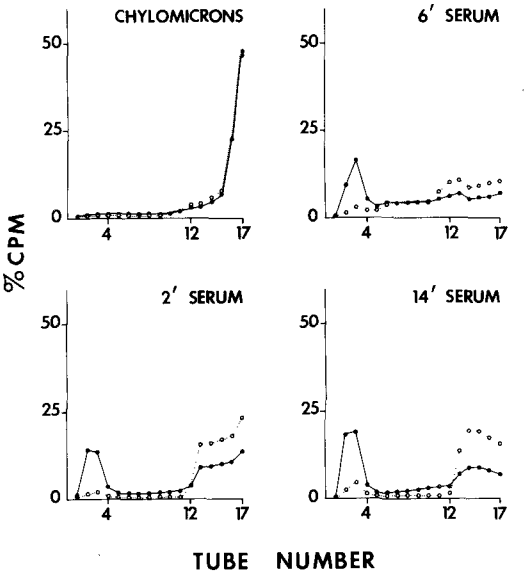

FIG. 4 Percent distribution of ^{14}C and ^{3}H in sucrose density gradients centrifuged simultaneously. Chylomicrons were doubly labeled with DDT-^{14}C and triglyceride-^{3}H. Samples consisted of: chylomicrons and serum from blood withdrawn from 1 rat 2 min, 6 min, and 14 min after chylomicrons were injected intravenously in vivo. Samples were introduced at the bottom of gradients. ●–●–● = ^{14}C. ○–○–○ = ^{3}H. CPM = total counts/min.

TABLE II

Percentage Distribution of Radioactivity after Sucrose Gradient Flotation of Chylomicrons Labeled with Dichlorodiphenyltrichloroethane-^{14}C and Triglyceride-^{3}H[a]

	Origin[b]		Top[c]	
	^{14}C	^{3}H	^{14}C	^{3}H
Chylomicrons	1.4 ± 0.3	NS[d]	93.7 ± 2.3	95.7 ± 2.3
Chylomicrons plus serum	26.3 ± 5.8	8.2 ± 4.1	54.0 ± 5.6	89.0 ± 5.1
Chylomicrons plus albumin	9.0 ± 1.3	NS	86.2 ± 2.4	96.5 ± 1.8

[a] n = 5.

[b] Bottom 4 tubes in a 16 tube gradient.

[c] Top 5 tubes in a 16 tube gradient.

[d] NS = no significant radioactivity.

The high efficiency of absorption of DDT-^{14}C from intestines suggests that DDT may have a high affinity for intestinal transport systems, either within the lumen or at the intestinal cell membrane. A high affinity for membrane lipids has not been demonstrated, although proton magnetic resonance studies indicate that DDT can complex with dipalmitoyl phosphatidylcholine, a lipid constituent of some membranes (22). As the molecular structure of DDT does not approximate that of any physiological molecule it is unlikely to be transported by specific active or facilitated membrane transport systems. However the passive transport of molecules into plant and gallbladder cells measured as permeation coefficients, increases with increasing hydrophobicity (23). Similarly the efficiency of absorption of a series of drugs from rat gastrointestinal tract has been shown to increase with increasing hydrophobicity (24). The apolarity of DDT may, therefore, account for its efficient absorption.

DDT-^{14}C was transported almost entirely in the oil phase of chylomicrons, but it remains possible that some were transported in the membrane which forms a small fraction of the mass of rat chylomicrons of this size (25). In lymph, despite the presence of other proteins, DDT-^{14}C remained in chylomicrons, but, on addition to serum or to plasma in vivo, DDT-^{14}C transferred rapidly to higher density proteins or lipoproteins. Rat chylomicrons take up proteins, predominantly lipoproteins, when exposed to blood serum in vitro (26). Such physical changes in the chylomicron membrane may have facilitated transport of DDT-^{14}C from chylomicron core lipid to extraneous proteins.

The independence of ^{14}C-DDT and ^{3}H-triglyceride clearance from plasma was unexpected, since studies of other hydrophobic lipids transported in the triglyceride core of chylomicrons, the cholesteryl esters, indicate that these do not transfer readily to other proteins in vitro (25) and their plasma clearance in vivo is probably dependent upon partial removal of chylomicron triglyceride before hepatic clearance of chylomicron cholesteryl esters in the rat (27).

In the intact animal, chylomicron DDT-^{14}C was cleared initially largely by liver and only to a minor extent by adipose tissue. Since long term feeding experiments to rats (1) and other mammals (2) show that DDT accumulates selectively in adipose tissue, this suggests that either DDT turnover in liver is much faster than in adipose tissue or that, over longer times, DDT is transported from liver to adipose tissue. The hepatic clearance of DDT is not specific to this tissue and may reflect only its high blood flow, since hepatectomy reduced but did not block plasma clearance of DDT-^{14}C.

Since DDT-^{14}C is a hydrophobic lipid with no structural resemblance to physiological lipids and was absorbed efficiently without metabolic change, its study may provide useful models for investigating factors controlling intestinal absorption and subsequent transport of other hydrophobic lipids.

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Gas Chromatographic Resolution of Homologous Monoacyl and Monoalkylglycerols

J.J. MYHER and A. KUKSIS, Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario, Canada M5G 1L6

ABSTRACT

Homologous series of 1 and 2 monoacyl and monoalkyl-sn-glycerols with 0-6 double bonds were resolved by gas liquid chromatography as the trimethylsilyl ethers and acetates using ethylene glycol-succinate siloxane, cyanopropylphenylsiloxane, and methylsiloxane liquid phases. The trimethylsilyl ethers gave effective separation of positional isomers and saturated and unsaturated derivatives of acylglycerols on both ethylene glycol-succinate siloxane and cyanopropylphenylsiloxane, columns. The corresponding acetyl esters were eluted on the basis of unsaturation, but positional isomers overlapped. The ethylene glycol-succinate siloxane and cyanopropylphenylsiloxane columns resolved the positional isomers and saturated and unsaturated species of the alkylglycerols when chromatographed as the acetates, while the corresponding trimethylsilyl ethers were separated on the basis of unsaturation only. On methylsiloxane columns essentially complete overlapping was observed among the positional isomers within each acyl and alkylglycerol series when chromatographed as the acetates. There was an effective resolution of the positional isomers of the acylglycerols as the trimethylsilyl ethers on methylsiloxane. From methylsiloxane, the alkenylglycerols were eluted earlier than the alkylglycerols of corresponding carbon numbers. These species were not separated on conventional ethylene glycol-succinate siloxane or cyanopropylphenylsiloxane columns.

INTRODUCTION

The composition of natural mixtures of monoacylglycerols commonly is assessed by thin layer chromatography (TLC) on borate treated silica gel, which resolves positional isomers, (1) and by gas liquid chromatography (GLC) on polyester phases, which separates the component fatty acids (2). GLC frequently is employed also for the identification of the aldehydes derived from alkenylglycerols (3), as well as for the analysis of intact alkylglycerols (4). None of these methods allows the simultaneous determination of acyl-, alkyl-, and alkenylglycerols.

Wood, et al., (5) has shown that the common monoacylglycerols can be resolved intact by GLC according to unsaturation and positional substitution, and Wood and Snyder (4) have demonstrated that the technique is also satisfactory for the separation of certain acyl- and alkylglycerol derivatives, provided these can be eluted at low temperatures.

The recent availability of polar liquid phases of moderate thermal stability has permitted an extension of the above separations to higher mol wt derivatives and has allowed improved resolution of the positional isomers within each homologous series. A mass spectrometric identification of the alkyl- and acylglycerols in the GLC effluents of polar and nonpolar siloxane columns has been presented elsewhere (6).

EXPERIMENTAL PROCEDURES

Materials

The palmitoyl and oleoylglycerols were 99% pure single isomers, while the linoleoylglycerols were cross-contaminated with ca. 10% other positional isomers. Synthetic 1 monocaproyl, 1 monomyristoyl, 1 monopalmitoyl, 1 monooleoyl, 1 monolinoleoyl, 1 monoarachidoyl, 2 monopalmitoyl, and 2 monooleoylglycerols were purchased (Serdary Research Laboratories, London, Canada) and were better than 95% single isomer. Mixtures of natural monoacylglycerols of better than 99% single isomer purity were prepared by Grignard degradation of corn, linseed, and cod liver oil triacylglycerols and TLC on borate treated silica gel.

Synthetic 2 monopalmityl and 2 monooleyl glycerols were obtained from Serdary Research Laboratories and were better than 99% single isomer. Grade II chimyl, batyl, and selachyl alcohols were obtained from Sigma Chemical Co., St. Louis, Mo. The main components in these materials were the 1 palmityl, 1 stearyl, and 1 oleylglyceryl ethers, respectively, but each sample contained small amounts of shorter and longer chain length homologues of the same positional isomer. Natural 1-alk-1-enyl glyceryl ethers were isolated from the phosphatidylethanolamine of rabbit skeletal muscle by lithium aluminum hydride degradation and TLC. The identity of the glyceryl ethers was

established by GLC and mass spectrometry (GC-MS) (6).

Methyl esters of standard fatty acids were obtained from Nu Chek Prep, Elysian, Minn. Octacosane and dotriacontane were provided by Distillation Products Industries, Rochester, N.Y.

Grignard Degradation of Triacylglycerols

Corn, linseed, and cod liver oils were treated with the Grignard reagent essentially as described by Yurkowski and Brockerhoff (7). Ca. 0.5 g each oil in 25 ml diethyl ether was treated at room temperature with 1 ml 3 M ethyl magnesium bromide in diethyl ether. After 25 sec, 0.5 ml acetic acid was added, followed by 5 ml water at 50 sec. The reaction was performed under nitrogen with sodium-dried ether. The ether layer was washed successively with 5 ml water, 5 ml aqueous 2% sodium bicarbonate, and 5 ml water. The ethereal solution was dried over sodium sulfate and evaporated to dryness. Smaller scale reactions were performed in an identical manner upon various subfractions of triacylglycerols isolated by TLC.

Lithium Aluminum Hydride Reduction

Natural mixtures of 1-alk-1-enyl glyceryl ethers were prepared by lithium aluminum hydride degradation (4) of the phosphatidylethanolamine of rabbit skeletal muscle which is rich in plasmalogens (8). A solution of $LiAlH_4$ in diethyl ether (10 mg/5 ml solvent) was added to ca. 100 mg lipid. The solution was heated in a sealed tube at ca. 40 C for 30 min. During this time the mixture frequently was shaken and occasionally the cap was opened to prevent any large pressure build-up. At the end of the reaction, the solution was cooled in an ice bath and water added dropwise until further addition did not give rise to a violent reaction. Water then could be added quickly and a total of 9 ml was used. To the aqueous solution, was added 9 ml 4% acetic acid in water. The lipid was extracted several times with diethyl ether, the extract dried with sodium sulfate, and a concentrated residue of the solution chromatographed on a TLC plate, according to Wood and Snyder (4), with diethyl ether-aqueous 30% ammonia 100:0.25.

Preparation of Derivatives

Diacetates of the various monoacyl- and monoalkyl-glycerols resolved on the TLC plates were prepared by treating the gel scrapings immediately with sufficient acetic anhydride and pyridine 10:1 to cover them (9).

For the preparation of the trimethylsilyl (TMS) derivatives, the TLC bands were eluted with diethyl ether-methanol 80:20 (v/v). Petroleum ether was added and the solution washed several times with water. The extracts were dried over anhydrous sodium sulfate and evaporated to dryness. The di-TMS ethers were obtained by treating the dry monoacyl- or monoalkylglycerols with pyridine-hexamethyldisilazane-trimethylchlorosilane in a ratio 12:5:2. The reaction was performed by adding the pyridine and hexamethyldisilazane to the lipid contained in a nitrogen filled vial at room temperature. After chilling the mixture on ice, trimethylchlorosilane was added and the fumes blown away with nitrogen. The reaction mixture was left at room temperature for 1/2-1 hr and the excess reagents evaporated under nitrogen. The TMS ethers were dissolved in petroleum ether and analyzed by GLC, as described below. The above routine and reagents allowed the preparation of TMS ethers of both 1 and 2 isomers of glycerol without interconversion. Other conditions and other common silylating reagents could not be employed effectively, because they led to extensive isomerization of the monoacylglycerols. Furthermore, the 1-alk-1-enylglycerols are known to be labile in both acidic and basic media (4).

Dimethylacetals of plasmalogenic phosphatidylethanolamine and 1-alk-1-enylglycerol or its TMS ether were prepared by heating the lipid with 10% sulfuric acid in anhydrous methanol (2).

TLC

Purified triacylglycerols were isolated by TLC on Silica Gel H (Merck & Co., Darmstadt, Germany) and spread on 20 x 20 cm plates in 0.5 mm thickness. The plates were developed with heptane-isopropyl ether-acetic acid 60:40:4 (v/v/v) (10). Positional isomers of monoacyl- and monoalkylglycerols were resolved by TLC on borate treated silica gel (1). Pure 1 and 2 isomers were obtained by collecting the appropriate section of the silica gel and eluting with chloroform. Monoacylglycerols and monoalkylglycerols of uniform degree of unsaturation were isolated by TLC of their acetates on Silica Gel G impregnated with 10% silver nitrate (10). The various fractions were eluted with chloroform. Phosphatidylethanolamine was isolated from rabbit skeletal muscle, as described previously (8).

GLC

The various glycerol derivatives were resolved on a Beckman GC-4 gas chromatograph (Beckman Instruments, Fullerton, Calif.) equipped with a modified on-column heater (11). The columns were stainless steel tubes (50

TABLE I

Separation Factors of Isomeric Monopalmitoyl and Monopalmitylglycerols upon Polar and Nonpolar Columns

Column[a]	Derivative[b]	Monopalmitoyl glycerol	Monopalmityl glycerol	Reference
	Isomer ratio:	1/2	2/1	
SILAR 5CP (3%, 180 cm)	TMS	1.13	1.0	This study
SILAR 5CP (3%, 180 cm)	Ac	1.0	1.07	This study
XE-60 (3%, 150 cm)	TMS	—	1.0	(4)
XE-60 (3%, 150 cm)	TFA	—	1.17	(4)
EGSS-X (10%, 180 cm)	TMS	1.11	1.0	This study
EGSS-X (10%, 150 cm)	TFA	—	1.21	(4)
EGSS-X (10%, 180 cm)	Ac	—	1.14	This study
DEGS (20%, 90 cm)	TMS	1.13	—	(5)
SE-30 (23%, 60 cm)	Ac	1.15	—	(12)
QF-1 (3%, 180 cm)	TMS	>1.0	—	(13)
SE-30 (3%, 150 cm)	Ac	1.0	—	This study
SE-30 (5%, 150 cm)	TMS, TFA	—	1.0	(4)
SE-30 (2.6%, 50 cm)	TMS	>1.0	—	((5)
OV-1 (3%, 60 cm)	TMS	>1.0	—	(14)
OV-1 (3%, 60 cm)	TMS	1.10	—	This study

[a]Percentage coating and column length in parentheses; temperatures and instruments, as given in original references. SILAR 5CP = cyanopropylphenylsiloxane, XE-60 = cyanoethylmethyl, EGSS-X = ethylene glycol-succinate siloxane, DEGS = diethylene glycol succinate, SE-30 = methylsiloxane, QF-1 = trifluoropropylmethylxiloxane, and OV-1 = methylsiloxane.

[b]TMS = trimethylsilyl ether, Ac = acetate, and TFA = trifluoroacetate.

cm x 0.2 mm inside diameter) packed with 3% OV-1 (a methylsilicone) on Gas Chrom Q (100-120 mesh), as supplied by Applied Science Laboratories, State College, Pa. Starting temperature was 170 C and the temperature was programed at 10 C/min.

The various glycerol derivatives were resolved by carbon number, unsaturation, functionality, and positional isomerism on an F&M Biomedical gas chromatograph (F&M Corp., Avondale, Pa.). The columns were glass U-tubes (180 cm x 2 mm inside diameter) packed with 3% SILAR 5CP (a cyanopropylphenylsiloxane) on Gas Chrom Q (100-200 mesh) or 3% EGSS-X (an ethylene glycolsuccinate methyl siloxane copolymer) on Gas Chrom Q (100-120 mesh), as supplied by Applied Science Laboratories. The SILAR 5CP columns were heated overnight at 270 C with the normal carrier flow (55 ml/min of helium). The EGSS-X columns were heated at 260 C for 2-3 hr prior to use. The SILAR 5CP columns gave stable base lines for operating temperatures up to 260 C, while the EGSS-X columns could be operated adequately up to 250 C for limited periods of time only.

The diacetates of corn, linseed, and cod liver oil monoacylglycerols and selected standards were run with nitrogen as carrier gas at a flow rate of 40 ml/min. All other samples were run with helium as carrier gas at a flow rate of 55 ml/min. Operating temperatures varied according to the nature of the sample and are shown in the appropriate figures and tables. The TMS ethers of the monoalkylglycerols required the lowest operating temperatures (210 C), while the monoacylglycerol acetates required the highest (250 C).

RESULTS AND DISCUSSION

Separation of Positional Isomers

Table I gives the GLC separation factors for various isomeric monopalmitoylglycerol derivatives on polar and nonpolar columns. Separation factors calculated from gas chromatograms or plots of retention times reported by other workers also have been included. These results are compared to those obtained for the corresponding palmitylglycerols. Effective separations of the isomeric acyl- or alkylglycerols are obtained only on the polar liquid phases and with specific types of derivatives. Although Huebner (12) reported good resolution of the isomeric palmitoyl glycerols as diacetates on nonpolar siloxane columns containing large amounts of liquid phase, the separation factors obtained for these derivatives on thin film siloxane columns are much lower and of little practical value. Likewise, Wood et al., (5) noted that the 2 isomer was not resolved from the 1 isomer when run as the TMS ether on a 45 cm 2.6% SE-30 (methylsiloxane) column. Kuksis and Breckenridge (15) reported a separation factor of 1.1 for the dibutyrates of the isomeric

palmitoylglycerols but failed to observe any separation for the corresponding dihexanoates. On the basis of examination of the GLC behavior of isomeric triacylglycerols of low mol wt, Watts and Dils (16) have concluded that differences in triacylglycerol polarity or constellation differences would not explain the separation of species of the same carbon number. Furthermore, there was no resolution of any of the isomeric diacetates of palmitoyl glycerol on the polar columns, while their TMS ethers were resolved effectively. These separations can be rationalized on the basis of a differential interaction of the ester and ether groups with the liquid phase, as well as an influence of the glycerol position upon the magnitude of this effect. Thus, the ester groups would be expected to interact more strongly with the polar liquid phase than the ether groups, and those in the primary would do so more effectively than those in the secondary position. The 2 monoacylglycerol derivatives would be thus eluted ahead of the 1 monoacylglycerol when chromatographed as the TMS ethers, as observed experimentally. This reasoning is supported by the observation that the isomeric alkylglycerols are resolved effectively on the polar columns only when run as the diacetates, as in the present study, or as the TFA esters, as reported by Wood and Snyder (4). In this instance, the determining factor in the separation is the interaction of the acyl groups with the liquid phase. A reverse effect would be predicted, however, since two acyl groups in the primary position would retard the migration rate more than a combination of one acyl group in the primary position with one in the secondary position. In this type of separation, therefore, the size of the acyl groups itself would have little influence, and one should not anticipate much difference in the retention times of the diacetates of isomeric palmitoylglycerols.

The separation factors are not the same for all carboxyl esters as seen from the less complete resolution of the isomeric palmitylglycerol diacetates in comparison to the isomeric palmitoylglycerol TMS ethers. The relative separation factors would, therefore, appear to depend upon the balance between the inherent polarities of the different functional groups as a result of the transfer between the primary and secondary positions of the glycerol molecule and the modifying effect of the glycerol position upon polarity.

The variability in the separation factors observed on the nonpolar columns (5,12,15) may be attributable to differences in the liquid phases themselves and the nature of the supports. The more recent studies have been conducted with the higher quality inert supports not available at the time of the earlier studies. Despite the high initial loading of the liquid phase, there may have existed considerable support-solute interaction, which may have been primarily responsible for the resolution of the isomers (12,15). In this connection, the same order of elution is seen for these isomeric monoacylglycerols and monoacylglycerol diacetates or dibutyrates on TLC (10). Furthermore, the possibility of an incorrect GLC peak identification also must be considered, since some of the reports, at least, were of a preliminary nature. In the present study, the identity of all the peaks was established by mass spectrometric examination of the appropriate GLC effluents (6). Snyder (4) previously has separated various isomeric 1 and 2 glyceryl ethers as the TFA esters on XE-60 (a cyanoethylmethylsiloxane) columns. In all instances the 2 isomers were eluted after the 1 isomers. There was no discernible resolution of the isomeric alkylglycerols when run as the TMS ethers. These observations are similar to the present findings with the alkylglycerol diacetates and TMS ethers on the SILAR 5CP column. Both columns contain cyanoalkylsiloxanes as the liquid phase, but the nature and proportions of the substituents differ, as do the total contents of the alkyl groups. Like the diacetates of isomeric alkylglycerol ethers, the ditrifluoroacetates also were not resolved on SE-30 or OV-1 columns. No separation of the isomeric alkylglycerols was obtained on EGGS-X, EGS, diethylene glycol succinate (DEGS), ethyleneglycol adipate (EGA) or ethylenesuccinate cyanoethylsiloxane (ECNSS-S) columns as the TMS ethers (4).

Separation of Homologues

Both polar and nonpolar liquid phases provide excellent resolution of monoacyl- and monoalkylglycerols on the basis of mol wt. Even relatively short columns yield complete resolution of homologues differing by one methylene unit. The separation factors are independent of the nature of the derivative, but vary with temperature of the column and with the liquid phase. Thus, the TMS ether and diacetate of the monostearoyl and monopalmitoyl glycerols give a separation factor of 1.81 at 220 C and 1.72 at 235 C, respectively. Similar separation factors are obtained for the acyl-, alkyl- and alkenylglycerols at any given temperature, with the highest separation factor always being noted at the lower temperatures. The magnitude of the effect is independent of the derivative employed. Since certain combina-

TABLE II

Relative Equivalent Chain Length (ECL) Values of Monoacyl- and Monoalkylglycerols on SILAR 5CP[a]

Carbon number	Trimethylsilyl ethers		Acetates			
	220 C[b]		220 C[b]		235 C[c]	
	1 alkyl	1 acyl	1 alkyl	1 acyl	1 alkyl	1 acyl
14	14.00	17.00	20.89	23.81	14.00	16.48
16	16.00	19.01	22.90	25.83	16.00	18.51
18	18.00	21.02	24.92	27.85	18.00	20.54
20	20.00	23.02	26.94	29.88		

[a]Experimental conditions, as given in text; average of three determinations. SILAR-5CP = cyanopropylsuccinate. ΔECL (1-acyl-1-alkyl) trimethylsilyl ethers = 3.01 (220 C); ΔECL (1-acyl-1-alkyl) acetates = 2.93 (220 C); ΔECL (1-acyl-1-alkyl) acetates = 2.51 (235 C); ECL of C_{28} hydrocarbon (octacosane) = 18.44 (220 C) relative to trimethylsilyl ethers of 1-alkylglycerols; ECL of C_{34} hydrocarbon (tetratriacontane) = 17.08 (235 C) relative to trimethylsilyl ethers of 1-alkylglycerols.

[b]Relative to the trimethylsilyl ethers of 1-alkylglycerols at 220 C.

[c]Relative to the trimethylsilyl ethers of 1-alkylglycerols at 235 C.

tions of derivatives and liquid phases allow the completion of the analysis at lower temperatures than others, they will be preferred for a practical separation because of the greater separation factors. However, the resolution of positional isomers, requires specific combinations of derivatives and liquid phases. Since the separations of the homologues may be required to be carried out under conditions which also allow a differentiation between positional isomers, it is obvious that it may not always be possible to take advantage of the full capabilities of resolution of a given derivative, liquid phase, or temperature.

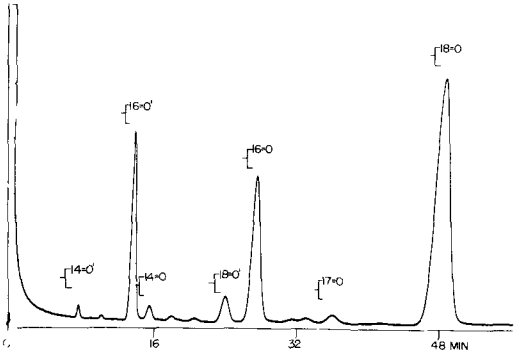

FIG. 1. Gas liquid chromatographic resolution of 1 monoacyl- and 1 monoalkylglycerols as diacetates on SILAR 5CP (cyanopropylphenylsiloxane). Peaks identified according to carbon number of acyl and alkyl (primed) radicals. Instrument: F and M Biomedical gas chromatograph equipped with 180 cm x 5 mm outside diameter glass columns containing 3% SILAR 5CP on Gas Chrom Q (100-120 mesh). Injector and oven heaters, 220 C; detector, 250 C. Carrier gas, helium (40 ml/min). Sample: 1 μliter of a 1% solution of the monoacyl- and monoalkylglycerols in petroleum ether. Attenuation 1/200 full sensitivity.

Table II gives the equivalent chain lengths (ECL) values for selected saturated alkyl- and acylglycerol derivatives at two different temperatures on SILAR 5CP columns. Maximum resolution of acyl- and alkylglycerol species is obtained at 220 C for either the TMS ethers or acetates. The acetates, however, run much later than the TMS ethers. At 235 C, the retention ratios of the acetates vs. TMS ethers are 6.1 and 8.0 for the monoacyl- and monoalkyl- glycerols, respectively. Figure 1 illustrates the resolution of a mixture of homologous acyl- and alkylglycerols as the acetates on SILAR 5CP. Both series of homologues are resolved with the same separation factors, but different absolute retention times. There is no overlap among any of the saturated species, since the glycerol ethers are eluted with retention times that differ sufficiently from those of any major glycerol esters present in common natural mixtures. Table II also gives the relationships among the retention values of saturated hydrocarbons and the TMS ethers and acetates of acyl- and alkylglycerols.

Effective separations of saturated and unsaturated species of acyl- and alkylglycerols have been obtained previously only on polyester columns (4,5,19). Due to thermal instability of the polyester liquid phases, these separations have been limited to the more volatile derivatives and low temperatures. Figure 2 shows the elution patterns recorded separately and in mixture for the TMS ethers of 1(3) and 2 monoacylglycerols derived from cod liver triacylglycerols by Grignard degradation. In all instances, the 2 monoacylglycerols are eluted ahead of the 1 monoacylglycerols, and, for most isomers, a clear-cut resolution is obtained for all saturated and unsaturated derivatives.

TABLE III

Relative Retention Data of Monoacylglycerols and Fatty Acids on SILAR 5CP[a]

Number of carbons and double bonds	Relative retention times[b]			Equivalent chain lengths		
	FA	Monoacylglycerols		FA	Monoacylglycerols	
	Me	TMS	Ac	Me	TMS	Ac
	185 C	220 C	248 C	185 C	220 C	248 C
14:0	0.230	0.318	0.393	14.00	14.00	14.00
16:0	0.482	0.558	0.627	16.00	16.00	16.00
16:1	0.543	0.630	0.699	16.31	16.41	
18:0	1.000	1.000	1.000	18.00	18.00	18.00
18:1	1.115	1.100	1.101	18.27	18.30	18.38
18:2	1.319	1.276	1.258	18.73	18.83	18.96
18:3	1.655	1.540	1.470	19.35	19.48	19.63
18:4	1.834	1.628	1.565	19.63	19.68	19.88
20:0	2.075	1.792	1.595	20.00	20.00	20.00
20:1	2.323	1.951	1.749	20.29	20.28	20.36
20:5	4.050	2.885	2.445	21.81	21.61	21.29
22:0	4.304	3.211	2.544	22.00	22.00	22.00
22:1	4.746	3.384	2.737	22.23	22.17	22.29
22:6	9.242	5.420	4.223	24.06	23.80	24.14

[a]Experimental conditions, as given in text; average of three determinations. For monoacylglycerols, the relative retention data also are averaged over the 1+3 and 2 isomers.

[b]FA = fatty acids, Me = methylesters, TMS = trimethylsilyl ethers, and Ac = acetates.

Exceptions are the 1 stearoyl and 2 oleoyl and 1 oleoyl and 2 linoleoyl species of monoacylglycerols which overlap to a greater or lesser extent depending upon the operating conditions. No resolution was seen for the positional isomers, when chromatographed as acetates, but separations based upon degree of unsaturation were excellent. In general, the resolution obtained on the polar siloxane phase is similar to that realized on a polyester column (5, 17), but there are certain differences. The SILAR 5CP column gives relatively higher separation factors for carbon number homologues and lower for double bond homologues than the EGSS-X column at comparable temperatures. As a result, all the unsaturated species of monoacylglycerols of a given carbon number are eluted ahead of the saturated species of the next higher carbon number homologues.

Figure 3 shows the resolution obtained for the saturated and monounsaturated 1 monoalkylglycerols when run as the TMS ethers on the polar siloxane column. There is an effective separation of the saturates and unsaturates of the same carbon number, which gives separation factors equivalent to those of the monoacylglycerols or, indeed, of the fatty acid methyl esters, when run at comparable temperatures. When chromatographed as the acetates, the saturated 2 isomers emerge between the saturated and monounsaturated 1 isomers. A complete base-line resolution, however, is not obtained.

On the SILAR 5 CP column, there was a complete overlap between the 1 alkyl and 1-alk-1-enylglycerols of corresponding carbon number and degree of unsaturation. The 1-alk-1-enyl function had no net effect upon the rate of migration of these molecules with this liquid phase. Figure 4 shows that the 1-alk-1-enyl ethers are eluted ahead of the alkyl ethers of corresponding carbon number when run either as the diacetates or TMS ethers on SE-30 columns. The difference in retention times is markedly larger than that anticipated from the mol wt and must reflect variations in molecular shape of the solutes to which this liquid phase is sensitive. Perhaps the lack of resolution on SILAR 5CP results from a mutual cancellation of two effects: a shape factor which favors an earlier elution and an unsaturation factor which favors a later elution of the alkenylglycerol molecule.

A comparison of the separation factors for various pairs of molecular species differing by one double bond shows that the resolution is not affected significantly by the type of derivative employed. On SILAR 5CP the monoene-saturate separation factor varies only from 1.09-1.11 for all the lipid classes examined. The variation is more apparent for comparisons involving polyunsaturated species, such as lino-

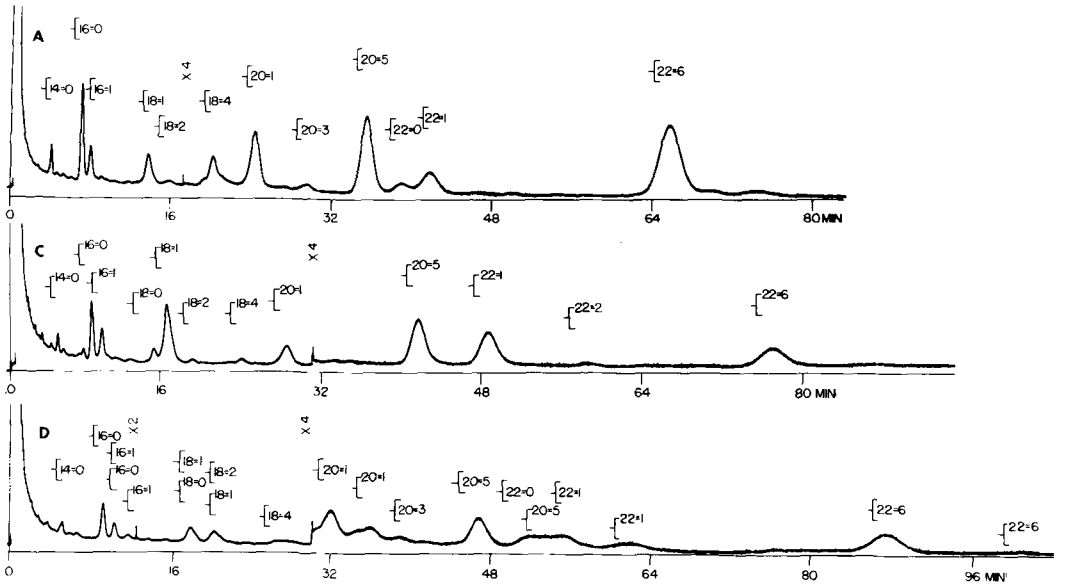

FIG. 2. Gas liquid chromatographic elution patterns of trimethylsilyl (TMS) ethers of monoacylglycerols of cod liver oil on SILAR 5CP (cyanopropylphenylsiloxane). (A) 2 monoacylglycerols (C) 1(3) monoacylglycerols and (D) ca. equal wt mixture of A and C. Peak identification, instrumentation, and operating conditions as in Fig. 1. Sample: 1 μliter of a 1% solution of TMS ethers of monoacylglycerols in reaction mixture.

lenoylglycerols (Table III). Relative to stearoylglycerol, there is a correlation between decrease in retention of the linolenate and the increase in temperature. This decrease in relative retention with temperature, however, is smaller than the corresponding decrease in retention noted for homologues differing by two carbons. The SE-30 provides a partial resolution of saturates and monounsaturates, but the order of elution is reversed from that seen on the polar liquid phases. Wood and Snyder (4) have reported that the Apiezon grease may have been used to separate saturated and monounsaturated monoacylglycerols. Due to the reverse order of elution of the saturates and unsaturates, this nonpolar liquid phase is of little value for analysis of complex mixtures of molecular species which contain significant amounts of saturated and unsaturated C_{16} and C_{18} monoacylglycerols of both types of configuration. Therefore, the SILAR 5CP liquid phase emerges as the best means of resolving homologues and positional isomers of monosubstituted glycerols at the present time.

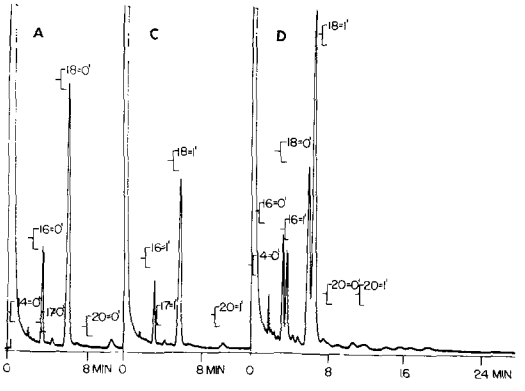

FIG. 3. Gas liquid chromatographic resolution of saturated and unsaturated monoalkylglycerols as trimethylsilyl ethers on SILAR 5CP (cyanopropylphenylsiloxane). (A) saturated 1 monoacylglycerols, (C) monounsaturated 1 monoacylglycerols, and (D) ca. equal wt mixture of A and C. Peak identification, instrument, and operating conditions as in Fig. 1. Sample: 1 μliter of an ca. 1% solution in reaction mixture.

Comparison of Retention Behavior of Different Derivatives of Monoacylglycerols and Fatty Acid Methyl Esters

Table III gives the relative retention times of the TMS ethers and acetates of the various acylglycerols examined on the SILAR 5CP column compared with fatty acid methyl esters. The values have been compiled from analyses of the monoacylglycerols of seed and cod liver oils, as well as of standards. Highly consistent and reproducible data are obtained with this liquid phase under a variety of conditions. The different molecular species of the monoacylglycerols could thus be accommodated on

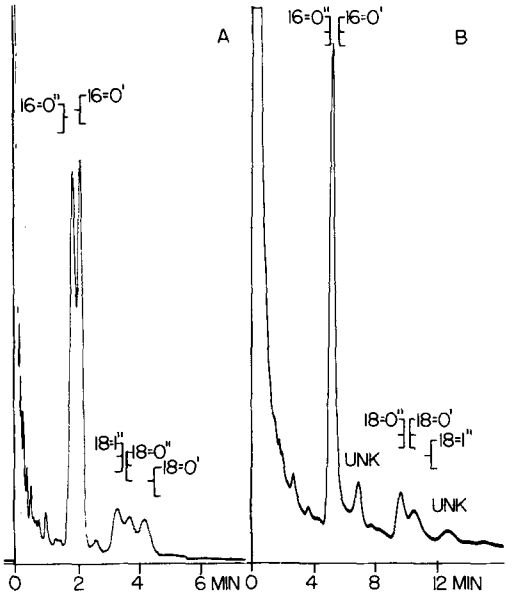

FIG. 4. Gas liquid chromatographic resolution of 1 monoalkyl and 1-monoalk-1-enyl-glycerols as TMS ethers on SE-30 (methylsiloxane) and SILAR 5CP (cyanopropylphenylsiloxane). (A) SE-30 and (B) SILAR 5CP. Peak identification as explained in text. Instrument and operating conditions: (A) Beckman GC-4 gas chromatograph equipped with a 60 cm x 3 mm outside diameter stainless steel column packed with 3% SE-30 of Gas Chrom Q (100-120 mesh). Carrier gas, nitrogen (80 ml/min). Injector, 200 C; oven, 180 C; detector, 250 C; (B) as in Figure 1. Sample: 1 μliter of a 1% solution of petroleum. UNK = unknown.

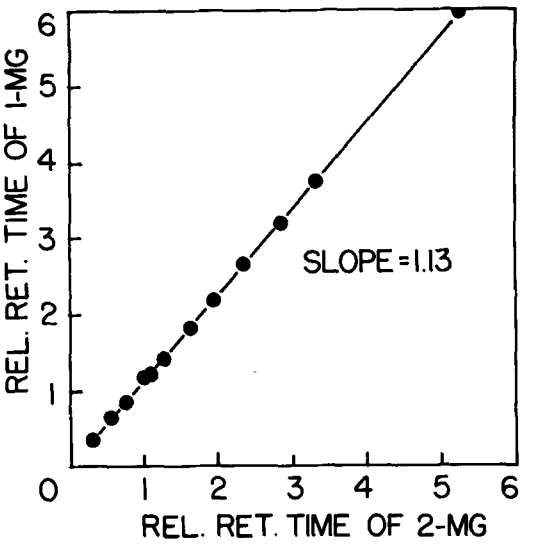

FIG. 5. Relationship of retention times of 1 and 2 monoacylglycerols (MG) of different mol wt and degree of unsaturation. The individual points represent, from left to right, in ascending order the following species of monoacylglycerols: 14:0, 16:0, 17:0, 18:0, 18:1, 18:2, 18:3, 20:1, 20:3, 20:5, 22:1, and 22:6. The retention times are relative to 2-monostearoylglycerol.

straight parallel lines like those commonly constructed for fatty acid methyl esters when plotting the log of the retention time vs carbon number. The slope of the straight line series for the monoacylglycerols, however, was not as steep as that seen for the fatty acid methyl esters. This was due to the decrease in the separation factors of the various species with increasing temperature of the column. At elevated temperatures, the fatty acid methyl esters were eluted with retention times that gave slopes comparable to those seen for the monoacylglycerols. In unpublished studies, we have shown that the separation factors for fatty acid methyl esters 20:0/18:0 decreased from 2.08 to 1.82 and 1.55 as the column temperature was increased from 185 to 220 and 248 C, respectively. Hence the decreased resolution of the longer retained derivatives and molecular species is not related to their mol wt or greater similarity in the boiling points but to the effect of temperature upon the properties of the liquid phase.

Table III also gives the ECL for various derivatives of the isomeric monoacylglycerols. These values were again found to be extremely reproducible and served as means of identification of unknown peaks in relation to known standards. Although complete resolution of all components could not always be obtained simultaneously, the separations realized on the SILAR 5CP columns were adequate for an accurate mass spectrometric identification of the natural monoacyl-, monoalkyl, and monoalkenylglycerols (6).

Figure 5 relates the retention times of the 2 monoacylglycerols to those of the 1 monoacylglycerols. A straight line is obtained, which indicates that the separation factors are the same for all species, regardless of the chain length, degree of unsaturation, and location of the fatty acid in the glycerol molecule. The effect of the positional placement of the fatty acid in the glycerol moiety is always the same upon the retention time of the molecule over the ranges of temperature investigated. Over the experimentally feasible range of temperature, the separation factors of saturates and unsaturates were relatively constant. This close relationship among the retention times of the different molecular species of the monoacylglycerols serves as a basis for an evaluation of the resolution of isomeric diacylglycerols on similar GLC columns.

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Biosynthesis of Monoacyl-sn-Glycerol 3-Phosphate by Rabbit Heart Mitochondria: Positional Specificity Differing from Liver Enzyme[1]

M.S. LIU, P.J. BROOKS, and K. JOE KAKO[2], Department of Physiology, Faculty of Medicine, University of Ottawa, Ottawa, Ontario, K1N 6N5, Canada

ABSTRACT

Rabbit heart mitochondria synthesize comparably more monoacyl- than diacylglycerol 3-phosphate as compared to heart microsomes. Since the fatty acid specificity of the biosynthesis by cardiac enzymes differs from that by hepatic enzymes, the positional specificity of monoacylglycerol 3-phosphate formation by mitochondria was investigated by using a method utilizing lysosomal phosphatidate phosphohydrolase and borate-impregnated thin layer chromatography. Contrary to the results of studies with liver particulates, ca. one-third of palmitate was found at position 2 of the glycerol moiety and the remainder at position 1, whereas one-third of oleate was bound at position 1. Only a slight, asymmetric distribution was observed with linoleate as the acyl donor. Thus, our study indicates that preferential acylation of individual fatty acids during the formation of monoacylglycerol 3-phosphate is not as distinct in the heart mitochondria as in the liver mitochondria and microsomes.

INTRODUCTION

In most naturally occuring phosphoglycerides, unsaturated fatty acids are esterfied preferentially at position 2 and saturated fatty acids at position 1 of the glycerol molecule (1). This asymmetric fatty acid distribution is thought to be of major importance in the functional and structural role of phospho- and glycerolipids in cellular processes. For instance, perturbation in the asymmetric distribution of the fatty acids of membrane phosphoglycerides can produce changes in cellular functions (2, 3). Thus, the enzyme reactions responsible for such positional specificity of fatty acid moieties are extremely critical (1, 4).

Early studies on the positional and substrate specificity of glycerophosphate acyltransferase (acyl-CoA:sn-glycerol-3-phosphate-O-acyltransferase, EC 2.3.1.15) in liver microsomal systems (5,6) indicated that glycerol 3-P(sn-glycerol 3-phosphate) was acylated in a nearly random manner, although marked positional specificity was exhibited in the esterification of 1- and 2-acylglycerol 3-P (4,7). However, several subsequent investigations with the enzyme from rat liver have shown that glycerol 3-P acylation proceeds in a nonrandom, asymmetrical manner, so that saturated fatty acids are esterified preferentially at position 1 of the diacylglycerol 3-P (8-14), and monoacylglycerol 3-P is an intermediate in this reaction (10-14).

In our studies reported elsewhere (15, 16), we examined the characteristics of mitochondrial and microsomal glycerophosphate acyl transferases of rabbit hearts. The mitochondrial enzyme could be differentiated from the microsomal one by the enzyme kinetics, reaction products, substrate specificity (15), and response to triiodothyronine administration (16). One of the characteristics of mitochondrial acylation of glycerol 3-P, in contrast to microsomal acylation, is the formation of monoacylglycerol 3-P disproportionately to diacylglycerol 3-P. This probably is due to the activity of the first acylation of glycerol 3-P which greatly exceeds that of the second acylation under our experimental conditions (16). Furthermore, our study revealed that the substrate specificity of the monoacylglycerol 3-P formation by cardiac mitochondria greatly differs from that by hepatic mitochondria (15). On the other hand, there is little information to indicate whether the selection mechanisms delineated in the liver during the glycerol 3-P acylation (9-14) are generally applicable to all tissues. Consequently, the positional specificity of this reaction has been examined and reported in this article.

EXPERIMENTAL PROCEDURES

Preparation of mitochondria: Albino male rabbits (Canadian Breeding Farm Laboratory, St. Constant, Quebec), weighing 2-3 kg, were anesthetized by an intraperitoneal injection of 2.5 ml 2% chloralose (trichloethylene-D-gluco-

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[2]Author to whom reprint requests should be addressed.

furanose) in 20% urethane (ethylcarbamate)/kg body wt. The hearts were excised, trimmed to remove fat, and were weighed and chopped into small pieces. The tissue then was homogenized with 4 volumes 0.25 M sucrose containing 0.02 M Tris-HCl, pH 7.4, in a Potter type homogenizer with a Teflon pestle rotated at 1000 rpm. The homogenization was standardized by performing two strokes up and down with a loose fitting pestle and then one stroke with a tight fitting pestle. The homogenate was centrifuged at 800 x g for 15 min, and the supernatant was decanted carefully and centrifuged at 10,000 x g for 15 min. The pellet was resuspended and centrifuged at 8000 x g for 15 min yielding a mitochondrial pellet, which was suspended in the homogenizing medium giving 8-10 mg protein/ml (15, 17, 18).

Monoacylglycerol 3-P biosynthesis: The reaction mixture contained, in a final volume of 2.0 ml, 100 μmoles Tris-phosphate buffer, pH 7.4; 50 μmoles borate buffer; 2.0 μmoles potassium palmitate; oleate or linoleate; 0.8 μmole coenzyme A (CoA); 12.0 μmoles adenosine 5′-triphosphate (ATP); 6.0 μmoles $MgCl_2$; 20 mg defatted bovine serum albumin; 6.0 μmoles sn-[U-^{14}C]glycerol 3-P (350 000 dpm/μmole, New England Nuclear Corp., Boston, Mass.); and a fresh enzyme preparation, containing ca. 5.0 mg mitochondrial protein. When palmitoyl-CoA served as the acyl donor, fatty acid, CoA, ATP, and $MgCl_2$ were replaced with 0.8 μmole palmitoyl-CoA (Sigma) and 10 mg, instead of 20 mg, bovine serum albumin. The purity of the ^{14}C-glycerol 3-P was verified by paper chromatography with phenol-water (5:2, w/v) as the solvent (15). The incubation was carried out in a metabolic shaker at 37 C for 10 min in air. The reaction was stopped by the addition of 3.0 ml 0.1 M boric acid-saturated butanol, followed by the addition of 4.0 ml butanol-saturated 0.1 M boric acid. After separation of the two phases, the upper phase was washed again with butanol-saturated boric acid and the lower phase with boric acid-saturated butanol (11,14,15,19). The butanol extract from 20-24 incubations was combined and was reduced to a small volume under N_2 gas. The residue was dissolved in chloroform:methanol (1:1, v/v) (20) and then chromatographed on Silica Gel G plates. The latter were prepared either by spraying of the commercial plates (Brinkman Instruments, Rexdale, Canada) with 0.4 M boric acid in methanol or by spreading of a slurry of 25 g Silica Gel G (E. Merck, Darmstadt, Germany) in 50 ml 0.4 M boric acid on the thin layer chromatographic (TLC) plates (21). The monoacylglycerol 3-P was separated by developing the plates with chloroform:methanol:acetic acid:water (65:25:8:4, v/v/v/v) (15). Lipids were localized by exposure to I_2 vapor and were compared to the authentic standards. The appropriate band of silica gel was scraped from the plate and eluted with chloroform:methanol (1:1, v/v). A 0.15 M potassium borate solution, containing 0.075 M ethylenediaminetetraacetic acid (EDTA), pH 6.8 (11), replaced water to induce the two phase system.

Treatment with phosphatidate phosphohydrolase: The chloroform phase which contained monoacylglycerol 3-P was dried under N_2 gas and was dispersed in 0.02 M Tris-HCl buffer, pH 7.4, by sonication (Biosonik, model B 10-II, Bronwill Scientific Co., Rochester, N.Y.). The resulting suspension then was treated with phosphatidate phosphohydrolase (EC 3.1.3.4) in an incubation mixture containing 0.05 M borate buffer, pH 7.4. After 60 min of dephosphorylation at 37 C, the reaction was stopped by the addition of boric acid (0.1 M)-saturated butanol as above, except that carriers 1- and 2-monoacylglycerol (Applied Science Laboratory, State College, Pa., and Supelco, Bellefonte, Pa.) were added to the butanol extracts. Finally, isomeric monoglycerides were separated by borate-impregnated TLC plates using a solvent system E by Thomas, et al. (21), containing chloroform:acetone:methanol (71:25:4, v/v/v). Appropriate bands were scraped off and their radioactivities determined by liquid scintillation spectrometry (15, 16). During the entire procedure, all possible precautions were taken to prevent acyl migration; these were: rapid handling of reaction products at low temperature until the final chromatograph was run, the use of borate buffer in the incubation mixture (3), performance of evaporation in a nitrogen atmosphere, the use of borate saturated butanol and borate-impregnated TLC plates (21), and the addition of 1- and 2-monoacylglycerol carriers to the final butanol extraction (14).

Preparation of phosphatidate phosphohydrolase: This was prepared according to the method of Wilgram and Kennedy (22). Male rats (20 rats) of Sprague-Dawley strain (Bio-Breeder, Ottawa, Ontario), weighing ca. 250 g, were killed by cervical dislocation. Their livers were removed rapidly and transferred to beakers immersed in crushed ice. Portions of the chilled livers were minced with scissors and homogenized in ice-cold 0.25 M sucrose (1:9, w/v) with a Potter type homogenizer fitted with a Teflon pestle. The homogenization was performed for ca. 30 sec. The homogenate was centrifuged at 600 x g for 12 min. Its supernatant fraction was centrifuged at 8500 x g for 12

min; the resulting supernatant then was removed with a Pasteur pipet. A layer of loosely sedimented material was removed from the mitochondrial pellet by careful washing with a small amount of cold sucrose solution added from a capillary pipet, without disturbing the bulk of the mitochondrial pellet. The mitochondrial pellet was washed once with homogenizing medium and recentrifuged. The fluffy lysosomal layer was collected and combined with that obtained from the first centrifugation. The fraction was dialyzed overnight against 0.02 M Tris buffer, pH 7.4, containing 0.005 M EDTA, then stored at -15 C. The activity of the phosphohydrolase preparation was 3.0-3.2 μmoles/mg hr, which agrees with the values reported by the previous workers (22,23); the activity remained unchanged during storage for up to 10 weeks. Although this crude preparation contained some lipids, no attempt was made to remove them, since their presence did not interfere with the hydrolysis of monoacylglycerol 3-P.

The activity of lysosomal phosphatidate phosphohydrolase was measured by the rate of release of inorganic phosphate from diacylglycerol 3-P (Pierce Chemical Co., Rockford, Ill.) (22). The stated fatty acid composition of the latter compound was: 36%, palmitate; 15%, stearate; 37%, oleate; and 12%, linoleate. The incubation mixture contained 160.0 μmoles Tris-acetate buffer, pH 7.4, 30.0 μmoles diacylglycerol 3-P, and enzyme in a final volume of 2.0 ml. The tubes were incubated for 1 hr at 37 C, after which the reaction was stopped by the addition of 2.0 ml 12% trichloroacetic acid. In control tubes, the enzyme fractions were incubated in the absence of diacylglycerol 3-P for 1 hr, after which the substrate was added, followed immediately by the trichloroacetic acid. Orthophosphate was estimated with aliquots of the trichloroacetic acid filtrates by the method of Berenblum and Chain (24).

RESULTS AND DISCUSSION

Monoacylglycerol 3-P synthesized by rabbit heart mitochondria was treated with phosphatidate phosphohydrolase, and its products were chromatographed on TLC plates; the latter were scanned by a radioactive scanner (Actigraph III, Nuclear-Chicago Corp., Des Plaines, Ill.). Three peaks invariably appeared; the first peak, which appeared at the origin, represented unreacted monoacylglycerol 3-P (Fig. 1). The R_f values of the two other peaks corresponded to those of the authentic 1-monoacylglycerol (0.54) and 2-monoacylglycerol (0.63). No radioactivity was detectable in the area corresponding to diacylglycerol, indicating that the isolated monoacylglycerol 3-P was free of diacylglycerol 3-P contamination (Fig. 1).

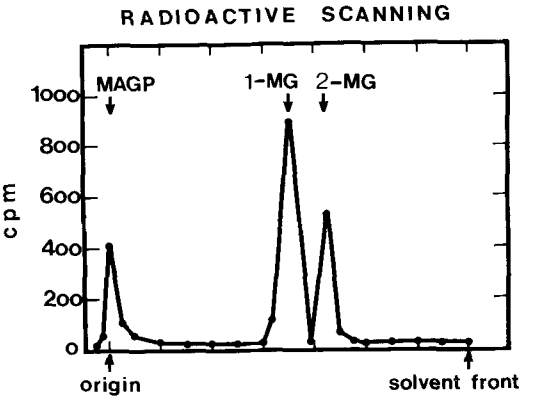

FIG. 1. Thin layer chromatography of the dephosphorylation reaction product from monoacylglycerol 3-phosphate (MAGP). MAGP synthesized by rabbit heart mitochondria with palmitate as the acyl donor was isolated by thin layer chromatography, pooled (29.8 nmoles), and then was dephosphorylated by incubation with lysosomal phosphatidate phosphohydrolase (4 mg protein/ml). The products were extracted and chromatographed with solvent system E of Thomas, et al. (21), as described in the "Experimental Procedures." The radioactivity of the developed plate was scanned by an Actigraph (Nuclear-Chicago Corp.). The full-scale of the latter equipment was set at 1000 cpm, and a scanning speed of 60 cm/hr was used. In order to maintain a high resolution, the slit width of the scanner was narrowed to 3 mm, and the response time was adjusted to 20 sec to suppress the noise level. The positions of 1- and 2-monoglycerides (1-MG, 2-MG) were identified with authentic standards.

The relative proportions of 1- and 2-monoglycerides, derived from 1- and 2-acyl-sn-glycerol 3-P synthesized by the heart mitochondria, are shown in Table I. Palmitic acid was preferentially, but not exclusively, acylated at position 1 of the glycerol molecule, whereas two-thirds of the oleoyl glycerol 3-P was 2-oleoyl-sn-glycerol 3-P and the remainder 1-oleoyl-sn-glycerol 3-P. Linoleic acid appeared to be less specific than the other two fatty acids tested but, nevertheless, showed a slight preference toward position 2.

The radioactive monoacylglycerol 3-P was hydrolyzed by the phosphohydrolase to an extent of 64-78%. These values are greater than those reported by some previous workers (11, 14) but less than those by others (80-90%) (3,13,25). Consequently, nonhydrolyzed monoacylglycerol 3-P was eluted from the TLC plates, concentrated, and treated a second time with phosphatidate phosphohydrolase in the manner described in "Experimental Procedures." The results are shown in Table II. The

TABLE I

Positional Specificity of Monoacylcerol 3-Phosphate Biosynthesis by Rabbit Heart Mitochondria

Acyl donor	Monoacylglycerol 3-phosphate used (nmoles)	Amount dephosphorylated (nmoles [%])		Relative amount of radioactivity found in 1-Monoglyceride(%)	2-Monoglyceride(%)
Palmitate	29.8	20.7	(70.)	63.8	36.2
Palmitate	35.8	27.6	(78.)	60.7	39.3
Oleate	20.7	13.2	(64.)	35.5	64.5
Oleate	25.0	18.0	(72.)	32.8	67.2
Linoleate	16.5	11.6	(71.)	42.8	57.2
Linoleate	18.0	13.5	(75.)	48.5	51.5
Palmitoyl-CoA	19.8	15.5	(78.)	69.9	30.1

first dephosphorylation produced results similar to those in the previous experiments shown in Table I. Relative distribution of palmitate and oleate in the monoacylglycerol 3-P, which had not been hydrolyzed in the first phosphohydrolase treatment, was almost identical (two rows in the middle, Table II) to the data already described, suggesting little specificity of the phosphatidate phosphohydrolase (26).

Although the second treatment by the phosphohydrolase was less effective (59% and 61% hydrolysis) than the first, an over-all dephosphorylation of 88% was achieved. A difference between this value and averages of hydrolysis (85% and 86% listed in Table II) is accounted by a loss which occurred during elution and evaporation in preparing monoacylglycerol 3-P, i.e. 81% and 86% recovery. The overall composition calculated from these twice incubated experiments (Table II, bottom two rows) confirms the results obtained in the experiments with a single incubation with phosphohydrolase shown in Table I. Thus, our results contrast those with liver particulates in which palmitate was almost exclusively esterified at position 1 (11-14); furthermore, a very small quantity of fatty acids was acylated to position 2 during the first step of the glycerol 3-P acylation process in the liver (11). The results using hepatic subcellular fractions were confirmed recently by the studies with partially purified hepatic enzymes (12, 13).

Our results reported previously demonstrated that the de novo biosynthesis of monoacylglycerol 3-P by rabbit heart mitochondria possessed only a moderate fatty acid specificity; the rate of synthesis was somewhat more rapid with oleic than palmitic acid and with palmitic than linoleic acid as the acyl donor (15). Therefore, it was postulated that, contrary to hepatic enzymes, the heart enzyme is capable of acylating both positions 1 and 2 of the glycerol 3-P moiety with little discrimination. Thus, both the substrate and positional specificity of cardiac glycerophosphate acyltransferase clearly differ from those of the liver enzyme, and the enzyme appears to be organ-specific. The reaction kinetics of the enzyme of heart mitochondria and its susceptibility to storage or to N-ethylmaleimide also differed from those of the liver enzyme (11, 15).

Recent evidence points to a nonrandom distribution of radioactivity from the major saturated and unsaturated fatty acids in the phosphatidic acid formation in rat brain (25). However, the asymmetry in the brain phosphatides is markedly different from that reported for rat liver phosphatides, supporting the organ specificity hypothesis proposed. Some reports indicate that both positions 1 and 2 are available for the first acylation even with enzymes of the liver or *Escherichia coli* (1,3,6,9,10,27). The second acylation, namely, the reaction catalyzed by acyl-CoA:monoacylglycerol 3-P acyltransferase, also is known to be selective with respect to individual fatty acids in the liver (4, 28), but no data have been available on the heart enzyme. Moreover, the role played by the acyl exchange reaction (1,4,6,7) must be assessed before the final molecular species of glycerolipids in an organ can be described more definitively.

Since acyl-CoA synthetase (EC 6.2.1.2, EC 6.2.1.3) activity is extremely high as compared to glycerophosphate acyltransferase activity in liver mitochondria (19) and even more so in heart mitochondria (29), it is unlikely that the results described are influenced by the recently reported specificity of acyl-CoA synthetase (30). The result obtained by using palmitoyl-CoA as the acyl donor (Table I) agrees with this conjecture. The influence of endogenous fatty acids in the mitochondria (15) again should not alter our conclusion.

TABLE II

Additional Analyses of the Positional Specificity of Monoacylglycerol 3-Phosphate (MAGP)

Acyl donor	Monoacylglyerol 3-phosphate used (nmoles)	Amount dephosphorylated (nmoles [%])	Relative amount of radioactivity found in 1-Monoglyceride(%)	2-Monoglyceride(%)
After the first treatment of MAGP with phosphatidate phosphohydrolase				
Palmitate	30.0	21.5 (72)	65.2	34.8
Oleate	26.7	18.7 (70)	36.0	64.0
After a second treatment of nonhydrolyzed MAGP with phosphatidate phosphohydrolase				
Palmitate	6.8[a]	4.0 (59)	71.0	29.0
Oleate	6.9[a]	4.2 (61)	31.6	68.4
Combined results of the two treatments				
Palmitate	30.0	25.5 (85)	66.0	34.0
Oleate	26.7	22.9 (86)	35.2	64.8

[a]Recovered from the first experiment, were 6.8 out of a theoretical value of 8.4 and 6.9 out of 8.0 (all in nmoles).

There seems to be little work dealing with the positional analysis of heart glycerides (31). A work which dealt with analyses of the glyceride compositions in pig organs revealed a striking difference in fatty acid distribution between hepatic and cardiac triglyceride, i.e. the latter contained considerably less palmitic acid at position 1 and more oleic and linoleic acids at positions 1 and 3 than did the liver triglyceride (32). In addition, it was reported earlier that the distribution of palmitic acid in positions 1 and 2 of the phosphatidylcholine in the heart was dissimilar to that in the liver (33). These results are, thus, compatible to those obtained in our study.

Although the first step of the glycerol 3-P acylation reaction by the cardiac mitochondria exhibited the selectivity in the study reported, the asymmetric distribution of fatty acids was not as pronounced as can be expected from the analyses of molecular classes of heart glycerides. Thus, a further question regarding the rearrangement and desaturation of fatty acid moieties which may take place (1, 4) after the formation of diacylglycerol 3-P, diglyceride or triglyceride in heart tissue is as yet to be investigated.

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Prostaglandins of Rat Testis

MARY P. CARPENTER, Biomembrane Research Laboratory, Oklahoma Medical Research Foundation, and Department of Biochemistry and Molecular Biology, University of Oklahoma School of Medicine, Oklahoma City, Oklahoma 73104

ABSTRACT

The purpose of the study was to determine whether prostaglandins were present in mammalian testis, a tissue that has a large concentration of polyenoic fatty acids that are potential precursors of prostaglandins. Acid-soluble lipids of rat testis were extracted, purified, and fractionated by thin layer and column chromatographies. ^{3}H-Prostaglandins were added as internal reference standards to monitor recoveries and facilitate identification. Initial identification of prostaglandin species was done by chromatography. Further identification was done by elution of the prostaglandin zones followed by rechromatographies (both thin layer and column), measurements of UV absorption spectra, and by gas liquid chromatography. The results of these analyses indicate that prostaglandin E_1, 11α,15(S)-dihydroxy-9-oxo-β-*trans*-prostenoic acid; prostaglandin E_2, 11α-15(S)-dihydroxy-9-oxo-5-*cis*-13-*trans*-prostadienoic acid; and prostaglandin F_1, 9α,11α,15(S)-trihydroxy-13-*trans*-prostenoic acid occur in rat testicular tissue and that prostaglandin $F_{2\alpha}$, 9α,11α,15(S)-trihydroxy-5-*cis*-13-*trans*-prostadienoic acid and prostaglandin E_2, 11α,15(S)-dihydroxy-9-oxo-5-*cis*-13-*trans*-prostadienoic acid may be the primary species of this tissue. Prostaglandin B_1, 15(S)-hydroxy-9-oxo-8(12),13-*trans*-prostadienoic acid and prostaglandin B_2, 15(S)-hydroxy-9-oxo-5-*cis*,8(12),13-*trans*-prostatrienoic acid also were detected, and some evidence was obtained for the presence of prostaglandin metabolites.

INTRODUCTION

Prostaglandins are known to be associated with the male reproductive tract but have been found primarily in seminal fluid and seminal vesicles (1-3). Testicular tissue has an unusually high concentration of polyenoic fatty acids derived from linoleic acid (4-6). In other tissues, certain of these fatty acids can act as biosynthetic precursors of prostaglandins. Attempts by others to detect prostaglandins in testis were not successful (7,8). Since the precursors are present, it seemed likely that these compounds would be found, and this study was initiated to detect them in rat testis. The present work is part of a study of the factors that are involved in the proliferation, differentiation, and maintenance of the mammalian testis. A preliminary report of this work has been published (9).

EXPERIMENTAL PROCEDURES

Materials

Solvents were either of ngrade or reagent quality. Ethyl acetate, methanol, benzene, dioxane, ether, and pyridine were redistilled prior to use. The latter was stored over KOH pellets after distillation. All other chemicals were of reagent quality.

For chromatography, 100 mesh silicic acid (Mallinckrodt, St. Louis, Mo.) and silica gel, extra pure (Brinkmann Instruments Co., Des Plaines, Ill.), were used. Thin layer glass plates precoated with a 250 μliter of silica gel with inert polymer binder were purchased from Mann Research Laboratories, New York, N.Y., and plates precoated with Silica Gel HR or containing 2.2% $AgNO_3$ from Analtech, Wilmington, Del. Diazomethane was freshly prepared before use from N,N^1-dinitrosoterephthal amide and diethylene monomethyl ether.

Materials used in gas liquid chromatography (GLC) obtained from Applied Science Lab., State College, Pa., included: methoxylamine hydrochloride, bis(trifluorosilyl)acetamide, saturated fatty acid mixture KF, methyl arachidate (99.8%), methyl tricosanoate (99.5%), and 3% OV-1, on Gas Chrom Q 100-200 mesh. The silylation reagent hexamethyldisilazone (HMDS):trimethylchlorosilane (TMCS):pyridine, 3:1:9 (v/v/v) was from Supelco Co., Bellefonte, Pa.

Prostaglandin reference standards: prostaglandin E_1, 11α,15(S)-dihydroxy-9-oxo-β-*trans*-prostenoic acid (PGE_1); prostaglandin E_2, 11α,15(S)-dihydroxy-9-oxo-5-*cis*-13-*trans*-prostadienoic acid (PGE_2); prostaglandin $F_{1\alpha}$, 9α,11α,15(S)-trihydroxy-13-*trans*-prostenoic acid ($PGF_{1\alpha}$); prostaglandin $F_{2\alpha}$, 9α,11α,15(S)-trihydroxy-5-*cis*-13-*trans*-prostadienoic acid ($PGF_{2\alpha}$) were obtained from Upjohn Co., Kalamazoo, Mich., and tritiated-prostaglandin E_1, 11α,15(S)-dihydroxy-9-oxo-

β-*trans*-prostenoic acid (^{3}H-PGE$_1$) was a gift. Tritiated-prostaglandin $F_{2\alpha}$, 9α,11α,15(S)-trihydroxy-5-*cis*-13-*trans*-prostadienoic acid (^{3}H-PGF$_2$) was purchased from New England Nuclear, Boston, Mass.

Extraction of Acid-Soluble Lipid

Holtzman-Sprague Dawley rats, bred in this laboratory were fed a control diet containing 20% casein (6) and maintained in animal care facilities fully accredited by the American Association for the Accreditation of Laboratory Animal Care. Animals of the same age, 5-6 weeks (immature) and 13 weeks or more (adult) were sacrificed, the testes excised, and the *tunica albuginea* removed. The tissue was weighed (40-80 g), homogenized in 4 volumes of ethanol, and filtered. Tissues were extracted as rapidly as possible and special precautions, such as N_2-covering, cold, and protection from light were taken. The ethanol extract was concentrated (10), extracted with petroleum ether (boiling point [bp] 30-60 C), acidified with 0.2 M citric acid (11) to pH 3, and partitioned into ethyl acetate (12). In some preparations, an equal volume of 0.9% NaCl was added to the concentrated ethanol extract (13) prior to further extraction. After washing to neutrality and evaporation, the acid-soluble lipid fraction was dissolved in 0.5-2.0 ml 2% methanol in ethyl acetate. Wt of the lipid was determined by weighing several 10 μliter aliquots on an electromicrobalance.

Addition of Internal Standard Radioactive Prostaglandins

To estimate recoveries by isotope dilution and to provide radioactive markers for chromatographic studies, most of the experiments were performed using ^{3}H labeled marker prostaglandins. An aliquot of 10 μliter ^{3}H-PGE$_1$ (8,9-^{3}H, 0.02 μCi, 40 ng) was added during the homogenization of the tissue. In some experiments, 10 μliter ^{3}H-PGF$_{2\alpha}$ (9,-^{3}H, 0.05 μCi, 1 ng) also was added. Aliquots taken at various stages of preparation were removed, placed in a scintillation cocktail containing Omnifluor (New England Nuclear) and toluene, and counted using a Packard TriCarb instrument. The counting efficiency was 25%.

Purification of Acid-Soluble Lipid

Several procedures, such as fractionation on columns of silicic acid, and chromatography on Sephadex LH-20, repartition into phosphate buffer, and thin layer chromatography (TLC) were utilized to eliminate contaminants from the tissue lipid preparations.

Silicic acid column chromatography: Columns were prepared in Pasteur pipets using 100 mg silicic acid for 5 mg lipid. Testis acidic lipid was applied to the column in ethyl acetate and aliquots of 2 ml collected during the successive elution of the column with 30% ethyl acetate in benzene (fraction I), 2% methanol in ethyl acetate (fraction II), and methanol (fraction III). The elution sequence $CHCl_3$, 4% methanol in $CHCl_3$, 10% methanol in $CHCl_3$, then methanol was not satisfactory for testis acid lipid, for phospholipids were distributed in all fractions.

Sephadex chromatography: Optimal conditions for separation of prostaglandins were established with a Sephadex LH-20 column (14), using methyl retinoate, the methyl esters of prostaglandin B_2, prostaglandin E_1, and prostaglandin $F_{2\alpha}$, and marker aliquots of ^{3}H-PGE$_1$ and ^{3}H-PGF$_{2\alpha}$ as references. The column was eluted with heptane, chloroform, ethanol, 10:10:1, using a flow rate of 0.25 ml/min, and 1 ml fractions were collected. Aliquots of the fractions were monitored in the UV spectral range using a Gilford-2400 spectrophotometer, and other aliquots were subjected to scintillation counting. On this column, the retention volumes relative to retinoate were 1.5 for PGB$_2$, 2.6 for PGE$_2$, and 3.5 for PGF$_{2\alpha}$. Fractions were pooled and evaporated prior to TLC and GLC analyses.

Repartition into phosphate buffer and ethyl acetate: The concentrated acidic lipid of testis was diluted to a volume of ca. 25 ml with ethyl acetate and then extracted 3 times into an equal volume of 0.15 M potassium phosphate buffer, pH 8.0. The phosphate buffer was extracted once with ethyl acetate, then acidified to pH 3.0 with 1 M citrate, and extracted 3 times with ethyl acetate. The acidified ethyl acetate fractions were pooled, washed to neutrality, concentrated to a volume of 1-2 ml, then aliquots weighed and stored under N_2. All the solvents used were redistilled just before use and equilibrated with the immiscible phases used during the extraction procedure.

TLC: Plates were prewashed with methanol prior to use. To ca. half of the plate, testis acidic lipid was applied in 10 μliter aliquots at the origin (0.3-0.4 ml preparation equivalent to 12-24 g testis tissue). Standard reference prostaglandins, and two separate additional aliquots of the testis acidic lipid, 10 μliter for scraping, elution, and counting and 30-50 μliter (representing 1.5-2.0 g tissue) for visualization also were applied to the same plate. The plates were developed using solvent system A I for free acids and M I for methylated preparations (15). After development, the area of the plate to be

visualized was sprayed with phosphomolybdate, a permanent record of the thin layer plate made, the zones corresponding to the reference prostaglandins were scraped, and the radioactivity counted. Analagous zones from the preparative portion of the plate were scraped carefully, eluted with methanol, and the supernates collected and stored under N_2 in the cold.

Prostaglandins in quantities of less than 1 μg can be detected on thin layer plates using ethanolic molybdate as a visualizing reagent (16). Studies in this laboratory indicate that 0.1 μg of $PGF_{2\alpha}$ can be visualized. When varying amounts of $PGF_{2\alpha}$ are applied to a thin layer plate, spots containing 750, 1500, and 3000 ng are visible within 2.5 min after heating, those containing 50-500 ng after 7 min; the 50 and 100 ng spots are faint.

Determination of PGEs by Chromophore Formation

An aliquot of the sample to be analyzed was placed in a cuvette (1 cm path length), and, after evaporation to dryness, methanol (0.25-1 ml) was added and the spectrum scanned between 400 and 200 mμ using a Cary model 14 recording spectrophotometer. Then an equal volume of 1 N KOH was added and the mixture heated for 15 min at 50 C. The sample then was scanned again using a similarly treated blank, and the amount of PGB was calculated from a standard curve prepared using PGE_1 as a reference.

GLC

Trimethylsilyl ether, trimethylsilyl ester (TMSi) and trimethylsilyl ether, methyl ester (TMSi-Me) derivatives of prostaglandin standard and of materials isolated from tissue were prepared. Prostaglandins of the E series were converted to 9 keto methoximes using a modification of the procedure of Vane and Horning (17). Ca. 50 μg sample and 0.5 mg methoxylamine hydrochloride were reacted overnight at room temperature, then dried under N_2. At this point in the procedure, 5 μliter internal standard containing a mixture of methyl arachidate (8.4 μg) and methyl tricosanoate (1.7 μg) was added. This was followed by the addition of 25 μliter silylating reagent and allowed to react for 45 min at room temperature. With the addition of 25 μliter CS_2, the sample was ready for analysis.

GLC was carried out using a Perkin Elmer model 881 instrument with a flame ionization detector and a 6-1/2 ft glass column (inside diameter 2.0 mm) packed with 3% OV-1 on 100-200 mesh Gas Chrom Q. The temperature of the injection chamber was 285 C, the column 210 C, and the detector chamber 250 C. Sample volumes injected varied from 4-8 μliter in different determinations. Linearity of the detector response under the same set of conditions was confirmed with a known mixture of saturated fatty acid methyl esters.

Appropriate derivatives of PGE_1, PGE_2, PGB_1, PGB_2, $PGF_{1\alpha}$, and $PGF_{2\alpha}$ were chromatographed, and the retention times, relative to tricosanoate, were determined. Since both C_{20} and C_{23} fatty acids were used as internal standards, the C value (18) was verified readily for each determination. Prostaglandins from testicular preparations tentatively were identified by comparison of the relative retention time and the equivalent chain lengths of the unknown with those of reference prostaglandins.

The amounts of the various prostaglandins were calculated from the area of the respective peaks and the area of the C_{23} internal standard. The detector response to varying amounts of appropriate derivatives of prostaglandin standards was linear over the range 0.25-5 μg.

RESULTS

Recovery of Acid-Soluble Lipid

The wt of the acidic lipid extracted from testes ranged from 0.6-2.5 mg/g tissue; there was no apparent relationship between yield and age of the animal. Determinations of the phosphorus content indicated that 20-60% of the total wt was phospholipid. After correction for phospholipid, the yields ranged from 0.39-0.82 mg/g. Since the total lipid isolated from rat testis is 22 mg/g (4), the above amounts reflect 14-37 μg acid-soluble lipid/mg of total lipid. Subsequent thin layer analyses of the acidic lipid confirmed the presence of nonpolar lipid, as well as phospholipid; the wts have not been adjusted for neutral lipid. The recovery of ^{3}H-prostaglandin in the acid lipid fraction was 84% ± 10 (mean ± standard deviation, average of 9 extractions). All the radioactivity could be accounted for in the experiments—7-11% remained in the ethanol, 5-12% was found in the petroleum ether wash, 2-10% in the water washes, and ca. 2% adherent to glassware.

Analyses of Acidic Lipid Fraction

The acidic lipid fraction subjected to alkaline treatment had a characteristic UV absorption spectrum with a maximum at 237 mμ and a shoulder at 278 mμ. Absorption at 278 mμ is characteristic of the dienone formed after alkali treatment of PGEs. Prior to treatment with KOH, some absorption was seen in this region, suggesting the presence of PGB compounds.

The peak at 237 mμ was not observed until after treatment with alkali and may represent dihydro-PGE_1, a product of the metabolism of PGE_1 (18).

TLC analyses of prostaglandins of the acid-soluble lipid preparations were performed. In solvent system A I, areas were visualized with R_f values corresponding to prostaglandin classes B, E, and F. Phospholipids were present at the origin and neutral lipid at the front.

Areas in the sample corresponding to reference prostaglandins were eluted and rechromatographed in solvent system M II on a $AgNO_3$ plate. The F area from the A I plate showed a single zone which corresponded to $PGF_{2\alpha}$, the area from the E zone had two spots with R_f values identical to PGE_1 and PGE_2, and the B zone a spot with an R_f value of 0.86.

A portion of the total acidic lipid isolated from the testis was methylated and the preparation chromatographed on both plain and $AgNO_3$ plates. Areas visualized included zones corresponding in R_f to prostaglandin compounds of the B, E, and F series, as well as spots of other as yet unidentified compounds. Zones corresponding to those of PGE and PGF were scraped, eluted, and rechromatographed. The rechromatographed E zone had compounds with the same mobility as PGE_1 and PGE_2 and an unknown with an R_f value of 0.53; the F zone had materials corresponding to $PGF_{1\alpha}$, $PGF_{2\alpha}$, and 3 unknowns that were more polar.

To determine whether the PGB compounds represented a mixture or a single species, an aliquot of the preparation of acid-soluble lipid was treated with alkali, extracted into ethyl acetate, and rechromatographed in the M III system (15). Zones were visualized that had R_f values of 0.85 and 0.71, identical to those of the PGB_1 and PGB_2 standards prepared by alkali treatment of PGE_1 and PGE_2.

Silicic Acid Column Chromatography of Testicular Acidic Lipid

Chromatography on small columns of silicic acid was effective in removing most of the neutral lipid and phospholipid from the acidic lipid. The concentrated eluate of fraction I developed in the M II system had neutral lipid material at the front and a spot with an R_f value of 0.92, corresponding to that of the PGB standard. After elution and rechromatography of this spot in an M III solvent system, a compound was visualized with a migration identical to that of PGB_2. To determine the neutral lipid present, fraction I was chromatographed on a silica gel thin layer plate along with reference nonpolar lipids, using the solvent mixture, petroleum ether:ethyl ether (90:10, v/v). Fraction I contained lipids that had R_fs identical to those of cholesterol ester, triacylglycerol, diacylglycerol, and fatty acids. Fraction II, chromatographed on a plain plate in the M II solvent system, had material at R_fs of 0.92, 0.75, and 0.58. On the same plate, reference standards of PGB, PGE, and PGF had R_fs of 0.92, 0.75, and 0.58, respectively. Fraction II also had unknown components at R_fs of 0.69, 0.64, 0.54, and several more small polar spots. The latter, on the basis of reaction with Zinzadze, ninhydrin, and Dragendorf sprays, were identified as phospholipids. Fraction III contained a large amount of phosphatidyl ethanolamine and smaller amounts of other phosphatides.

Analyses of Silicic Acid-Separated Testicular Lipid

After silicic acid chromatography, the ethyl acetate fractions had an average wt of 50-60 μg/g tissue. The recovery of ^3H marker prostaglandins from the columns was 90 ± 2% (n=6). Ca. 15% radioactivity was in fraction I, 70-80% in fraction II, and 2-6% in fraction III. Samples of fraction I chromatographed in the A I solvent system routinely showed a radioactive spot that had the same R_f and PGB, suggesting that PGBs were present in this fraction and that the ^3H-PGE_1 might have become degraded. Standard ^3H-$PGF_{2\alpha}$ and ^3H-PGE_1 checked in the A I system for radiopurity confirmed that the ^3H-PGE_1 marker had degraded and that ca. 33% counts were eluted in fraction I. PGEs are known to be labile, particularly during silicic acid column chromatography. A characteristic separation of a sample of fraction II in the M I system showed compounds with migrations identical to those of standard PGF, PGE, PGB, and cholesterol. An aliquot of the sample applied to the plate for counting was scraped and counted. Radioactivity was associated with material in the sample in areas coincident with the R_f values of PGE, PGF, and PGB. In fraction I, 86% cpm applied to the plate was in the B zone, and, in fraction II, 85% cpm was in the E and F zones.

Partition of Testis Acidic Lipid into Phosphate Buffer

Because PGEs were degraded and neutral lipid was difficult to remove on silicic acid columns, repetitive partition of the testicular acid-soluble lipid into phosphate buffer and ethyl acetate was tested as a procedure for cleaning the acid-soluble lipid. When aliquots of ^3H marker PGE_1 and $PGF_{2\alpha}$ were subjected to the separation, 93% radioactivity was partitioned from ethyl acetate into the buffer. During the extraction of testis lipid, washing the buffer with ethyl acetate prior to acidifi-

TABLE I

Gas Liquid Chromatography of Prostaglandin Standards[a]

Prostaglandin[b]	Derivative	Relative retention time[c]	C Value
PGE_1	MO-TMSi-Me[d]	1.80[f], 1.51	24.6[f], 23.9
PGE_2	MO-TMSi-Me[d]	1.66[f], 1.38	24.6[f], 23.9
PGB_1	TMSi-Me[e]	1.32	23.9
PGB_2	TMSi-Me[e]	1.30	23.9
$PGF_{1\alpha}$	TMSi-Me[e]	1.66	24.6
$PGF_{2\alpha}$	TMSi-Me[e]	1.47	24.2

[a]Chromatography was performed on a 3% OV-1 column.

[b]PGE_1 = prostaglandin E_1, 11α,15(S)-dehydroxy-9-oxo-β-*trans*-prostenoic acid; PGE_2 = prostaglandin E_2, 11α,15(S)-dehydroxy-9-oxo-5-*cis*-13-*trans*-prostadienoic acid; $PGF_{1\alpha}$ = prostaglandin $F_{1\alpha}$, 9α,11α,15(S)-trihydroxy-13-*trans*-prostenoic acid; $PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$, 9α,11α,15(S)-trihydroxy-5-*cis*-13-*trans*-prostadienoic acid; PGB_1 = prostaglandin B_1 15(S)-hydroxy-9-oxo-8(12),13-*trans*-prostadienoic acid; and PGB_2 = prostaglandin B_2, 15(S)-hydroxy-9-oxo-5-*cis*,8(12),13-*trans*-prostatrienoic acid.

[c]Relative retention time is the ratio of the retention time of the peak relative to that of tricosanoate.

[d]MO-TMSi-Me indicates a trimethylsilyl ether, trimethylsilyl ester, 9 keto methyloxime derivative.

[e]TMSi-Me indicates a trimethylsilyl ether, trimethylsilyl ester derivative.

[f]Indicates the major peak.

cation removed a large amount of neutral lipid and phospholipid; ca. 17% ^3H was removed after washing 3 times with ethyl acetate. After acidification of the buffer and partition into ethyl acetate, the final recovery of ^3H was 78%, and the overall recovery beginning with homogenization was 61-64%. Purified acid-soluble lipid recovered by this procedure varied from 4-20 μg/g tissue. TLC of material isolated from both immature and adult testes indicated the presence of material corresponding to PGF, PGE, and PGB; a small amount of phospholipid was observed at the origin and cholesterol at the front. Most of the radioactivity in the preparations (90%) was associated with compounds in the testis samples that had the same mobilities as PGE and PGF.

Separation of Purified Testis Acidic Lipid into Prostaglandin Classes

The prostaglandins of the testicular lipid preparations were separated into classes by chromatography on columns of lipophilic Sephadex or on thin layer plates prior to GLC.

Partition on Sephadex LH-20

A LH-20 column separation of standards was performed using methyl esters of 20 μg retinoate, 22 μg PGB_2, 16.8 μg PGE_2, and 20 μg $PGF_{2\alpha}$ and 122 μg phosphatidyl ethanolamine. On the basis of UV absorption of the eluants, 81.5% of PGE_2 and 87% of PGB_2 were recovered. The PGFs were monitored qualitatively by TLC. In a quantitative separation of a mixture of standard prostaglandins (9.4 μg PGB_1, 6.6 μg PGE_2, 4.4 μg $PGF_{2\alpha}$, 10 μliter aliquots ^3H-PGE_1, and ^3H-$PGF_{2\alpha}$, 36,400 cpm), 78% ^3H was recovered from the silicic acid column and 82% from LH-20. The overall recovery was 64%, 38% for the E zone eluant and 34% for the F zone eluant. Radioactivity also was present in the B zone and pre-B zone eluants. GLC of the eluants and quantification of the peaks indicated that the recovery of PGBs was less than 10%. Of the PGE_2 applied to the column, 74% was recovered and, of the $PGF_{2\alpha}$, only 23%.

Methylated testicular total acid-soluble lipid did not partition successfully on the Sephadex column; therefore, preparations recovered from silicic acid columns were used. The recovery of ^3H-PGE_1 and ^3H-$PGF_{2\alpha}$ from purified testis lipid preparations applied to such columns was 84% ± 7.5 (n=7). In a typical experiment, silicic acid purified (70% recovery) lipid from 44 g adult testis was separated on LH-20. The radioactivity of the eluants was as follows: 5.8% in the pre-PGB, 10.5% in the PGB, 24% in the PGE, and 59% in the PGF eluant. Aliquots chromatographed on a thin layer plate showed that the radioactivity of the PGE and the PGF eluants coincided with the mobility of the appropriate standards and that materials corresponding to PGF, PGE, and PGB were visualized.

Partition on Thin Layer Plates

Lipid preparations after silicic acid chroma-

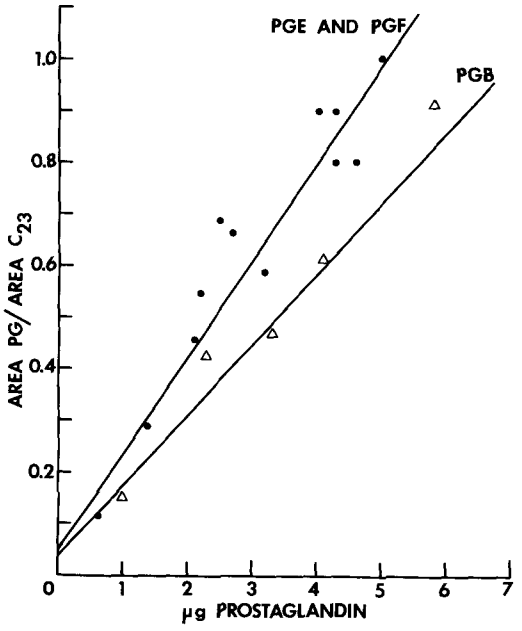

FIG. 1. Relationship between the areas of varying amounts of prostaglandin reference standards resolved by gas liquid chromatography and the area of a constant amount of the internal reference, tricosanoate. The ratios are shown on the ordinate and the amount of prostaglandin on the abscissa. The upper line represents the response to prostaglandin E and prostaglandin F and the lower to prostaglandin B.

tography and after phosphate repartition were chromatographed on thin layer plates in the A I or M I solvent systems for group separation, prior to elution and GLC. Standard reference prostaglandins chromatographed on the same plates were used to determine recoveries. The total recovery of ^3H from thin layer plates ranged from 91-92%; the specific recoveries of ^3H-PGE_1 and ^3H-$PGF_{2\alpha}$ on the basis of radioactivity were 72-95%. Estimation of recoveries of eluted standards after GLC gave the following range of values: PGE_1, 78-95%; PGE_2, 70-75%; $PGF_{1\alpha}$, 85-93%; $PGF_{2\alpha}$, 75-95%; and PGB, 44%.

GLC Analysis of Testis Prostaglandin Preparations

GLC of standard reference prostaglandins (0.2-5.0 µg) gave peaks that had reproducible retention times relative to the tricosanoate internal standard. These values and the carbon numbers are shown in Table I. Methyloxime derivatives of the PGEs separate into syn- and anti-isomers on GLC (19), and two peaks were observed for both PGE_1 and PGE_2. The ratios between the area of varying amounts of standard reference prostaglandins and that of the internal standard, tricosanoate, were determined. The slope and y intercept of the regression lines shown in Figure 1 were determined using these values and a computer program. The ratios between the areas of PGE_1, PGE_2, $PGF_{1\alpha}$, and $PGF_{2\alpha}$ and the internal standard fit the same line, but the response to the PGBs is significantly different.

Total acid-soluble lipid extracted from testis was not a satisfactory preparation for GLC, since it contained large amounts of tissue lipids. Testis lipid, after purification on a silicic acid column, was characteristically resolved into two major peaks with C values of 24.2 and 24.8. These preparations also showed peaks that had long relative retention times, 2.5-5.0. One of these peaks had the same retention time as cholesterol; the others may represent testicular steroids and other nonpolar lipids. Chromatography of testicular lipid purified by partition from phosphate showed two peaks having C values of 24.2 and 23.9 and little contamination by compounds having retention times from 2.5-5.0. The purified testis lipid preparations have peaks that have C values similar to those of prostaglandin standards, but, since there is overlap between these values, it is difficult to identify individual prostaglandins.

After class separation, resolution was improved. The peaks resolved from the E zone eluant of adult testis from a LH-20 column had C values of 24.8, 24.6, 24.3, and 23.9, the same C values as the primary and the secondary peaks of PGE_1 and PGE_2. The major component had a C value of 24.6, the primary peak of PGE_2. The F zone of a sample of immature testis lipid eluted from a thin layer plate had a major peak with a carbon number of 24.2, as well as a small peak, C value 23.7. The major peak has the same retention time as $PGF_{2\alpha}$, and there is no indication of the occurrence of $PGF_{1\alpha}$.

Prostaglandins do not appear to be stored in tissue in significant amounts, but they apparently can be synthesized and released during the processing of tissue. In the present work, care was taken to remove the testes quickly and to homogenize the tissue in ethanol immediately. The amounts of prostaglandins after this procedure would then tend to be minimal.

To determine whether more of these compounds could be isolated under conditions that would tend to promote biosynthesis and release, a pool of frozen adult testes was extracted (85 g). Since the tissue had to be thawed, the *tunica* removed, and the tissue weighed, an interval of time elapsed prior to ethanol homogenization. The tissue was extracted using the phosphate purification proce-

TABLE II

Recoveries from Preparative Plates[a]

Fraction	Applied, cpm	Recovered		
		Radioactivity		Gas chromatography, percent
		cpm	Percent	
Standard	7336	5850	79.5	
F zone	4104	3600	87.7	$F_{1\alpha}$, 92.5 $F_{2\alpha}$, 74.8
E zone	3202	1500	69.6	E_1, 56.6 E_2, 58.5
above E zone		750		E_1, 6.1 E_2, 6.3
B zone				B_1, 43.0
Sample	7606	7550	99.3	
F zone		5250		
E zone		1700		
above E zone		600		

[a]Acid-soluble lipids from adult testes and prostaglandin standards were chromatographed in the M I system. Zones were eluted and aliquots counted to determine the recovery of radioactivity. Other aliquots were subjected to gas liquid chromatography and the recoveries estimated as described in the "Experimental Procedures" section.

dure and the isolated lipid subjected to group separation on a preparative thin layer plate. Zones of both the sample and standards corresponding to prostaglandins were eluted and derivatives prepared for gas chromatography.

Recovery of radioactivity from the sample and standards after elution, and the quantification of the eluted standards after GLC are shown in Table II. Two major peaks having carbon numbers of 24.2 and 23.7 were observed in the eluted F zone of the testes samples. The former corresponds to that of the reference $PGF_{2\alpha}$, and, thus, this peak tentatively has been identified as $PGF_{2\alpha}$. The unknown compound with a C value of 23.7 may represent the spot with an R_f value more polar than $PGF_{2\alpha}$, which was visualized in the F zone of the sample and may be a metabolite. The eluted E zone of the sample had a major peak with an equivalent chain length of 24.3, a shoulder at 23.9, and a small peak at 24.8. Identification on the basis of the C values of the reference prostaglandins indicates that these peaks probably correspond to primary and secondary peaks of PGE_1 and PGE_2. Material from tissue was visualized in the zone above the E zone, R_f value of 0.67, and also was observed as a secondary spot of the PGE_1 standard and is believed to be a degradation product. GLC of this area from the tissue sample shows peaks with C values similar to those of the secondary peaks of PGE_1 and PGE_2. Similar peaks were seen in the material from the corresponding area of the standard (Table II). It appears that the compounds migrating in the E zone and the zone above it may represent a mixture of PGE_1, PGE_2, and unknown compounds, perhaps metabolically modified prostaglandins.

The amounts of prostaglandins that can be isolated from testicular tissue appear to be quite small. For this reason, resolution and quantification of these compounds using GLC with a flame ionization detector are difficult. When relatively large pools of tissue are processed, prostaglandin peaks which appear to be PGE_1, PGE_2, $PGF_{2\alpha}$, and PGB are resolved. Quantitative studies of the $PGF_{2\alpha}$ peak of rat testis (Table III) indicate more $PGF_{2\alpha}$ in immature than in adult testis and that the amount found in the frozen-thawed adult pool was four times higher than that obtained by direct ethanol extraction of fresh tissue. Studies currently in progress in the laboratory using radioimmunoassay to quantify testicular prostaglandins corroborate the relative amounts of $PGF_{2\alpha}$ reported in Table III; for example, radioimmunoassay of the adult, frozen preparation, indicates 120-140 ng/g tissue (Carpenter, unpublished results).

DISCUSSION

Evidence outlined in this article supports the

TABLE III

Prostaglandin $F_{2\alpha}$ of Rat Testis[a]

Tissue	$PGF_{2\alpha}$
	μg/g
Adult	0.03
Adult	0.04
Adult, frozen	0.16
Immature	0.16
Immature	0.25
Immature	0.20

[a]The amount of $PGF_{2\alpha}$ was estimated by gas liquid chromatography of the F zones of preparations of acid soluble lipid from pools of adult and immature testes.

occurrence of prostaglandins in rat testis. The most expedient procedure for preparation of fractions of testicular acid-soluble lipid was repetitive partition followed by group separation by TLC. Chromatography on silicic acid columns resulted in considerable degradation of the PGEs; and, during subsequent chromatography on lipophilic Sephadex, degradation compounds were distributed in several fractions, preventing quantitative recovery of the ^{3}H marker prostaglandins.

The evidence for the isolation of prostaglandins from testicular tissue includes the observation that the separated compounds partitioned along with the ^{3}H marker prostaglandins and that their behavior on TLC and GLC appeared to be identical to that of standard reference prostaglandins. These observations have led to the tentative identification of PGE_1, PGE_2, and $PGF_{2\alpha}$ from testis. A spot with the same R_f value as $PGF_{1\alpha}$ was seen in thin layer systems, but no $PGF_{1\alpha}$ was found after GLC. There also are indications that other prostaglandins, perhaps metabolites, are present. Thus, spectral studies suggest that dihydro-PGE_1 is found in testis and analyses of a frozen-thawed testis pool showed the presence of compounds in the prostaglandin fraction whose properties in TLC and GLC were different from those found in fresh, ethanol homogenized tissue. Preparations of pig testes have been reported to contain 15 hydroxy dehydrogenase and prostaglandin-Δ13-reductase (20) and rat testes homogenates incubated with PGE_1 to form the 15 keto derivative (21). A recent publication (22) reports that swine testis contains PGE_1, PGE_2, $PGF_{1\alpha}$, and $PGF_{2\alpha}$.

The presence of prostaglandins and apparent prostaglandin metabolites in testis preparations suggests that probably prostaglandins are synthesized and metabolized in this organ. Apparently, small amounts of PGE_1 can be formed nonenzymically from Δ8,11,14 eicosatrienoic acid (23). The specificity of the type of prostaglandin found in testis makes nonenzymic conversion unlikely. An alternative is that the prostaglandins found in testis might be synthesized in another tissue and transported to the testis; however, there is no evidence to support this possibility. Furthermore, prostaglandins appear to be synthesized and released at their site of action. Eliasson (3) observed that incubation of homogenates of ram seminal vesicle in buffer led to increased quantities of prostaglandin, and Jouvenaz, et al., (24) found little prostaglandin in rat organs if they were homogenized in ethanol, whereas there were larger amounts if the tissues were homogenized in aqueous saline. In the work reported here, the tissue usually was homogenized in ethanol immediately after removal. The quantities observed were larger when the testes were frozen and thawed before homogenization.

The concept that prostaglandins are synthesized in testis is supported by isolation of radioactive prostaglandins from testis after the intratesticular injection of precursor fatty acid (25). 1-^{14}C Δ9,12-Linoleic acid injected into testis is readily elongated and desaturated, and ^{14}C labeled PGE_1, PGE_2, and $PGF_{2\alpha}$ have been identified by TLC of the tissue extract.

Testis has an unusually large concentration of polyenoic fatty acids that are potentially available for prostaglandin biosynthesis. The major fatty acyl groups of testis are arachidonate and docosapentaenoate. These fatty acids are present, not only in phosphatides, but also in triacylglycerol. In the phospholipid fraction of the adult rat testis, $C_{20:4}$ and $C_{22:5}$ are each 16% of the total fatty acyl groups and in the triacylglycerol fraction, $C_{20:4}$ is 4%, and $C_{22:5}$ is 20%. Studies of the distribution of phosphatides and of the fatty acid composition of each phosphatide show that potential prostaglandin precursor fatty acids are highest in the phosphatidyl ethanolamine fraction (4). In addition to a high concentration of polyenoic fatty acids, testicular tissue has a remarkable conservation mechanism for arachidonic acid which includes an active retroconversion of $C_{22:5}$ to $C_{20:4}$ (26). Both the triacylglycerol and phospholipid fractions have the potential to serve as substrates for prostaglandin synthesis.

Prostaglandin species that occur in tissue have been postulated to reflect the fatty acid composition of the tissue. The renal medulla, rich in arachidonic acid, yields primarily PGE_2 (27), and the seminal vesicle with a high content of eicosatrieonic acid produced PGE_1 (28,29). Testicular tissue has a high content of arachidonic acid, the biosynthetic precursor of PGE_2 and $PGF_{2\alpha}$, and $PGF_{2\alpha}$ and PGE_2 appear to be the predominant species of testis. PGE_1 also is found but no firm evidence was obtained for the occurrence of $PGF_{1\alpha}$. The precursor fatty acid of these prostaglandins, eicosatrienoic acid, also occurs in testis, but in much smaller amounts than arachidonate.

Quantitative determinations of the amounts of prostaglandins in tissue are extremely difficult, because the amounts found are small and the compounds are readily metabolized by and released from the tissues. Very few data are available in regard to quantification of these compounds from tissue. The amount of PGE_1 reported for brain is 0.3 μg/g (30), from epi-

didymal fat pad 12.5-106 ng/g (13) and 24-29 ng/g tissue (31) and of PGEs from a variety of tissues of the rat, 10-480 ng/g (24). The amount of $PGF_{2\alpha}$ reported for rat uterine tissue during stages of the estrous cycle is 85-185 ng/g (32) and from various other tissues of the rat from 10-280 ng/g (33). The amounts of $PGF_{2\alpha}$ observed in testicular tissue appear to fall within the ranges reported for other tissues.

In the present study, PGBs were found associated with the nonpolar lipid fraction. GLC of purified neutral lipid and phospholipid isolated from testis gave no evidence for the presence of prostaglandins. This would seem to confirm previous observations that prostaglandins are not associated with intact phosphatides (28,29).

The biological significance of the presence of prostaglandins in mammalian testis can, at the present, only be conjectured. In this context, it may be relevant that the polyenoic fatty acid compositions of ovarian tissue, Graafian follicle (34) and corpus luteum (35) are similar to those of testis. This suggests a possible functional role in gametogenesis for linoleate derived fatty acids, which are necessary for prostaglandin production. Prostaglandins administered in vivo have a luteolytic effect (36) and in vitro stimulate steroidogenesis (37). The prostaglandin effect upon steroid synthesis in ovarian tissue appears to be similar to that of pituitary gonadotropins and to be mediated via adenyl cyclase. Testicular adenyl cyclase also has been shown to be stimulated by pituitary gonadotropins (38). That prostaglandins could function in the regulation of cell differentiation is supported by the observations that in cultures of chick embryo skin, they stimulate epidermal proliferation (39) and in cultures of hamster ovary cells promote normal fibroblast development (40). Elucidation of the role of prostaglandins in mammalian testis requires further study.

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Bile Acid Metabolism in Mammals: VI. Effect of Ethionine upon Bile Acids of Rat Bile

I.M. YOUSEF[1], G. KAKIS, and M.M. FISHER, Departments of
Medicine and Pathology, University of Toronto, Toronto, Ontario, Canada M5S 1A8.

ABSTRACT

Sex differences in the effect of ethionine upon rat liver metabolism prompted our investigation into possible sex differences in the effect of ethionine upon bile acid metabolism. The bile ducts of 24 rats, 12 male and 12 female, were cannulated. After 1 hr of bile collection, 6 rats of each sex were given ethionine, 1 mg/g body wt, by feeding tube. The bile acid composition of the bile collected during the subsequent 4 hr was analyzed by combined thin layer and gas chromatography. Ethionine induced a reduction in bile flow (3rd and 4th hr) and in bile acid concentration (4th hr) in female rats. The amino acid had no effect upon bile flow but did increase biliary secretion of bile acids (1st and 2nd hr) in male rats. Cholic acid accounted for the bulk of the reduction in total bile acid secretion in the female studies. The increase in total bile acid secretion in the male studies involved all bile acids. The effects of ethionine upon bile acid secretion were delayed in the female studies, immediate in the male. The changes in bile acid secretion involved only the taurine conjugates in the female studies, both taurine and glycine conjugates in the male. There are substantial sex differences in the effect of ethionine upon bile acid metabolism in the rat.

INTRODUCTION

The acute administration of large doses of ethionine to female rats produces a dramatic response pattern in the liver. An early, profound decrease in hepatic adenosine 5'-triphosphate (ATP) occurs, and this is followed by a reduction in ribonucleic acid (RNA) synthesis, a reduction in protein synthesis associated with the disaggregation of liver polyribosomes, and the accumulation of triglyceride in the liver (1-4). The acute administration of similarly large doses of ethionine to male rats produces disturbances in liver metabolism which are much less marked than those found in female rats (5,6). Songster, et al., (7) documented an influence of ethionine upon bile acid metabolism in male rats. The interest of our laboratory in the sex differences in bile acid metabolism (8) led us to explore the possible existence of a sex difference in the effect of ethionine upon bile acid metabolism in the rat.

MATERIALS AND METHODS

Wistar rats (High Oak Ranch, Toronto, Canada) weighing 225 ± 2.3 g were used. The animals were maintained on a commercial diet (Rockland Mouse and Rat Diet) and fasted overnight before use. The bile ducts of 24 such animals, 12 male and 12 female, were cannulated under ether anesthesia, and the animals then allowed to recover in individual metabolism cages. After 1 hr of bile collection, 6 rats of each sex were given ethionine, 1 mg/g body wt in physiological saline, and the other 6 rats of each sex were given appropriate volumes of saline alone. The solutions were administered by feeding tube. Bile was collected in the dark in tubes chilled in ice, and in hourly aliquots, for a further 4 hr. The bile acids of the bile were identified and analyzed quantitatively by the combined system of thin layer and gas chromatography (TLC-GLC), described previously by this laboratory (8). The column used in the gas chromatograph was 3% OV 210 on 80-100 mesh Chromosorb W (Chromatographic Specialties, Brockville, Canada), and 122 cm in length, inside diameter 3.18 mm.

RESULTS

The volumes of bile secreted by the animals in this study are presented in Table I. There was no significant sex difference in the volume of bile secreted in the control animals. Female rats secreted significantly less bile during the 3rd and 4th hr after the administration of ethionine but the amino acid had no significant effect upon the volume of bile secreted by male rats.

The biliary secretion of bile acids on an hourly basis is presented in Table II. Although female rats secreted more bile acids than did male rats during the 1st hr of base-line collection, the difference was not statistically significant. Female rats treated with ethionine se-

[1]I.M. Yousef is a Scholar of the Canadian Hepatic Foundation.

TABLE I

Bile Volume

	Bile produced during hourly period[a] ml ($\bar{x}$ ± standard error)				
	0 - 1	1 - 2	2 - 3	3 - 4	4 - 5
Controls					
Female[b]	0.61 ± 0.02	0.79 ± 0.07	0.86 ± 0.05	0.90 ± 0.03	0.81 ± 0.05
Male[b]	0.73 ± 0.05	0.86 ± 0.06	0.91 ± 0.05	0.76 ± 0.06	0.70 ± 0.04
Ethionine					
Female[b]	0.58 ± 0.04	0.89 ± 0.09	0.81 ± 0.07	0.53 ± 0.07	0.46 ± 0.06
Male[b]	0.87 ± 0.04	1.10 ± 0.10	1.00 ± 0.10	0.89 ± 0.09	0.79 ± 0.09
P Values[c]					
Control F vs control M	NS	NS	NS	NS	NS
Control F vs ethionine F	NS	NS	NS	<0.002	<0.002
Control M vs ethionine M	NS	NS	NS	NS	NS
Ethionine F vs ethionine M	<0.001	NS	NS	<0.02	<0.02

[a]A biliary fistula was created at time 0. After 1 hr of bile collection, ethionine (1 mg/g body wt) was administered in physiological saline by feeding tube. Control animals received appropriate volumes of saline.

[b]n = 6.

[c]Significant differences were determined by t-test. No significant difference (NS) = P>0.05.

creted significantly less bile acids than did control female rats during the 3rd and 4th hr following administration of the amino acid. Male rats, on the other hand, responded with a marked increase in bile acid secretion during the first 3 hr following the administration of ethionine, and their total biliary secretion of bile acids during the 4 hr following administration of the amino acid was more than twice that of the controls.

The biliary secretion of bile acids in terms of mass/unit volume is recorded in Table III.

TABLE II

Bile Acid Secretion

	Total bile acids secreted during hourly period[a] μmoles ($\bar{x}$ ± standard error)				
	0 - 1	1 - 2	2 - 3	3 - 4	4 - 5
Controls					
Female[b]	6.50 ± 0.28	5.90 ± 0.58	4.26 ± 0.30	3.61 ± 0.08	4.63 ± 0.26
Male[b]	4.02 ± 1.79	7.21 ± 0.61	5.05 ± 0.53	3.84 ± 0.37	3.88 ± 0.36
Ethionine					
Female[b]	6.68 ± 0.45	5.85 ± 0.70	3.50 ± 0.21	1.70 ± 0.18	1.73 ± 0.21
Male[b]	4.54 ± 1.61	15.16 ± 1.62	20.37 ± 2.49	5.73 ± 0.95	2.36 ± 0.55
P Values[c]					
Control F vs control M	NS	NS	NS	NS	NS
Control F vs ethionine F	NS	NS	NS	<0.001	<0.001
Control M vs ethionine M	NS	<0.002	<0.001	NS	NS
Ethionine F vs ethionine M	NS	<0.001	<0.001	<0.01	NS

[a]A biliary fistula was created at time 0. After 1 hr of bile collection, ethionine (1 mg/g body wt) was administered in physiological saline by feeding tube. Control animals received appropriate volumes of saline.

[b]n = 6.

[c]Significant differences were determined by t-test. No significant difference (NS) = P>0.05.

TABLE III

Biliary Secretion of Bile Acids

	Bile acids secreted during hourly period[a] μmoles/ml ($\bar{x}$ ± standard error)				
	0 - 1	1 - 2	2 - 3	3 - 4	4 - 5
Controls					
Female[b]	10.66 ± 0.48	7.47 ± 0.71	4.95 ± 0.34	4.01 ± 0.08	5.72 ± 0.33
Male[b]	5.58 ± 2.48	8.38 ± 0.17	5.55 ± 0.19	5.05 ± 0.13	5.54 ± 0.14
Ethionine					
Female[b]	11.52 ± 0.76	6.57 ± 0.80	4.32 ± 0.27	3.21 ± 0.37	3.76 ± 0.32
Male[b]	5.22 ± 1.86	13.78 ± 1.47	20.37 ± 2.50	6.44 ± 1.07	2.99 ± 0.71
P Values[c]					
Control F vs control M	NS	NS	NS	<0.002	NS
Control F vs ethionine F	NS	NS	NS	NS	<0.01
Control M vs ethionine M	NS	<0.01	<0.001	NS	<0.01
Ethionine F vs ethionine M	<0.02	<0.01	<0.001	<0.05	NS

[a]A biliary fistula was created at time 0. After 1 hr of bile collection, ethionine (1 mg/g body wt) was administered in physiological saline by feeding tube. Control animals received appropriate volumes of saline.

[b]n = 6.

[c]Significant differences were determined by t-test. No significant difference (NS) = P>0.05.

Ethionine induced no significant change in the biliary concentration of bile acids in the female animals, apart from a decrease during the 4th hr following its administration. On the other hand, ethionine induced a significant increase in the concentration of biliary bile acids in male rats during the first 2 hr following its administration. It is of interest that no significant choleresis was associated with this increased secretion of bile acids.

The biliary output of individual bile acids in the 4 hr following administration of ethionine is presented in Table IV. In female rats, the decrease in the total bile acid secretion following treatment with ethionine was accounted for by the decrease in cholic acid secretion. In fact, the biliary secretion of chenodeoxycholic acid and deoxycholic acid by these animals actually increased. In male animals, the increase in total bile acid secretion following treatment with ethionine involved most of the bile acids. Although cholic acid accounted for more than 50% of the total increase, the percent increase of cholic, β-muricholic, deoxycholic, chenodeoxycholic, and 3β,12α-dihydroxycholanoic acids was about the same.

The hourly secretion of individual bile acids by the female rats is presented in Table V and by the male animals in Table VI. The secretion of individual bile acids in the female studies was not altered significantly during the 1st hr after ethionine administration. However, in the 2nd hr following the administration of ethionine, there was a dramatic decrease in cholic acid secretion and a significant increase in the secretion of chenodeoxycholic, deoxycholic, and hyodeoxycholic acids. In the 3rd and 4th hr following the administration of ethionine, the secretion of cholic acid continued to be depressed, while that of the dihydroxy bile acids decreased to values lower than those found in the control animals. There was no striking difference from control animals in the biliary secretion of β-muricholic, 3β,12α-dihydroxycholanoic, and lithocholic acids by the ethionine treated female rats. The generalized increase in bile acid secretion by the male rats treated with ethionine took place during the first 3 hr following administration of the amino acid.

In terms of total biliary bile acid secretion, ethionine treatment did not influence the relative degree of glycine and taurine conjugation, Table VII. Although a significant decrease in the biliary secretion of taurine conjugated bile acids occurred in the female studies, the decrease was not sufficient to alter the taurine/glycine ratio. In the male studies, a significant increase occurred in the biliary secretion of both glycine and taurine conjugated bile acids, but the increases were such that the taurine/glycine ratio was not altered.

DISCUSSION

Cholic acid and chenodeoxycholic acid are

TABLE IV

Biliary Secretion of Individual Bile Acids[a]
μmoles/4 hr ($\bar{x}$ ± standard error)

	Cholic acid	β-Muricholic acid	Chenodeoxycholic acid	Deoxycholic acid	Hyodeoxycholic acid	3β,12α dihydroxy-cholanoic acid	Lithocholic acid
Control							
Female[b]	10.14 ± 0.39	0.65 ± 0.03	2.06 ± 0.08	2.40 ± 0.08	2.03 ± 0.08	0.48 ± 0.02	0.52 ± 0.05
Male[b]	7.42 ± 0.85	2.12 ± 0.26	2.86 ± 0.30	3.65 ± 0.33	3.07 ± 0.25	0.21 ± 0.05	0.32 ± 0.05
Ethionine							
Female[b]	3.99 ± 0.19	0.63 ± 0.02	2.74 ± 0.10	2.98 ± 0.10	1.68 ± 0.21	0.42 ± 0.02	0.54 ± 0.02
Male[b]	20.25 ± 0.35	4.91 ± 0.60	6.85 ± 1.10	6.63 ± 0.88	3.92 ± 0.38	0.74 ± 0.08	0.31 ± 0.05
P Values[c]							
Control F vs control M	<0.05	<0.001	<0.05	<0.01	<0.01	<0.001	<0.05
Control F vs ethionine F	<0.001	NS	<0.001	<0.02	NS	NS	NS
Control M vs ethionine M	<0.001	<0.01	<0.01	<0.02	NS	<0.001	NS
Ethionine F vs ethionine M	<0.001	<0.001	<0.001	<0.01	<0.001	<0.01	<0.01

[a]One hr after creation of a biliary fistula, ethionine (1 mg/g body wt) was administered in physiological saline by feeding tube. Control animals received appropriate volumes of saline. Bile was collected for 4 hr following this administration.

[b]n = 6.

[c]Significant differences were determined by t-test. No significant difference (NS) = P>0.05.

TABLE V

Effect of Ethionine upon Hourly Secretion of Individual Bile Acids by Female Rats

	Bile acids, μmoles/hr ($\bar{x}$ ± standard error)						
	Cholic acid	P[a]	β-Muricholic acid	P	Chenodeoxy-cholic acid	P	Deoxycholic acid	P	Hyodeoxy-cholic acid	P	3β,12α-Dihydroxy-cholanoic acid	P	Lithocholic acid	P
1-2 hr[b]														
Control[c]	3.18 ± 0.30	NS	0.36 ± 0.04	NS	0.58 ± 0.08	NS	0.66 ± 0.12	NS	0.52 ± 0.08	NS	0.24 ± 0.02	NS	0.29 ± 0.07	NS
Ethionine[c]	3.18 ± 0.37		0.41 ± 0.05		0.66 ± 0.07		0.63 ± 0.09		0.51 ± 0.08		0.25 ± 0.03		0.32 ± 0.31	
2-3 hr														
Control	3.00 ± 0.22	<0.001	0.02 ± 0.01	<0.05	0.43 ± 0.02	<0.001	0.39 ± 0.04	<0.001	0.27 ± 0.02	<0.01	0.10 ± 0.01	NS	Trace	---
Ethionine	0.07 ± 0.01		0.06 ± 0.01		1.28 ± 0.09		1.46 ± 0.08		0.52 ± 0.05		0.08 ± 0.01		0.09 ± 0.01	
3-4 hr														
Control	1.82 ± 0.04	<0.001	0.13 ± 0.01	<0.001	0.44 ± 0.05	NS	0.57 ± 0.01	NS	0.52 ± 0.02	<0.001	0.08 ± 0.01	<0.01	0.06 ± 0.01	NS
Ethionine	0.34 ± 0.03		0.08 ± 0.01		0.42 ± 0.09		0.43 ± 0.06		0.29 ± 0.04		0.05 ± 0.01		0.10 ± 0.02	
4-5 hr														
Control	2.14 ± 0.14	<0.001	0.14 ± 0.01	<0.001	0.61 ± 0.04	<0.01	0.78 ± 0.04	<0.002	0.73 ± 0.05	<0.001	0.06 ± 0.01	<0.001	0.18 ± 0.02	<0.001
Ethionine	0.39 ± 0.04		0.08 ± 0.01		0.37 ± 0.05		0.46 ± 0.06		0.36 ± 0.04		0.03 ± 0.01		0.04 ± 0.01	

[a]Significant differences between control and ethionine groups for each bile acid for each hr were determined by t-test. No significant difference (NS) = $P > 0.05$.

[b]A biliary fistula was created at time 0. After 1 hr of bile collection, ethionine (1 mg/g body wt) was administered in physiological saline by feeding tube. Control animals received appropriate volumes of saline. Bile was collected in hourly aliquots, and the values in the table represent the bile acid composition for each hr after ethionine administration.

[c]$n = 6$.

TABLE VI

Effect of Ethionine upon Hourly Secretion of Individual Bile Acids by Male Rats

	Bile acids, μmoles/hr ($\bar{x}$ ± standard error)						
	Cholic acid	β-Muricholic acid	Chenodeoxy-cholic acid	Deoxycholic acid	Hyodeoxy-cholic acid	3β,12α-Dihydroxy-cholanoic acid	Lithocholic acid
	P[a]	P	P	P	P	P	P
1-2 hr[b]							
Control[c]	2.28 ± 0.26	0.55 ± 0.12	1.39 ± 0.29	1.47 ± 0.41	1.32 ± 0.26	0.08 ± 0.01	0.15 ± 0.01
	<0.05	<0.01	NS	NS	NS	<0.02	<0.02
Ethionine[c]	7.03 ± 1.76	1.48 ± 0.22	2.17 ± 0.31	2.55 ± 0.72	1.54 ± 0.38	0.27 ± 0.06	0.06 ± 0.03
2-3 hr							
Control	2.09 ± 0.29	0.50 ± 0.10	0.55 ± 0.15	0.78 ± 0.18	0.86 ± 0.13	0.17 ± 0.02	0.08 ± 0.01
	<0.01	<0.01	<0.002	<0.05	<0.05	<0.05	<0.01
Ethionine	9.31 ± 1.58	2.37 ± 0.47	3.24 ± 0.52	3.05 ± 0.83	1.82 ± 0.29	0.33 ± 0.05	0.16 ± 0.04
3-4 hr							
Control	1.38 ± 0.23	0.43 ± 0.05	0.59 ± 0.13	0.74 ± 0.15	0.51 ± 0.15	0.17 ± 0.02	0.02 ± 0.01
	<0.05	NS	NS	NS	NS	NS	NS
Ethionine	2.51 ± 0.43	0.61 ± 0.15	1.10 ± 0.24	0.82 ± 0.14	0.56 ± 0.13	0.08 ± 0.03	0.04 ± 0.03
4-5 hr							
Control	1.51 ± 0.05	0.59 ± 0.12	0.37 ± 0.04	0.72 ± 0.12	0.44 ± 0.10	0.17 ± 0.02	0.09 ± 0.01
	<0.05	NS	NS	<0.05	<0.02	<0.05	NS
Ethionine	0.97 ± 0.18	0.34 ± 0.05	0.47 ± 0.06	0.34 ± 0.06	0.08 ± 0.04	0.07 ± 0.03	0.07 ± 0.02

[a]Significant differences between control and ethionine groups for each bile acid for each hr were determined by t-test. No significant difference (NS) = $P > 0.05$.

[b]A biliary fistula was created at time 0. After 1 hr of bile collection, ethionine (1 mg/g body wt) was administered in physiological saline by feeding tube. Control animals received appropriate volumes of saline. Bile was collected in hourly aliquots, and the values represent the bile acid composition for each hr after ethionine administration.

[c]n = 6.

TABLE VII

Effect of Ethionine upon Conjugation of Biliary Bile Acids

	Female			Male		
	Control[a]	Ethionine[a]	P	Control[a]	Ethionine[a]	P
Taurine conjugated bile acids (μmole)	15.90 ± 1.41	10.37 ± 1.10	<0.02	14.14 ± 1.17	29.65 ± 1.98	<0.001
Glycine conjugated bile acids (μmole)	2.38 ± 0.24	1.94 ± 0.18	NS	5.50 ± 0.47	13.96 ± 1.10	<0.001
Taurine/glycine ratio	6.69 ± 0.54	5.36 ± 0.49	NS	2.57 ± 0.29	2.13 ± 0.39	NS

[a]n = 3; no significant difference (NS) = P>0.05. The values are the means ± standard error of the total conjugated bile acid secretion of the 3 rats in the 4 hr after the administration of saline or ethionine by feeding tube.

quantitatively the most important primary bile acids synthesized from cholesterol by the liver of the rat. Lithocholic acid and muricholic acid also are synthesized from cholesterol, but these are quantitatively much less important (9). In the intestinal tract, these bile acids undergo biotransformations under the influence of the intestinal microflora. These modifications include removal of the 7α-hydroxyl group with the result that deoxycholic acid is produced from cholic acid, and lithocholic acid is produced from chenodeoxycholic acid. All of these bile acids are absorbed to some degree into the portal circulation and returned to the liver where they undergo further changes. Deoxycholic acid undergoes 7α-hydroxylation to cholic acid; lithocholic acid undergoes 7α-hydroxylation to chenodeoxycholic acid; and chenodeoxycholic acid is converted to the muricholic acids following 6β-hydroxylation (10). The bile acids are confined to this enterohepatic circulation, and de novo synthesis of bile acids from cholesterol compensates for the fecal loss of bile acids.

Under normal circumstances, a balance between synthesis and excretion of bile acids is maintained with the synthesis regulated by the concentration of bile acids returning to the liver in the portal blood. Normally the fecal excretion of bile acids is small, and the bile acids in the portal blood effectively suppress the de novo synthesis of bile acids by the liver. Shefer, et al., (11) demonstrated that there is very little de novo synthesis of bile acids during the early phase of bile collection from a male rat with a biliary fistula. The bile acids in the bile secreted in at least the first 12 hr by such a preparation represent the pool of preformed bile acids, plus those produced by suppressed de novo synthesis. However, the newly synthesized bile acids contribute only ca. 5% of the total output during this period. From 12-36 hr, the de novo synthesis of bile acids continues to be suppressed, and, because the preformed pool has been largely depleted, very little bile acid secretion takes place. After ca. 36 hr, the feedback inhibition of bile acid synthesis is released, de novo synthesis increases 10-15-fold, and bile acid secretion increases appropriately. Studies in our laboratory have documented a similar sequence of events in the female rat with a biliary fistula (unpublished observations, I.M. Yousef and M.M. Fisher). Therefore, the experiments reported in this article deal largely with the metabolism of previously synthesized bile acids, and the results are little affected by the de novo synthesis of bile acids.

The decrease in bile volume induced by ethionine in the female rat may be a consequence of the generalized derangement of cellular metabolism in the liver of this animal and particularly with the reduction in available energy, as manifested by reduced hepatic ATP levels (12). However, it is also possible that the reduction in bile flow is more specifically the consequence of the reduction in the secretion of cholic acid, a bile acid of considerable choleretic potential. The explanation for the dramatic decrease in the secretion of cholic acid is not yet available, but defects in the intestinal absorption, hepatic uptake, and hepatic metabolism of the bile acid are all possible. Whatever the site of the biochemical lesion, it is certainly a specific one, the biliary secretion of all other bile acids remaining unchanged or actually increasing.

In male rats with a bile fistula, the administration of ethionine was associated with an early and generalized increase in the biliary secretion of bile acids. Unpublished observations in this laboratory suggest that the bile acid pool in the liver of the rat is less than 5 μmoles. Therefore, the ethionine induced increase in biliary bile acid secretion of more than 20 μmoles must be associated with a stimulation in the flux of bile acids from the intestinal tract to the liver.

It is clear that ethionine induces significant alterations in the metabolism of bile acids in

the rat and that a striking sex difference is involved. This sex difference is qualitatively so disparate in terms of the biliary secretion of bile acids that it seems unlikely to be explicable on the basis of a sex difference in the intestinal absorption and hepatic uptake of ethionine itself. Studies on the distribution of ethionine 4 hr after its gastric administration also support this contention (paper in preparation). We found no sex difference in the total hepatic concentration of ethionine (free plus bound), ca. 10 μmoles/g liver, or in the amount of ethionine secreted in the bile, ca. 6 μmoles. Furthermore, we found no sex differences in the plasma concentration of ethionine, ca. 4.5 μmoles/ml. These studies suggest that our results concerning the biliary secretion of bile acids probably are not associated with any sex difference in the intestinal absorption or hepatic uptake of ethionine. However, the studies in the male animals indicate that there must be a sex difference in the effect of ethionine upon the enterohepatic circulation of bile acids. Whether this effect is related to an increase in substrate availability or to a stimulation of the intestinal absorptive process itself remains to be investigated.

The selective reduction in cholic acid secretion in the female rat and the generalized increase in bile acid secretion independent of bile flow in the male suggest that ethionine may be a useful experimental tool in the study of some of the control mechanisms involved in bile acid metabolism.

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Mass Determination of Lipids with Automated Thermogravimetric Analytical Procedure

J. LEERKAMP and A.N. SIAKOTOS, Department of Pathology, Indiana University School of Medicine, Indianapolis, Indiana 46202

ABSTRACT

An automated thermogravimetric system for the microdetermination of lipids is described. A study of the analytical variability experienced with this method revealed that samples can be assayed with considerable accuracy (±3%) down to 100-200 μg, while samples less than this range exhibit variations of ±7-10%. In the absence of other more sensitive methods, even this experimental error is acceptable. In addition, multiple applications of very dilute samples to achieve a lipid mass with an acceptable coefficient of variation is possible. No significant variations were observed with samples assayed on the same day and on different days, indicating a high degree of control of nonoperator errors. A careful application of the method to different lipid classes revealed no significant weighing losses under the experimental conditions of this study, except for cholesterol; however, application of the method to mixtures of lipids containing significant amounts of cholesterol did not reveal abnormal errors.

INTRODUCTION

Ideally, the quantitation in the analysis of lipids should be possible by a simple mass determination, since lipids, a group of compounds classified by their common solubility in fat solvents (1), are purified readily by Sephadex column partition chromatography (2). Unfortunately, instrument technology has not been available to determine lipids by a simple gravimetric method at the microlevel. In the past, the determination of total wt of lipids and lipid-like materials often has required specific chemical analysis for polar components, such as phosphorus (3), sugars (2), sphingosine (4), or the use of general reactions unique to the hydrocarbon nature of lipids, e.g., oxidation of chromate (5), quantitative densitometry (6), etc. Because of the structural diversity found in lipid classes, these techniques are not applicable as a universal mass analytical system.

Although a procedure has been available for the thermogravimetric mass determination of lipid mixtures by applying discrete columes of dissolved lipids to a microbalance pan, followed by evaporation of the solvent with heat and then cooling until a constant wt was obtained (7), this procedure required considerable operator skill and time to provide reproducible and true values. These drawbacks have inhibited acceptance of this approach to quantitation for lipids and lipid mixtures.

Our approach to this problem has been to employ a commercial thermogravimetric apparatus (TGA) modified to provide highly reproducible results without constant supervision and with neglibible operator error. The same constant reproducibility is obtained under a variety of atmospheric conditions and is independent of the skill of the technician. In comparison with a previously published procedure (7), equivalent experimental coefficients of variation (cv) at the same concentrations/unit volume were obtained, but without the excessive technician time required by the other method (7). Although the cv increased with the decreasing sample size, multiple applications were successful in achieving lower cvs characteristic of larger lipid samples. Reproducibility was independent of sample volumes and was inversely related to sample wt.

MATERIALS AND METHODS

Sphingomyelin, cholesterol sulfate (sodium salt), and L-α-lecithin were purchased from General Biochemical Industries, Chagrin Falls, Ohio; Applied Science Laboratories, State College, Pa., supplied lecithin (plant), stearic acid, and tripalmitin. Bovine cerebrosides, phosphatidyl inositol, and cholesterol were obtained from Supelco, Bellefonte, Pa. The system employed was a Cahn RG 2045 electrobalance

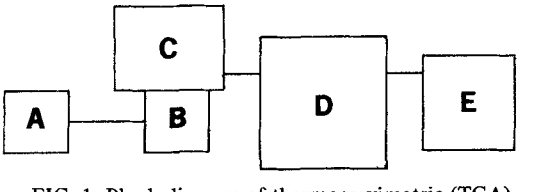

FIG. 1. Block diagram of thermogravimetric (TGA) system. (A) 120 volt variable resistance variac, (B) 2006 Little Gem TGA accessory, (C) 2008 Weighing Assembly, (D) 2054 RG Electrobalance Control Unit, and (E) 1-millivolt recorder.

TABLE I

Analytical Variation of Thermogravimetric Procedure with Sequential Samples Varying in Lipid Concentration but with Constant Volume[a]

Absolute value (μg)	Experimental value (μg)					Mean	Coefficient of variation
	1	2	3	4	5		
27	25	27	31	27	30	28	10.00
43	53	46	49	40	43	46	10.97
99	94	99	90	103	86	94	7.42
212	209	206	213	218	215	212	2.24
324	333	332	328	330	333	332	0.81
730	763	753	747	747	756	753	0.89

[a]Volume (20 μliter) applied to sample pan.

TABLE II

Analytical Variation with Five Multiple Applications of Dilute Sample[a] to Same Sample Pan

Absolute value (μg)	Experimental value (μg)					Mean	Coefficient of variation
	1	2	3	4	5		
50	52	59	60	57	50	56	7.78

[a]0.5 μg/μliter applied in 20 μliter aliquots.

(Ventron Instruments Corp., Paramount, Calif.), with a TGA analysis accessory and a Minneapolis Honeywell Electronik 19 recorder (Fig. 1). The hand-down wire of the TGA accessory was modified by using a wire of three connected sections, rather than the single straight wire, to avoid deformation of the straight wire during pan and sample manipulations.

When the system was used continuously, power to the electrobalance, furnace, and recorder was maintained to avoid a prolonged warm-up stabilization period. The recorder was adjusted to 0% and the recorder span adjusted to 100% with a calibrated external source (1mr). For optimum sensitivity, the mass dial of the RG control unit was set to 10 mg and the recorder range adjusted to 1 mg. The stirrup pan was suspended from the hang-down wire. The hand-down tube was replaced, and the tare wt were added to the counterbalance pan to equal the hang-down wire and stirrup mass. For increments of wt below 1 mg, the mechanical coarse 0 control on torque motor axis was adjusted, moving the recorder pen to near 0. The electrobalance and recorder then were activated, and the recorder pen was adjusted to 0% using the mass dial range and the mass control on the RG control console. Calibration was achieved with a 1 mg wt on the sample pan and setting the recorder scale to 100% with the recorder calibrate control on the RG console. The calibrating wt then was removed and the recorder adjusted to 0% with the RG mass control (0/10). The calibration procedure was repeated until no change was observed. Next, 5-20 μliter sample in chloroform/methanol was added with a 25 μliter[3] syringe (Hamilton Co., Whittier, Calif.). The capacity of the balance pan is 25 μliter. The hang-down tube was replaced carefully, and the heater was elevated to 1 in. above the sample pan and heated for 1 hr with the variac preset for 70-80 C. The balance and recorder system was activated; at least 1 hr was required to reach equilibrium. When the slope of the wt loss reached a minimum (equilibrium), the residual wt was noted. The TGA heater was lowered, the hang-down tube removed, and the sample pan was detached and heated directly in the oxidizing flame tip of a

TABLE III

Analysis of Sequential Determinations Varying in Volume but with Constant Wt[a]/Determination

Volume (μliter)	5	10	15	20
Determination				
1	304	283	295	313
2	298	298	295	314
3	286	296	307	328
4	306	290	306	324
5	312	297	308	308
Mean	301	293	302	317
Coefficient of variation	3.25	2.15	2.19	2.61

[a]Pure sphingomyelin (324 μg).

TABLE IV

Thermogravimetric Apparatus Data for Analysis of within Days and between Days Variations

Days	Replicates					Mean	Coefficient of variation
1	3461	335	336	340	333	338	1.52
2	337	328	323	324	333	329	1.81
3	338	330	320	332	331	330	1.97
4	330	328	334	340	331	333	1.40
5	335	332	328	330	333	335	0.81
Mean	337	331	332	333	332		
Coefficient of variation	1.72	0.90	2.02	2.06	0.33		

bunsen burner. The pan then was replaced, and the recorder pen zeroed with the RG mass dial (0/10), after which a second aliquot was added until 3 replicate aliquots had been assayed. The delivered sample volume should carefully be noted, since this was a major source of error.

RESULTS

With the automated TGA system described (Fig. 1), the analytical variation for five replicates of pure sphingomyelin varying in lipid concentration is shown in Table I. In this experiment the volume of solvent containing the solute was held constant at 20 μliters. The cv among 5 levels of solute varied from 10.00% (27 μg sample) to 0.89% (730 μg sample). Variations experienced with very dilute samples, such as an extract of a lipid separated by thin layer chromatography (TLC), were determined and are expressed in Table II. Five aliquots of a sphingomyelin sample (0.5 μg/μliter) were applied in sequence to the same sample pan and the solvent allowed to evaporate at room temperature between each addition. After the final addition, the hang-down tube was replaced, and the TGA system was started as described for the standard system. A cv of 7.78% was obtained, and this error compares favorably with the cvs of 10.97% for samples in a similar wt range (43 μg) in Table I. The described method was compared with the published method of Rouser, et al., (7) with a pure sphingomyelin sample in our laboratory. The analytical variation observed in our laboratory with the method of Rouser, et al., (7) was 3.40% for 5 sequential replicate determinations, using a Cahn Electrobalance, model G-2. This cv is similar to data obtained by our automated procedure (Tables I, II).

When the solvent was varied by the sample (solute mass) held constant, the results obtained are, nevertheless, highly reproducible (Table III). Cvs varied from 2.15-3.25% with a sample load of 324 μg. These results are within the experimental error shown in Tables I, IV, and V where samples of constant solvent volume were employed.

An analysis of variance to determine the reproducibility of this method from day to day and with replicates obtained within the same day by the same operator are shown in Table IV. The one-way analysis of variance seen in Table V results in an F ratio of 2.2675 which is not significant at the 1% level for differences between days and samples analyzed within the same day. Obviously, this method is highly reproducible and is not subject to fluctuations due to mechanical, electronic, or human vagaries.

Table VI depicts the experimental mean values obtained for a series of lipid classes. In the third column, the percent difference between absolute value and the experimental mean value is given. Essentially, these data are within experimental variation with the possible exception of cholesterol. The coefficient of variation for five replicates of the various lipid classes ranges from 0.81-3.54%.

DISCUSSION

The gravimetric method described by

TABLE V

One Way Analysis of Variance with Days and between Days Variations[a]

Source of variance	DF	SS	MS
Among days	4	241.8125	60.4531
Within days	20	533.2187	26.6609
Total	24	775.0312	

[a]Total mean = 332.28 F = 2.2675 (not significant), S = 5.6267, DF = degrees of freedom, SS = sum of squares, and MS = mean squares.

TABLE VI

Variation in Data Obtained by Thermogravimetric Analysis of Difference Lipid Classes[a]

Lipid Class	Absolute value	Mean μg/20 μliter	Difference	Coefficient[b] of variation
Normal human brain (total lipids)	480	471.8	-1.71%	1.79
Stearic acid	367	367.0	0	3.54
Cholesterol	314	292.6	-6.82%	3.55
Bovine cerebrosides	303.8	294.4	-3.09%	2.18
Tripalmitin	300	285.2	-4.93%	2.53
Cholesteryl sulfate	306	296.8	-3.01%	3.54
Phosphatidyl inositol	212	217.2	+2.45%	2.79
L-α-lecithin	286	287.0	+0.35%	2.64
Plant lecithin	350	349.6	-0.80%	1.45
Sphingomyelin	324	332.0	+2.47%	0.81

[a]Samples applied in 20 μliter 2/1 chloroform:methanol.

[b]Coefficient of variation between replicate sample aliquots.

Rouser, et al., (7) is clearly highly reproducible with a coefficient of variation of 3.40%. However, the procedure is tedious and is successful only with a highly motivated technician. In spite of considerable effort, only 3-4 samples can be processed in an 8 hr day. In addition, the successful application of this method requires the complete attention of the operator, as well as considerable attention to detail. In contrast, the method described in this report is automated and requires the operator's attention only when applying samples. An efficient technician can continue with other tasks simultaneously.

Analytical variation has been shown to be related directly to the mass of lipid applied to the balance; however, this variation is a statistical variation and is not due to the method as shown by the data in Table I. Even the dilute samples can be assayed without increasing the variability of the method over the cv expected for the total mass applied, whether applied in multiple applications to the sample pan (Table II) or in a single application (Table I). That the experimental variation is due only to solute mass and not solvent volume is shown in Table III. With dilute solutions or small lipid samples, a variation of ±10% is acceptable in the absence of a more refined and sensitive instrumental approach.

Another advantage of the method is the lack of operator influence or day-to-day variation (Tables IV, V). No significant differences were observed between aliquots of a sample assayed within the same day or on five different days. Therefore, the method is highly reproducible from day to day.

The TGA analysis of different lipid classes is consistent, and highly reproducible results can be obtained (Table VI). Significant losses are experienced only with relatively volatile lipids, such as cholesterol; however, when these lipids are encountered in mixtures, such as normal human brain extracts, their mixed melting isotherms preclude significant losses.

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In Vivo and In Vitro Biosynthesis of Free Fatty Alcohols in *Escherichia Coli* K-12

WILLIAM F. NACCARATO, Department of Biochemistry, Faculty of Arts and Sciences, and **JOHN R. GILBERTSON** and **ROSE A. GELMAN**, Department of Pharmacology and Physiology, School of Dental Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

ABSTRACT

In vivo studies have indicated that exogenous free fatty acids may serve as precursors of the free fatty alcohols of *Escherichia coli* K-12. Following disruption of the cells, the enzymatic activity capable of catalyzing the reduction of long chain fatty aldehydes to fatty alcohols was localized in the 100,000 x g supernatant fraction. Nicotinamide adenine dinucleotide phosphate, reduced form, was the required cofactor. The product of the reaction was characterized rigorously as 1-hexadecanol when hexadecanal was the substrate. Three independent, but complementary, assay methods were developed to assay the aldehyde reductase activity. By employing these methods, an equivalence between nicotinamide adenine dinucleotide phosphate, reduced form, oxidation and 1-hexadecanol synthesis was established. Two protein fractions catalyzing the reduction of fatty aldehydes to fatty alcohols were detected in the 100,000 x g supernatant fraction following ammonium sulfate fractionation and diethylaminoethyl-cellulose chromatography. Enzymatic activity (70%) applied to the diethylaminoethyl-cellulose column was eluted at a phosphate concentration of 0.115 M. The remaining 30% was eluted at a concentration of 0.23 M. Following sephadex chromatography, it was observed that the enzyme eluting at 0.115 M phosphate had an apparent mol wt of 250,000 Daltons while that eluting at 0.23 M had an apparent mol wt of 62,000 Daltons. The enzymes were similar with respect to substrate specificity, pH optima, ionic strength optima, and stability with respect to thiol inhibitors, suggesting different sized aggregates of similar subunits.

INTRODUCTION

Long chain, nonisoprenoid fatty alcohols have been characterized in bacteria (1-5) and their biosynthesis studied (1, 5-13). However, the biosynthesis of the fatty alcohols in *Escherichia coli* has not been investigated.

This article provides information concerning the partial purification and characterization of an nicotinamide adenine dinucleotide phosphate, reduced form, (NADPH) linked aldehyde reductase from *E. coli* catalyzing the synthesis of long chain fatty alcohols from free fatty aldehydes. Evidence is presented to establish that this enzyme is not the alcohol dehydrogenase previously reported in this microorganism (14).

METHODS

Materials: [1-C^{14}] Hexadecanoyl CoA and [1-C^{14}] hexadecanoic acid were purchased from New England Nuclear, Boston, Mass. [1-C^{14}] Hexadecanal was synthesized from [1-C^{14}] hexadecanoic acid (15) and the specific activity adjusted with unlabeled hexadecanal. Gas liquid chromatography (GLC) analysis of the product purified by thin layer chromatography (TLC) (5) showed the major component was hexadecanal, greater than 95% total, with small amounts of octadecanal. All of the unlabeled aldehyde substrates, except acetaldehyde and butyraldehyde, were synthesized from the corresponding alcohol (16) and purified by TLC. GLC analysis of the unlabeled aldehydes showed that 95-100% total was one chain length. Acetaldehyde and butyraldehyde were generated from ethanol and butanol using yeast alcohol dehydrogenase (17). This reaction was carried out for 10 min at 23 C. At the end of this time period, an aliquot was removed to measure NADH absorption at 340 nm, and then the reaction mixture was adjusted to pH 5. This latter step served to stop the reaction and also prevented the yeast alcohol dehydrogenase from reoxidizing any ethanol or butanol which might be generated by the reductase studied here. After the pH 5 precipitation, the reaction mixture was kept at 4 C, and 0.2 ml aliquots from this solution were used immediately as substrates. [1-C^{14}] Hexadecanol was synthesized by $LiAlH_4$ reduction of [1-C^{14}] hexadecanoic acid. The [1-C^{14}] hexadecanol was purified by TLC. GLC analysis of this compound showed no other contaminants.

The pyridine nucleotides used were purchased from Sigma Chemical Co., St. Louis, Mo. All solvents were the highest grade com-

mercially available and, with the exception of methanol, were redistilled prior to use.

Bacteria: *E. coli* K-12 were grown on trypticase soy broth (TSB), as described in previous publications (5). For experiments measuring incorporation of [1-C^{14}] hexadecanoic acid, the acid was solubilized with albumin (18), sterilized by ultrafiltration, and 5.5 μmoles (1.5 x 10^6 dpm) added to each culture flask immediately before inoculation.

Lipid extraction, purification, and analysis: Lipids were isolated, purified, and analyzed, as described previously (5). In this study, fatty alcohols were quantitated via GLC (19). A linear relationship was observed between the amount of material injected into the gas chromatograph and the detector response over a range of 0.25-1.0 nmoles 1-hexadecanol. Peak areas were measured by triangulation. When 0.25 nmoles hexadecanol were injected, the average peak area observed was 99.2-100.4% individual peak areas obtained in 5 separate injections. The sensitivity of the gas chromatograph was such that 1 nmole hexadecanol corresponded to a full scale deflection on a 1 millivolt recorder.

When stream splitting was carried out on the gas chromatograph, the split ratio of the detector to splitter port was 1:3 as determined with [1-C^{14}] hexadecanol.

Preparation of cell free system: All procedures were carried out between 0-4 C. *E. coli* cells were suspended in 0.075 M potassium phosphate buffer (pH 7.4) containing 5 mM mercaptoethanol; cells:buffer = 1:4 (w/v). The cells were sonicated for fifteen 2 min periods with 3 min cooling intervals and the sonicate centrifuged at 12,000 x g for 10 min to remove unbroken cells and debris. The particulate fraction was sedimented from the 12,000 x g supernatant using a Beckman L2-65B ultracentrifuge at 100,000 x g for 60 min. The protein concentration of the supernatant was 19-20 mg/ml. Throughout this article, the 12,000 x g supernatant will be referred to as the crude sonicate, the 100,000 x g supernatant as supernatant, and the 100,000 x g particulate as particulate.

The supernatant was adjusted to a protein concentration of 12-13 mg/ml with 0.075 M potassium phosphate buffer containing 5 mM mercaptoethanol. Ammonium sulfate was added to give 55% saturation, and, after standing 15 min, the precipitated protein was sedimented at 12,000 x g for 20 min. The pellet was redissolved in 0.01 M potassium phosphate buffer containing 5 mM mercaptoethanol and stored at -20 C. The activity remained stable for a period of 3 months.

Enzyme assays: Three independent methods were developed for assaying the reduction of hexadecanol to 1-hexadecanol. Methods A and B are based upon measuring the formation of 1-hexadecanol, and method C involves spectrophotometric determination of NADPH utilization. Identical assay conditions were used for all three methods, and, unless otherwise indicated, the concentration of assay components in a final volume of 2.0 ml were: 50 mM potassium phosphate buffer (pH 7.4), 0.125 mM NADPH, 0.100 mM hexadecanal dispersed in Tween 20 (final concentration = 1 μg/ml) and an appropriate amount of protein. The reactions were carried out in 15 ml glass-stoppered conical tubes or 3.0 ml glass cuvettes for 8 min at 37 C.

Method A: In this method, 1-hexadecanol formation was measured by determining the radioactivity incorporated into the fatty alcohols from the labeled substrates. The assays were carried out as described and the incubation mixture extracted according to the method of Bligh and Dyer (20). Carrier 1-hexadecanol had been added to the extracting solvent, such that ca. 0.5 μmole was added to each assay at the time of extraction. The extracted lipid was concentrated under N_2 in a 30 ml centrifuge tube and chromatographed by TLC using two solvent systems (I and II) to purify the 1-hexadecanol. I. Hexane:Chloroform:Methanol (73:25:2) II. Hexane:Ethyl Ether:Acetic Acid (30:70:1).

The purified 1-hexadecanol was dissolved in 0.3 ml n-heptane; 0.1 ml was radioassayed and 1 μliter analyzed by quantitative GLC to determine carrier recovery. Carrier recovery was estimated from the ratio of the peak area of 1-hexadecanol in the sample to the peak area of the 1-hexadecanol originally present in the extracting solvent. The latter area was determined by adding the same amount of carrier hexadecanol to the same volume of solvent as used in the assay tube, concentrating and examining the lipid residue in exactly the same manner as the lipid from the assay. In this instance, the peak area of 1-hexadecanol was considered to represent 100% recovery. Nmoles of 1-hexadecanol synthesized were calculated by correcting the raw cpm for carrier recovery and counting efficiency and dividing total dpm calculated by the specific activity of the substrate.

Method B: This method measured 1-hexadecanol formation directly, i.e. without carrier addition, by GLC. The technique involved extracting the assay mixture as before, except 20 nmoles 2-octadecanol were added as an internal standard to the extracting solvent. The

lipid residue was dissolved in 30 μliter $CHCl_3$, immediately transferred to a capillary tube to prevent evaporation and 1 μliter analyzed by GLC. The amount of 1-hexadecanol formed was determined by triangulation of the peak area generated by 1-hexadecanol and then relating this value to a calibration curve. Losses incurred upon extracting were estimated by measuring the recovery of 2-octadecanol in the same way as 1-hexadecanol recovery in method A. The value measured for the amount of 1-hexadecanol then was corrected for losses using recovery of 2-octadecanol.

For methods A and B, an assay mixture incubated under the same conditions as the sample, but in the absence of protein or NADPH, served as a control.

Method C: The third method involved following NADPH oxidation in a Gilford 2400 spectrophotometer by continuously monitoring the decrease in absorbance at 340 nmeters. Nmoles of NADPH oxidized was calculated using the observed absorbancy and an extinction coefficient for NADPH = 6.25×10^3 L/mole cm. An assay system incubated in the absence of hexadecanal served as a control.

Radioactivity measurements: The scintillation fluid used consisted of Liquifluor with toluene mixed to a concentration of 0.4% 2,5 diphenyloxazole and 0.1% 1,4 bis-2 (5'phenyloxazole) benzene. The radioactive sample was dissolved in 0.1-0.5 ml n-heptane and added to 10 ml scintillation fluid. Radioactivity was assayed with a Packard Tri Carb liquid scintillation counter, model 3320 (efficiency = 84%).

Protein determination: Protein was measured using the biuret method with crystalline bovine serum albumin as a standard or in more dilute solutions by determining the A_{280}/A_{260} (21, 22).

Diethylaminothyl (DEAE)-cellulose chromatography: DEAE cellulose (Whatman DE32, microgranular) was prepared for chromatography by treating with 0.5 N HCl, then with 0.5 N NaOH, and washed with water until the pH of the supernatant was 7.4. The cellulose was stored at 4 C with 0.001 M sodium azide until needed. Just prior to column chromatography, CO_2 was removed from the cellulose suspension by vacuum, and the cellulose poured into a column 2.5 x 80 cm. The packed column was washed with 0.01 M phosphate buffer (pH = 7.4) containing 5 mM mercaptoethanol until the pH of the effluent was 7.4. The phosphate content of the eluate from this column was determined colorimetrically (23).

Sephadex chromatography: Sephadex G-200 was equilibrated with 0.075 M potassium phosphate buffer (pH = 7.4 and 5 mM mercaptoethanol) in a column 2.6 x 80 cm. D-1 enzyme (4 Dml) (9 mg/ml protein) was layered onto the G-200 bed as a 10% sucrose solution. Elution was carried out with the above buffer at a pressure of 10 cm. Fractions (5 ml) were collected and assayed spectrophotometrically for enzyme activity. Protein was assayed in each fraction by determining the optical density at 280 nmeters. The void volume of the column was 120 ml, as determined with Blue Dextran-2000. The column was calibrated by measuring the elution volume of aldolase (158,000), ovalbumin (45,000), chymotrypsinogen (25,000), and ribonuclease (13,700). A plot of log M.W. vs. V_e/V_o, where V_e = elution volume of protein and V_o = void volume, was constructed. A separate column, 2.5 x 40 cm, containing sephadex G-100 was prepared and calibrated in a similar manner. The void volume of this column was 72 ml, as determined by aldolase. The enzyme mol wt was determined using the specific calibration curve and the observed V_e of the enzyme. D-2 enzyme (4.0 ml) (4 mg/ml protein) was layered onto the column bed as a 10% sucrose solution and the column developed as before. Fractions (2.5 ml) of the effluent were collected and assayed for enzyme activity and protein content as before.

RESULTS

In vivo incorporation of fatty acids into E. coli lipids: The lipids extracted from *E. coli* grown in the presence of [1-C^{14}] hexadecanoic acid were resolved by column chromatography and the column fractions radioassayed. In two separate experiments, 80-82% radiolabel, 7.3 x 10^6 dpm, initially added to the incubation medium was detected in the phospholipid fraction and 1.5-2% in the neutral lipids. Of the total radioactivity in the neutral lipid fraction, 70-76% was detected in the free fatty acids, none in the free fatty aldehydes, and 4-6% in the free fatty alcohols after purification of these lipid types by TLC. The results of the analysis indicate that exogenous fatty acid is incorporated into the intracellular free fatty acid pool and also is reduced to free fatty alcohols. While the free fatty aldehydes did not contain detectable radioactivity, they were present in trace amounts (0.142 μmoles/100 mg lipid). GLC of the free fatty acid methyl esters performed in conjunction with stream splitting indicated that all of the 7×10^3 dpm recovered were eluted as methyl hexadecanoate ruling out extensive catabolism of the fatty acid. Radiopurity of the free fatty alcohols was assessed in a similar fashion after conversion of the alcohols to the acetoxy alkanes (24). Following

TABLE I

In Vitro Synthesis of Fatty Alcohols by *Escherichia Coli* K-12

Substrate	Protein mg	Fatty alcohol dpm	Fatty alcohol synthesized nmoles
1-C^{14}-Hexadecanoic[a,c]	8.04	213	0
acid	0	200	0
1-C^{14}-Hexadecanoyl[b,c]	0.50	240	0.06
CoA	0	121	0
1-C^{14}-Hexadecanal[c]	0.43	115,600	21.3
	0	4,200	0
1-C^{14}-Hexadecanal[c]	0.43	105,100	19.5
	0	4,500	0

[a]Adenosine 5'-triphosphate (4.7 mM), coenzyme A (CoA) (1.2 mM), Mg^{+2} (1 mM), and reduced form of glutathione (3.5 mM) were included in this assay. Concentration of 1-C^{14}-hexadecanoic acid (1.5×10^6 dpm/μmole) = 4.4×10^{-4} M. Final volume of reaction mixture was 2.0 ml. Incubations were carried out at 37 C for 8 min.

[b]Concentration of 1-C^{14}-hexadecanoyl CoA (1×10^6 dpm/μmole) = 1×10^{-4}M.

[c]Both nicotinamide adenine dinucleotide, reduced form, and nicotinamide adenine dinucleotide phosphate, reduced form, were present in 1 mM concentration. Hexadecanal present in 1×10^{-4} M concnetration (specific activity = 5.2×10^6 dpm/μmole).

acetylation, all of the radioactivity initially present as the fatty alcohol migrated as the acetoxy alkane during TLC. GLC of the acetoxy alkanes and stream splitting of the effluent indicated again that all of the radioactivity injected was associated with the component eluting as 1-acetoxy hexadecane.

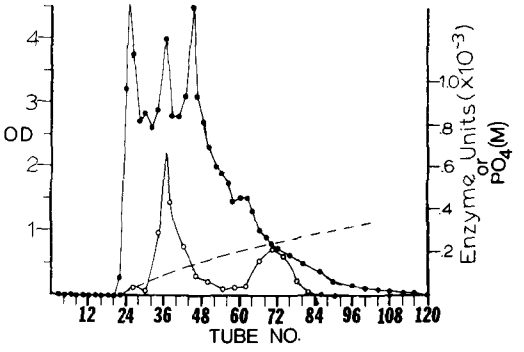

FIG. 1. Diethylaminoethyl-cellulose chromatography of the 0-55% ammonium sulfate fraction. Protein (1.5-1.6 g) in 0.01 M phosphate buffer pH 7.4, containing 5.0 mM mercaptoethanol, was applied to the column and the column washed with the same buffer until no UV absorbing material was detected in the effluent. At this point, a gradient was started with 0.075 M phosphate and 0.50 M phosphate. Both buffers were at pH 7.4 and contained 5 mM mercaptoethanol. The flow rate was 50 ml/hr; 10 ml fractions were collected. Protein concentration (—•—•—) was measured by determining the absorbance 280 nm. Phosphate content of the eluate (---) was assayed colorimetrically (23). Enzyme activity (-O O-) assessed by methods B and C in the same assay mixture. Tubes containing activity were combined; the protein was precipitated with $(NH_4)_2SO_4$; and precipitate was redissolved in 0.075 M potassium phosphate buffer (pH 7.4 and 5 mM mercaptoethanol). OD = outside diameter.

In vitro biosynthesis of free fatty alcohols: A crude sonicate of *E. coli* K-12 was prepared and assayed according to the radioassay method. The data presented in Table I show that this extract was unable to catalyze the reduction of [1-C^{14}] hexadecanoic acid or [1-C^{14}] hexadecanoyl CoA to hexadecanol but that hexadecanal served as a substrate. These data are not consistent with the findings of the in vivo experiments that indicated reduction of a fatty acid to a fatty alcohol. However, the in vitro experiments are not considered contradictory for reasons discussed later.

Product characterization: Radio purity of the radio labeled fatty alcohols isolated from the previous incubation experiments was established in two ways. First, a known amount of the radio labeled fatty alcohol was converted to the acetoxy alkane (24), and this derivative was purified by TLC using solvent system II. The lipids migrating as known 1-acetoxy hexadecane were eluted from the chromatographic plate and radioassayed. In two separate experiments, 96 and 98% radioactivity initially present as a fatty alcohol, 51 x 10^3 dpm, was recovered as the acetoxy alkane derivative. The radio purity of [1-C^{14}] hexadecanol was evaluated further by GLC. Unlabeled fatty alcohols tetra, penta, hepta, and octadecanol were added to the C^{14} labeled fatty alcohols and the column effluent stream split, collected, and radioassayed for each component. The amount of radio label in the 1-hexadecanol peak was 90% total radioactivity recovered. It was concluded,

TABLE II

Pyridine Nucleotide Requirement for Fatty Aldehyde Reductase in 0-55% $(NH_4)_2SO_4$ Fraction[a]

Component varied	Hexadecanol formed/8 min nmoles
Complete	42.6
NADPH and NADH omitted	3.0
NADPH omitted	3.5
NADH omitted	40.8
Protein omitted	1.9
Boiled protein	2.0
Heated protein	21.0

[a]Reactions were assayed according to method B. The complete assay mixture included: 0.7 mg protein, 250 nmoles each of nicotinamide adenine dinucleotide, reduced form, (NADH) and nicotinamide adenine dinucleotide phosphate, reduced form, (NADPH), 200 nmoles hexadecanal and 100 μmoles potassium phosphate buffer (pH 7.4) in a final volume of 2.0 ml. Incubations were carried out at 37 C for 8 min. The boiled protein was boiled for 5 min, and the heated protein was heated at 55 C for 5 min.

therefore, that the reduction [1-C^{14}] hexadecanal to hexadecanol did take place.

The product identified as 1-C^{14} hexadecanol was characterized further by combined GLC-mass spectrometry (MS). Mass spectra were taken on either side and center of the 1-hexadecanol peak. The fragmentation patterns were all identical to the spectra of known 1-hexadecanol (25) and contained no indications of impurities. In brief, the key fragmentations involve loss of H_2O (m/e 224) coupled with the loss of ethylene (m/e 196) and cleavage between carbon atoms 1 and 2 to give an ion at m/e 31. The hydrocarbon fragmentation is that of the corresponding olefin. This occurs because the parent molecule readily loses H_2O, generating the olefinic ion, which then fragments (25).

Effect of time and protein concentration upon rate of fatty alcohol biosynthesis: The relationship between time of incubation and nmoles of fatty alcohol synthesized was evaluated over an incubation interval varying from 1-14 min with two different assay procedures. It was observed that fatty alcohol biosynthesis was linear with respect to time of incubation over the interval studied and that similar results were obtained by either the GLC or radioassay procedure.

The relationship between the amount of supernatant protein incubated and the nmoles of fatty alcohol synthesized was evaluated using the GLC and radioassay procedures. In this instance, fatty alcohol synthesis was linear with respect to protein concentration over a range 0.05-0.30 mg/ml and no difference was observed with respect to the assay procedure employed.

Ammonium sulfate fractionation and cofactor requirements: Using method B, cofactor requirements and the effect of heating were determined for a 0-55% $(NH_4)_2SO_4$ fraction containing 80-85% activity initially present in the crude sonicate. The results from these studies are presented in Table II. The enzymatic activity had an absolute requirement for NADPH. Boiling completely destroys the enzymatic activity, while heating at 55 C for 5 min reduces the total activity, as well as protein, twofold. Thus, heating does not serve as a purification step, although some degree of heat stability is present.

Correlation of NADPH oxidation and 1-hexadecanol formation: The amount of NADPH oxidized, as measured spectrophotometrically (method C), was compared to the amount of fatty alcohol produced (method B), using the 0-55% $(NH_4)_2SO_4$ fraction as the enzyme source. The spectrophotometric assay was performed, as described in "Methods." At the end of 8 min incubation intervals, the contents of the cuvette were extracted with organic solvent, and the amount of fatty alcohol synthesized was evaluated via GLC. From the data presented in Table III, it is apparent that similar results are obtained when either NADPH oxidation or 1-hexadecanol formation is employed to assess enzyme activity.

DEAE-cellulose chromatography: Figure 1 shows the results that were obtained following DEAE-cellulose chromatography of the protein participitating at 0-55% saturation with $(NH_4)_2SO_4$. These results indicate that 95-100% activity applied to the column was recovered in two separate fractions. One protein fraction eluted with 0.115 M phosphate (D-1 enzyme) contained 70% recovered activity, the remaining 30% activity was found in a

TABLE III

Quantitative Correlation of Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form, (NADPH) Oxidation with 1-Hexadecanol Formation[a]

Experiment	Nmoles NADPH oxidized	Nmoles 1-hexadecanol formed
1	38.0	40.0
2	37.0	39.5

[a]Assay mixtures contained 0.40 mg protein, 250 μmoles NADPH, 200 nmoles hexadecanal, and 100 μmoles potassium phosphate buffer (pH 7.4) in a final volume of 2.0 ml. NADPH oxidation was monitored continuously at 340 nm; after an incubation interval of 8 min, the contents of the cuvette were extracted and the 1-hexadecanol content measured according to method B.

protein fraction that eluted with 0.23 M phosphate (D-2 enzyme). The aldehyde reductase activity in each fraction was established by measuring NADPH oxidation spectrophotometrically and 1-hexadecanol formation via GLC in the same assay mixture. The two independent assay methods were found to give essentially the same values for aldehyde reductase activity. A summary of the degree of purification of the reductase studied here is presented in Table IV.

Characterization of D-1 and D-2 enzyme fractions: The D-1 and D-2 enzyme fractions both exhibited an absolute requirement for NADPH. Nicotinamide adenine dinucleotide, reduced form, (NADH) was neither active nor inhibitory at the same concentration as NADPH.

The substrate specificity of the D-1 and D-2 enzymes was measured using aldehyde substrates of different chain lengths and degrees of unsaturation. The spectrophotometric procedure was employed to assay enzyme activity, and all substrates were present at the concentration previously established to give their optimal activity (zero order kinetics). In the case of the unsaturated aldehydes, GLC determination of alcohol formation also was employed to evaluate the possibility of double bond reduction. This effect was not observed with either enzyme fraction. Figure 2 shows that D-1 and D-2 enzymes catalyzed the reduction of a number of aldehydes, saturated and unsaturated. However, it is significant to note that acetaldehyde

TABLE IV

Summary of Purification of Fatty Aldehyde Reductase from *Escherichia coli*

Fraction	Protein mg	Activity[a]	Recovery	Specific activity[b]	Fold purification
10^5 x g Supernatant	2600	13,500	100	5.2	---
Ammonium sulfate (0-55%)	1765	13,410	99.3	7.6	1.46
Diethylaminoethyl-cellulose					
D-1	478	7887[c]	58.4	16.5	3.17 (5.4)[d]
D-2	84	3427[c]	25.4	40.8	7.84 (31.0)[c]
Sephadex G-200					
(D-1)	65	7887	58.4	121	23.0 (39.9)[d]

[a]Unit of activity = That amount of enzyme which catalyzes formation of 1 nmoles of hexadecanol or oxidation of 1 nmole of nicotinamide adenine dinucleotide phosphate, reduced form, in 1 min.

[b]Specific activity = units/mg protein.

[c]The values do not indicate the total number of units of D-1 and D-2 recovered in their respective fractions. Some tubes containing low activity were not included.

[d]Actual specific activities of D-1 and D-2 cannot be measured separately in the 100,000 x g supernatant. The value measured is the combined specific activity of enzyme(s) D-1 and D-2. The theoretical specific activities of D-1 and D-2 can be calculated by multiplying the total units of enzyme activity measured in the supernatant by the fraction that the activity observed in either D-1 or D-2 represents of the sum of the individual activities in both fractions. The resulting values will give the theoretical total units of D-1 and D-2. The theoretical specific activities then can be calculated using these values. The number in parenthesis is the fold purification calculated on the basis of the theoretical specific activity, assuming the same fractional distribution of both enzymes in the 100,000 x g supernatant.

gave no measurable reaction, nor is there any remarkable difference in the specificity of the two enzyme fractions. Separate experiments have shown that there was no enzyme activity when 100 μM 2-pentadecanone was used as the substrate for the D-1 or D-2 enzyme. It was not possible to measure the kinetic parameters of these enzymes with the various substrates or NADPH, since the reaction rate was linear for only a very short time interval at low reactant concentrations, making it difficult to measure the initial velocity accurately.

The enzymatic activity at different pHs was determined for both the D-1 and D-2 enzymes. Both enzymes were active over the pH range, 5.5-10.0. The D-1 fraction had two pH optima at 7.5 and 9.0, while the D-2 enzyme also had two pH optima at 8.0 and 9.5. Considering the enzyme preparations are still impure, a difference in pH optima of 0.5 units was not considered significant.

The effect of ionic strength of the buffer upon the aldehyde reductase activity also was investigated. There was no observed change in the activity of the D-1 or D-2 enzymes when the ionic strength was varied between 0.005 M phosphate and 0.50 M phosphate (pH = 7.4).

The activities of both aldehyde reductases were measured in the presence of thiols and thiol inhibitors. Addition of 5 mM mercaptoethanol of 5 mM glutathione to the assay mixture had no effect upon the activity of either enzyme. However, the activity of both enzyme fractions was inhibited by preincubation with mercuric ions, and this inhibition was reversed when mercaptoethanol was added to the protein heavy metal preparation (Table V). Preincubation of the enzyme fraction with NADPH or hexadecanal failed to protect against inhibition by the metal ions. Similar

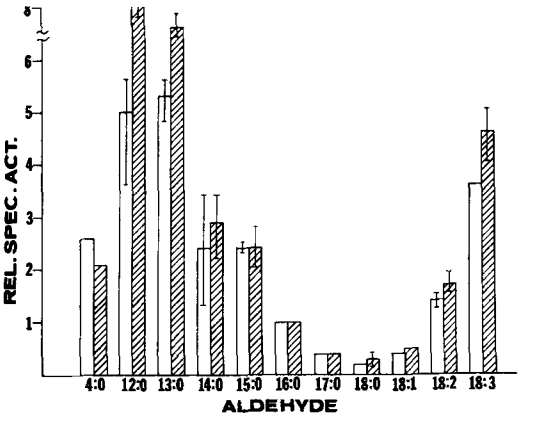

FIG. 2. Histogram depicting the relative specific activities of the D-1 and D-2 enzymes with aldehydes substrates of different chain lengths and degree of unsaturation. Enzyme activity was assessed spectrophotometrically. The range of specific activities obtained from three separate assays is indicated by the vertical bar (I). If no bars appears, then there was no variation. The shorthand notation used for the aldehyde can be interpreted as follows: number of carbon atoms = number of double bonds. The unsaturated 18 carbon aldehydes were oleyl, linoleyl, and linolenyl aldehyde. □ D-1 ▨ D-2

results were observed when the D-1 and D-2 enzyme preparations were preincubated with Zn^{+2} ions. In this instance, a 10-fold higher zinc concentration was required to achieve the same degree of inhibition.

The D-1 and D-2 protein fractions were chromatographed on Sephadex G-200 and G-100, respectively, as described in the "Methods." All of the D-1 enzyme activity applied to the Sephadex G-200 column was eluted in a volume of buffer that gave a V_e/V_o value of 1.5. After plotting this value on the calibration curve obtained by standardizing this column

TABLE V

Effect of Thiol Inhibitiors upon Activity of D-1 and D-2 Enzymes[a]

Hg^{+2} mM	D-1 NADPH oxidized nmoles	D-1 Inhibition %	D-2 NADPH oxidized nmoles	D-2 Inhibition %
0	6.7	0	10.3	0
.01	2.7	60	7.2	30
.025	0	100	0.9	91
.10	0	100	0	100
.025 + .025 mM SH	6.7	0	9.7	6

[a]Inhibitiors were preincubated for 15 min at 37 C with 0.25 mg D-1 enzyme or 0.27 mg D-2 enzyme in the concentrations given in the table. The inhibition of the enzyme activity was reversed by incubating an aliquot of the inhibited protein with mercaptoethanol for 15 min at 37 C. 0.1 M Tris Buffer was used in this experiment because the heavy metals formed insoluble salts with phosphate. The assays were carried out in glass cuvettes for 8 min at 37 C, and nicotinamide adenine dinucleotide phosphate, reduced form, (NADPH) oxidation was measured spectrophotmetrically (3). Each assay mixture contained 0.25 mg D-1 enzyme or .27 mg D-2 enzyme, 200 nmoles hexadecanal, 125 nmoles NADPH, and 200 μmoles Tris Buffer (pH 7.4) in a final volume of 2.0 ml. The control sample included all of the components except hexadecanal.

TABLE VI

Oxidation of Fatty Alcohol Substrates Using the 1 x 10^5 xg Supernatant Fraction[a]

Protein mg	dpm	Corrected dpm	Hexadecanoic acid/8 min nmoles
0	766	0	0
.2	4025	3259	0.34
.4	7322	6556	0.68

[a]The reactions were carried out for 15 min at 37 C. Activity was assessed using method A. The assay mixtures included: 1 μ oxidized form and nicotinamide adenine dinucleotide, 100 nmoles 1-C^{14}-hexadecanol (9.6 x 10^6 dpm/μmole) and 100 μmoles potassium phosphate buffer (pH 7.4). Fatty acids were isolated by thin layer chromatography.

with ribonuclease, chymotrypsin, ovalbumin, and alsolase, a mol wt of 250,000 was calculated for the D-1 protein.

The D-2 enzyme activity was eluted from the Sephadex G-100 column in a volume of buffer that gave a V_e/V_o value of 1.25. Using the calibration curve established for this column, a mol wt of 62,000 was calculated for the D-1 protein.

It should be noted here that only 25% aldehyde reductase activity applied to the G-100 column was detected in the active fraction. The lost activity could not be restored upon recombination of any of the column fractions. Furthermore, this loss in activity was not due to mechanical or absorptive losses of the protein since 95% protein applied to the G-100 column was recovered in the effluent, as judged by 280/260 ratios (21). It was concluded from these results that the D-2 enzyme was unstable upon gel filtration.

Reversibility of reaction: Method A was used to investigate the oxidation of 1-hexadecanol to hexadecanal and hexadecanoic acid by the supernatant fraction. The assay mixture contained 1 mM NADP and 1 mM nicotinamide adenine dinucleotide (NAD) and 1.0 mM [1-C^{14}] hexadecanol $\frac{9.6 \times 10^6 \text{ dpm}}{(\mu\text{moles})}$ and 0.2 or 0.4 mg protein in a final volume of 2.0 ml. Samples were incubated for 8 min at 37 C and extracted, as described previously. Carrier hexadecanal, hexadecanoic acid, and hexadecyloctadecanoate were added to the extract and these moieties isolated by TLC and radioassayed. Radioactivity was found in only the hexadecanoic acid (Table VI).

DISCUSSION

In vivo studies reported here indicate that free fatty acids and alcohols are metabolically interconvertible. Free fatty aldehydes, while present in trace amounts in *E. coli*, were not radio labeled, when either [1-C^{14}] palmitate or [1-C^{14}] cetyl alcohol were substrates. This finding contrasts to studies with *Clostridium butyricum* and *M. tuberculosis* in which the free fatty aldehyde was a transient intermediate (12, 13). However, the inability to isolate radio labeled fatty aldehyde is not sufficient evidence to exclude the aldehyde as an intermediate in this reaction since it may have remained bound to the enzyme complex in vivo. In other in vitro enzymatic systems catalyzing the reduction of fatty acids to fatty alcohols, the aldehyde intermediate could not be isolated, unless it was trapped as the hydrazone (26). When similar in vitro trapping studies were attempted at the pH of the incubation medium (pH 7.4), the hydrazone did not form with fatty aldehyde concentrations less than 5 nmoles/ml. With the small amount of endogenous fatty aldehyde present here, such a trapping experiment was unsuccessful. In separate in vitro experiments, unlabeled octadecanal was incorporated into the lipid extracting solvent and added to the medium at the end of the incubation period. Fatty aldehydes were isolated and the presence of hexadecanal evaluated by the GLC and radioassay procedures. Hexadecanal was not detected by either method. Considering the sensitivity of the assay procedures employed, these results are not sufficient to exclude the fatty aldehyde as an intermediate, especially since the exogenous aldehyde is metabolized readily.

The fact that [1-C^{14}] palmitate or [1-C^{14}] palmitoyl coenzymee A (CoA) was not reduced to a fatty alcohol in vitro does not indicate an absence of appropriate enzymes. Previous studies have shown that the enzymatic reduction of B-hydroxy- B-methyl glutaryl CoA to mevalonic acid or aspartic acid to homoserine requires an initial activiation of the carboxyl group (27-30). *E. coli* grown as described here have a low acyl CoA synthetase activity (31-33). Since these cells also have a high thioesterase activity (34-36), one would expect

a cell-free system to contain only a small fraction of any exogenous fatty acid in the activiated form. A similar difficulty in demonstrating the reduction of exogenous fatty acyl CoA in the presence of a high thioesterase activity has been noted before in plants (26).

Evidence that fatty aldehydes are metabolically active in *E. coli* is based upon their rapid reduction to the corresponding fatty alcohol when added in vitro. Two protein fractions catalyzing this process have been purified partially from the 100,000 x g supernatant fraction and characterized.

At present, it is not possible to decide with certainty whether the two protein fractions designated D-1 and D-2 represent separate enzymes or whether one is an aggregate composed of a greater number of subunits than the other. The similarities in the characteristics of each protein fraction argues for the concept that the D-2 enzyme is a dissociation product of the D-1 enzyme.

The observation that acetaldehyde is not a substrate for either enzyme and that NADH was noot a required cofactor indicates that this enzyme is not identical with the alcohol dehydrogenase of *E. coli* (14).

The high enzyme activities observed when the polyunsaturated fatty aldehyes were employed as substrates were unexpected, since the corresponding fatty alcohols were not detected in *E. coli*. The similarity in the activities observed with the polyunsaturated 18 carbon aldehydes and dodecanal and tridecanal may be explained by the structures of the polyunsaturated chains. The *cis*-double bonds in the latter moieties lead to a shortening of the effective chain length. Because of this effect, the length of linolenyl aldehyde will be similar to dodecanal; however, the polyunsaturated moiety is fatter because of the bend in the molecule and more polar. This latter effect may account for the fact that linolenyl aldehyde is not quite as active a substrate as dodecanal.

The observation that 2-pentadecanone is not a substrate for either the D-1 or D-2 enzyme fractions indicates that these enzymes are not involved in the biosynthesis of the secondary alcohols in *E. coli* and that the carbonyl group must be present as such for enzyme activity.

Previous studies from this laboratory have indicated that the fatty alcohols of *E. coli* are both 1- and 2-alkanols (5). While a direct isolation of the secondary alcohols was not attempted, it is considered that only primary fatty alcohols were formed in vivo from exogenous fatty acids or in vitro from palmitaldehyde. This is based upon the observation that the secondary alcohols of *E. coli* are all less than 16 carbon atoms in chain lengths, and radioactivity was detected in only hexadecanol when the alkyl acetates were analyzed via GLC and the effluent stream split.

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Embryonic vs Tumor Lipids: II. Changes in Phospholipids of Developing Chick Brain, Heart, and Liver

RANDALL WOOD, Division of Gastroenterology, Departments of Medicine and Biochemistry, University of Missouri School of Medicine, Columbia, Missouri 65201

ABSTRACT

Brain, heart, and liver tissues were excised from embryos and chicks 10, 13, 16, 19, 22, 27, and 53 days after incubation was initiated and the lipids extracted. The quantitative distribution of the phospholipids and the fatty acid composition of the individual phosphatides were determined for each time period. Each tissue exhibited a distinct phospholipid composition that differed from the composition of egg. Elevated concentrations of particular phosphoglycerides that characterize certain mature tissues were observed at the earliest time period. As development progressed, some phospholipid classes in all tissues showed dramatic change, while others remained relatively constant. Brain showed the most stable composition, while the phosphatides of liver were the most dynamic. Each phospholipid class exhibited a characteristic fatty acid profile that was unique for each tissue. All of the phospholipid classes showed a change in fatty acid composition as development progressed, and, in some tissues, the change was dramatic. The fatty acid composition of brain phosphoglycerides showed the least change, while liver showed the greatest fluctuation. Docosahexaenoic acid and, in most cases, arachidonic acid decreased in the phosphoglycerides with increased development. The decrease in docosahexaenoic acid correlated well with the decreasing mitotic indices of heart and liver cells as development progressed. Comparison of observed abnormal lipid patterns between mature and neoplastic tissue with embryonic tissue lipid profiles suggest that some of the observed abnormalities of neoplasms probably are due to changes in lipid metabolism associated with rapidly proliferating cells, whereas other abnormalities appear to be associated with neoplasia.

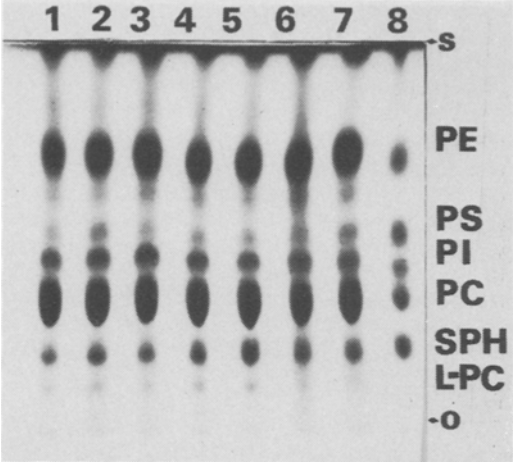

FIG. 1. A thin layer chromatoplate depicting the distribution of phospholipids derived from embryo and chick heart at: lane 1, 10 days; lane 2, 13 days; lane 3, 16 days; lane 4, 19 days; lane 5, 22 days; lane 6, 27 days; and lane 7, 53 days after incubation was initiated. Standard phospholipids resolved by the solvent system chloroform-methanol-acetic acid-0.9% saline, 50:25:8:4 (v/v) are given in lane 8. Abbreviations are: PE = phosphatidylethanolamine, PS = phosphatidylserine, PI = phosphatidylinositol, PC = phosphatidylcholine, SPH = sphingomyelin, L-PC = lyso-phosphatidylcholine, O = origin, and S = solvent front. The material running near the solvent front is labeled SF in Table II.

INTRODUCTION

Differences that have been found to exist between neoplasms and normal mature tissues, with which they usually are compared, are not necessarily related directly to neoplasia but may have resulted from changes in metabolism associated with rapid growth and cell proliferation. Since most embryonic tissues undergo rapid cell proliferation, they probably represent more closely ideal control tissues for comparison with neoplastic tissue. Therefore, a detailed examination of the structure and metabolism of embryonic tissues could further our understanding of neoplasms.

The possible implications an error in lipid metabolism could have upon the important biological processes of a cell and the similarities in the lipid structure and metabolism of neoplastic and embryonic tissues have been discussed previously (1). The first article of this series (1) contained data on the dry wt, total lipids, total phospholipids, total neutral lipids, and the neutral lipid class composition of brain, heart, and liver at various stages of the develop-

TABLE I

Percentage Phospholipid Composition of Chick Brain at Various Stages of Development[a]

Phospholipid class[b]	Days after incubation initiated							
	Egg	10	13	16	19	22	27	53
Origin	0.3	0.3	0.6	0.6	0.2	0.4	0.7	0.9
Lyso-PC	2.6	1.3	2.4	2.3	0.9	1.4	2.4	1.7
SPH	3.4	2.0	2.4	3.6	2.1	3.7	5.3	8.2
PC	67.2	32.4	27.6	33.2	22.3	29.0	24.1	36.4
PI	3.4	6.7	7.8	6.4	6.5	5.8	6.4	4.5
PS	2.9	10.4	11.1	10.1	11.7	9.7	10.0	7.9
PE	18.7	42.8	44.3	40.6	52.8	47.0	47.1	35.6
SF	1.5	4.1	3.9	3.2	3.5	3.1	4.0	4.8

[a]Percentages represent the mean of duplicate phosphorous analyses. Values have not been corrected for differences in mol wt of the various classes.

[b]Lyso-PC = lyso-phosphatidylcholine, SPH = sphingomyelin, PC = phosphatidylcholine, PI = phosphatidylinositol, PS = phosphatidylserine, PE = phosphatidylethanolamine, and SF = material running near the solvent front.

ing chick. This article describes quantitatively the changes that occur in the individual phospholipid classes and fatty acids of these three tissues during development.

MATERIALS AND METHODS

Brains, hearts, and livers were obtained from embryos and chicks 10, 13, 16, 19, 22, 27, and 53 days after incubation was initiated, and lipids were extracted and fractionated into neutral and phospholipid fractions, as described previously (1). Phospholipids were resolved into individual classes by thin layer chromatography (TLC) on adsorbent layers of Silica Gel HR developed in a solvent system of chloroform-methanol-acetic acid-0.9% saline 50:25:8:4, v/v, when the relative humidity was below 35%. Phospholipid classes resolved by TLC were quantified by the phosphorous determination method of Rouser, et al. (2). TLC plates used for quantitative or qualitative determinations were sprayed with sulfuric acid, charred, and documented by photography (Fig. 1). Class identifications were based upon the use of phosphorous and ninhydrin spray reagents (3), gas liquid chromatography (GLC) of derived esters, and cochromatography with authentic phospholipid standards in two or more TLC solvent systems. Adsorbent layers containing the resolved phospholipid classes were scraped directly into teflon lined screw cap culture tubes (16 x 100 mm) and converted to methyl esters by refluxing 2 hr (4 hr for sphingomyelin) with 3 ml 2% sulfuric acid in anhydrous methanol. After transesterification was complete, an equal volume of water was added, the sulfuric acid was neutralized with an excess of ammonium hydroxide, and the methyl esters were extracted thrice with hexane.

GLC analyses were carried out with an Aerograph model 2100 instrument. Esters were analyzed on 180 cm x 2 mm (inside diameter) pyrex columns packed with 10% ethylene glycol succinate polysiloxane (EGSS-X) coated on 100-120 mesh Gas Chrom P. Column temperature was programed from 140-200 C at either 2 or 4 C/min. Quantitation was achieved with an Autolab digital integrator. Identity of esters is based upon analyses before and after hydrogenation and cochromatography with commercially available standards. The use of classical names for unsaturated fatty acids does not imply that the double bond positions and configurations were determined.

Percentages represent the mean of duplicate analyses of a single composite sample. Agreement between values of the duplicate analyses was usually ± 5% for major components and ± 10% for low concentrations of components.

RESULTS

Class Composition

The chromatoplate shown in Figure 1 depicts the resolution of the phospholipid classes derived from chick heart at various stages of development and a standard phospholipid mixture. All the major phospholipid classes, except diphosphatidylglycerol which migrated into the solvent front, were resolved. Except for liver, an unidentified minor component that migrated just below phosphatidylethanolamine (Fig. 1) was not always resolved sufficiently from phosphatidylethanolamine to permit separate quantification. The chromatoplate shown in Figure 1 is typical of the type of resolution also obtained for brain and liver phospholipids.

TABLE II

Percentage Phospholipid Composition of Chick Heart at Various Stages of Development[a]

Phospholipid class[b]	Days after incubation initiated							
	Egg	10	13	16	19	22	27	53
Origin	0.3	0.4	0.9	1.1	0.4	0.4	0.2	0.4
Lyso-PC	2.6	2.6	3.3	3.1	2.0	1.9	1.6	1.0
SPH	3.4	5.0	6.7	5.1	5.9	7.5	5.1	4.7
PC	67.2	46.9	44.5	36.7	44.6	45.4	33.1	36.3
PI	3.4	6.6	6.8	7.7	6.7	5.6	6.5	6.0
PS	2.9	3.7	4.4	3.8	3.3	2.9	3.4	3.9
PE	18.7	25.7	27.6	34.8	32.8	31.4	37.0	36.1
SF	1.5	8.9	5.7	7.8	4.3	4.9	9.7	11.5

[a]Percentages represent the mean of duplicate phosphorous analyses. Values have not been corrected for differences in mol wt of the various classes.

[b]See Table I for definitions of abbreviations.

The quantitative percentage distributions of the various phospholipid classes found in brain, heart, and liver at the various stages of development are given in Tables I-III, respectively. The class composition of eggs selected at random prior to incubation also is given in each table for comparison. A graphical representation of the percentage distributions of six phospholipid classes for the three tissues at each time period is given in Figure 2. Phosphatidylcholine and phosphatidylethanolamine represented more than two-thirds of the total phospholipids found in the three tissues at all stages of development. Brain was characterized by its high levels of phosphatidylserine and phosphatidylethanolamine. Brain phosphatidylcholine levels fluctuated and phosphatidylinositol values remained constant and sphingomyelin percentages increased as development progressed. Heart contained the highest level of phosphatidylcholine of the three tissues at the earliest time period and the level decreased with time, while phosphatidlethanolamine values increased proportionately. Liver showed the reverse trend: phosphatidylcholine levels increased as development progressed, while phosphatidylethanolamine values decreased. Liver contained the highest level of phosphatidylinositol and the highest solvent front values (primarily diphosphatidylglycerol) at the early time periods. Liver sphingomyelin concentrations, like brain, increased with increased development.

Fatty Acid Composition of Phospholipid Classes

The fatty acid compositions of phosphatidylcholine derived from brain, heart, and liver at various developmental stages and also of egg are given in Table IV. Each tissue exhibited a

TABLE III

Percentage Phospholipid Composition of Chick Liver at Various Stages of Development[a]

Phospholipid class[b]	Days after incubation initiated							
	Egg	10	13	16	19	22	27	53
Origin	0.3	0.8	0.6	0.6	1.0	0.5	1.0	1.0
Lyso-PC	2.6	1.7	0.9	1.7	3.1	2.6	3.1	2.7
SPH	3.4	3.8	3.4	3.7	6.1	5.1	10.0	7.3
PC	67.2	22.3	32.2	32.7	34.5	38.9	35.7	42.6
PI	3.4	10.5	9.4	10.9	9.3	7.7	8.1	8.5
PS	2.9	1.9	2.7	4.2	4.0	3.4	2.4	4.5
X[c]	—	4.2	1.6	1.6	4.0	3.1	1.9	—
PE	18.7	44.2	41.5	33.6	27.9	32.7	34.0	28.9
SF	1.5	10.5	7.7	11.0	10.1	6.2	4.8	4.5

[a]Percentages represent the mean of duplicate phosphorous analyses. Values have not been corrected for differences in mol wt of the various classes.

[b]See Table I for definitions of abbreviations.

[c]Unidentified phosphorous containing compound migrating between phosphatidylserine and phosphatidylethanolamine.

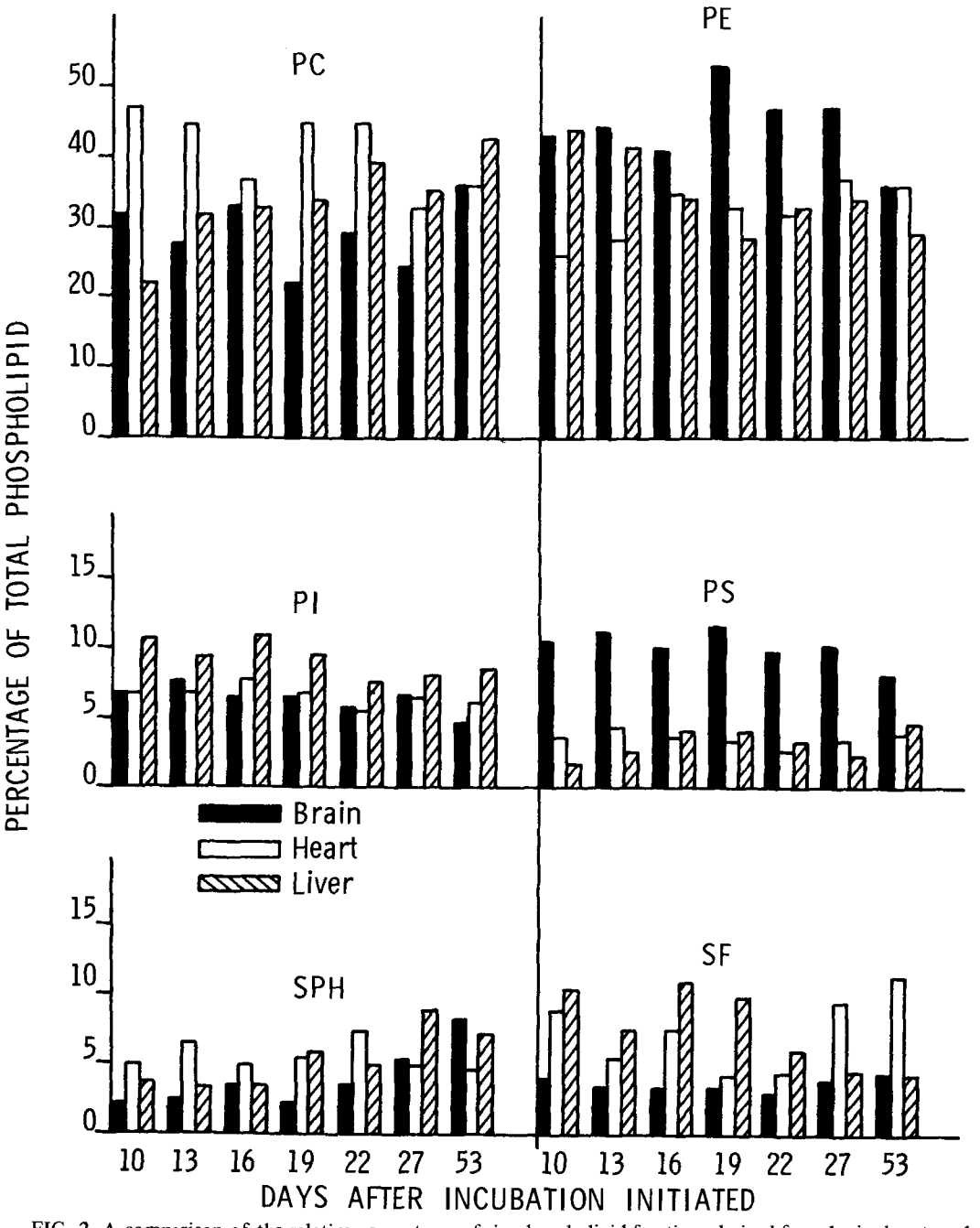

FIG. 2. A comparison of the relative percentages of six phospholipid fractions derived from brain, heart, and liver at various stages of the developing chick embryo and chick. Abbreviations are the same as those given in Figure 1.

fatty acid profile different from that found in egg. Brain phosphatidylcholine contained the highest percentages of palmitic, palmitoleic, and oleic acids and, except for palmitoleic, the values remained relatively constant during development. Heart contained less monoenoic acids and more polyunsaturated acids than brain. Linoleic acid concentration increased in heart phosphatidylcholine as development progressed. Liver contained the lowest percentages

TABLE IV

Fatty Acid Composition of Phosphatidylcholine Derived from Brain, Heart, and Liver at Various Stages of Development

Days after incubation initiated	Fatty acid percentages[a]										
	14:0	15:0	16:0	16:1	18:0	18:1	18:2	20:4 + 22:1	22:4 + 24:1	22:5	22:6
						Brain					
10	2.9	0.4	45.9	8.8	5.3	22.7	2.1	5.7	0.8	0.5	3.3
13	2.9	0.9	44.8	8.4	6.0	20.7	3.2	5.3	1.2	0.5	3.3
16	2.3	0.6	54.2	6.6	6.3	23.1	1.1	1.9	0.3	0.2	1.6
19	1.3	—	53.1	5.8	7.6	24.7	1.7	2.9	—	—	—
22	1.2	0.4	53.8	3.8	7.7	24.8	1.6	3.2	0.4	0.3	1.6
27	1.0	0.6	48.0	3.2	9.8	25.0	2.4	4.6	0.8	0.7	1.9
53	1.0	0.5	52.9	2.5	10.4	26.8	0.9	2.0	0.5	0.2	0.5
						Heart					
10	0.6	0.5	40.5	1.9	14.9	19.5	4.4	10.9	0.7	1.5	3.5
13	0.4	0.4	41.0	1.2	15.1	19.9	5.5	11.7	0.7	0.8	2.8
16	0.4	0.5	38.7	0.9	13.8	17.2	8.9	13.7	0.6	1.0	3.6
19	0.8	1.2	38.1	1.2	15.1	17.3	8.8	13.8	0.2	0.8	2.0
22	0.6	0.9	40.6	—	17.2	19.1	7.7	10.6	0.4	0.8	1.0
27	0.7	1.8	38.6	0.8	15.7	15.6	10.5	10.3	1.3	1.0	0.4
53	1.3	3.2	35.2	—	15.6	13.4	18.1	7.2	1.5	2.0	—
						Liver					
10	T[b]	T	35.7	—	19.6	4.9	2.4	23.8	T	1.4	11.9
13	T	—	34.5	T	12.7	5.7	5.1	30.8	T	1.2	8.8
16	T	—	30.3	T	15.2	7.3	12.3	24.3	—	1.4	8.3
19	T	—	25.7	T	21.3	6.5	12.4	21.2	—	1.7	10.5
22	—	—	27.0	T	21.0	7.2	11.3	19.2	0.5	1.8	12.0
27	T	—	47.4	—	16.3	20.4	6.6	5.5	0.4	0.5	2.2
53	T	T	28.6	T	21.5	7.0	21.1	11.2	1.2	2.1	4.7
Egg	—	—	32.5	2.0	13.1	25.5	16.1	4.5	T	2.3	3.8

[a]Percentages represent the mean of duplicate analyses. Agreement between percentages for the major components was ±5% and ±10% for minor components.

[b]T = detectable amounts that represent less than 0.25%.

of monoenoic acids and the highest concentrations of polyunsaturated acids. Liver phosphatidylcholine fatty acids showed the most fluctuation, particularly at the two earliest times and after hatching. Docosahexaenoic acid concentrations were the highest in early development in all three tissues.

As indicated in Table V, phosphatidylethanolamine of all three tissues contained only one-third to one-half of the palmitic acid found in phosphatidylcholine. Furthermore, phosphatidylethanolamine contained much higher levels of C-20 and C-22 fatty acid than phosphatidylcholine. All three tissues had a significant level of C-16 and C-18 aldehydes, thus indicating that in some cases up to one-third of the phosphatidylethanolamine fraction consisted of plasmalogens. The stearate percentages in Table V also contained the 18:1 aldehyde, which could not be resolved from stearate under the chromatographic conditions used. However, analyses of the aldehydes and esters after hydrogenation indicated that the 18:1 aldehyde values were ca. one-third to one-half the stearaldehyde percentages. Brain phosphatidylethanolamine contained higher percentages of monoenoic acids and lower levels of 20:4 than heart and liver. The ca. 10% higher level of stearate in liver before hatching distinguished it from heart percentages. Except for the longest time period, brain fatty acid percentages showed little change with development, while both heart and liver exhibited significant changes. Heart phosphatidylethanolamine 22:6 and 16:0 percentages decreased, while 20:4 levels increased as development progressed. The percentage of 22:6 in liver phosphatidylethanolamine also decreased with increased development, and 18:2 levels showed a corresponding increase. The fatty acid composition of egg phosphatidylethanolamine, also shown in Table V, differed from the composition of all three tissues at all time periods.

The fatty acid composition of sphingomyelin derived from each of the tissues at each time period is given in Table VI. Only saturated and monoenoic acids were found in significant amounts. The composition of all three tissues differed from each other and from that of egg. All three tissue sphingomyelins showed dra-

TABLE V

Fatty Acid Composition of Phosphatidylethanolamine Derived from Brain, Heart, and Liver at Various Stages of Development

Days after incubation initiated	Fatty acid percentages[a,b]											
	14:0	16:0A + 15:0	16:0	16:1	18:0A + 17:0	18:0	18:1	18:2	20:4 + 22:1	22:4 + 24:1	22:5	22:6
						Brain						
10	1.4	7.4	14.6	1.8	4.1	19.6	11.4	1.4	10.2	3.3	3.6	19.1
13	1.2	6.8	14.0	1.4	3.5	22.7	10.5	1.2	11.1	3.4	3.5	19.3
16	1.2	8.5	13.6	1.2	4.8	25.8	10.3	2.0	10.0	3.1	2.4	14.8
19	1.1	7.9	10.8	1.2	5.6	23.9	9.1	1.4	11.3	3.4	3.0	18.4
22	0.5	6.4	9.7	0.6	6.3	27.4	10.0	0.7	10.5	4.3	2.6	17.6
27	0.9	7.1	8.1	1.2	8.9	25.8	12.0	1.3	9.3	4.7	2.8	11.5
53	1.3	5.4	8.2	1.2	7.8	25.6	17.0	4.2	7.7	6.2	1.9	5.9
						Heart						
10	2.1	5.4	12.9	—	5.2	30.4	8.9	4.0	18.2	1.7	2.2	7.8
13	1.0	4.4	12.2	—	4.6	30.9	6.8	2.9	23.0	1.8	2.6	8.6
16	0.9	4.9	10.9	—	5.1	30.4	6.2	4.1	26.0	1.4	1.7	6.7
19	0.8	5.0	8.8	—	5.8	29.5	5.2	4.3	30.4	1.3	2.0	5.8
22	0.7	4.6	9.3	—	5.5	39.3	4.9	4.2	27.4	0.5	1.0	1.9
27	0.7	4.2	8.0	—	4.5	40.1	4.6	4.5	30.3	0.8	0.7	0.7
53	0.8	5.6	6.0	—	6.3	35.8	2.9	4.1	34.7	1.5	0.6	0.4
						Liver						
10	0.3	1.5	11.8	—	3.4	41.6	3.6	2.3	22.7	0.8	2.0	9.8
13	0.4	1.1	11.8	0.4	1.8	39.3	4.0	3.3	25.4	0.7	1.4	8.4
16	0.3	1.2	9.5	0.3	1.7	41.0	4.4	6.6	25.6	0.6	1.1	6.8
19	0.4	1.9	9.8	—	2.8	40.8	5.7	5.5	25.5	0.6	1.1	5.4
22	0.6	0.9	11.8	0.5	1.3	42.7	4.9	5.2	23.5	0.7	1.2	6.2
27	0.4	1.0	17.3	0.4	1.4	38.7	8.1	7.6	18.4	1.0	1.3	4.0
53	0.6	1.3	21.5	0.4	1.9	41.6	4.2	8.9	11.8	1.3	1.2	2.9
Egg	—	—	17.5	—	—	29.8	16.3	8.7	12.2	1.3	4.0	8.0

[a]Percentages represent the mean of duplicate analyses. Agreement between percentages for the major components was ± 5% and ± 10% for minor components.

[b]16:0A and 18:0A represent aldehydes which eluted as double peaks with 15:0 and 17:0 methyl esters, respectively. The aldehydes represent more than 75% of the combined percentages in all cases.

matic change as development progressed. Up to the sixteenth day, five days before hatching, the level of C-20 and higher fatty acids remained low; after this time, the percentages of these acids increased significantly. Stearate levels in brain sphingomyelin increased from 24% of the total acids at the earliest time period to 75% by the fifty-third day, and 16:0 levels decreased by more than 80%. In contrast, stearate levels of heart sphingomyelin remained constant during development, but the percentage of palmitate fell more than 50%, the difference being made up by increased levels of C-20 and higher fatty acids. Liver stearate and palmitate percentages of sphingomyelin showed abrupt changes shortly before and after hatching; however, the sum of the percentages of these two acids remained relatively constant during this time.

The fatty acid compositions of brain and liver phosphatidylinositol obtained at various stages are given in Table VII along with values for egg. This phosphoglyceride class was characterized by high levels of stearate and arachidonate at most time periods. Brain contained much higher levels of monoenoic acids than liver, and, with minor exceptions, the percentages of all brain fatty acids remained constant until a few days after hatching. On the other hand, liver phosphatidylinositol showed some dramatic changes during development. Arachidonate and docosahexaenoate percentages increased up to the nineteenth day of incubation and then decreased, while phosphatidylinositol stearate and palmitate showed the inverse relation. Except for the high level of 18:2 in egg phosphatidylinositol, the fatty acid patterns compared well with some time periods of brain and liver.

Table VIII shows the fatty acid compositions of brain and liver phosphatidylserine and heart phosphatidylserine plus phosphatidylinositol for each of the time periods. Phosphatidylserine of the tissues contained a high level of palmitate and stearate, which represented 60-80% of the total acids in most cases. Brain was easily distinguishable from heart and liver by the high level of docosahexaenoate which

TABLE VI

Fatty Acid Composition of Sphingomyelin Derived from Brain, Heart and Liver at Various Stages of Development

Days after incubation initiated	Fatty acid percentages[a,b]									
	14:0	16:0	16:1	18:0	18:1	20:0	22:0	23:0	24:0	24:1
					Brain					
10	2	46	6	24	13	2	2	---	---	---
13	2	29	10	33	11	2	2	---	T[c]	---
16	2	28	3	45	4	2	1	---	1	---
19	T	18	T	70	4	2	3	---	T	---
22	T	15	2	69	2	2	1	T	1	1
27	T	15	2	57	4	3	2	T	2	3
53	T	7	T	75	T	2	3	T	1	5
					Heart					
10	2	51	T	28	2	2	3	---	1	2
13	1	48	T	31	1	4	4	T	1	3
16	2	46	T	26	1	3	4	T	1	4
19	2	40	T	26	1	4	6	1	2	6
22	1	35	T	26	1	4	9	3	4	8
27	2	32	T	29	1	4	8	3	4	5
53	2	25	T	21	1	7	16	4	7	4
					Liver					
10	T	39	—	42	3	3	2	T	1	1
13	—	55	—	26	2	3	5	T	T	2
16	—	54	—	25	2	1	5	2	3	2
19	—	42	—	14	1	2	10	7	5	12
22	—	55	—	8	1	1	11	6	4	9
27	—	22	—	43	1	1	11	4	4	8
53	—	41	T	11	T	2	17	7	8	8
Egg	T	75	—	14	5	2	2	---	2	2

[a]Percentages represent the mean of duplicate analyses. Agreement between percentages for the major components was ± 10%.

[b]Difference between the sum of the percentages in any row and 100% represents the sum of percentages of other fatty acids that do not appear here.

[c]T = detectable levels that represent less than 0.5%.

decreased in this fraction after the nineteenth day of incubation. The high level of arachidonate in the heart phosphatidylserine plus phosphatidylinositol fractions, probably resulted from the phosphatidylinositol, because brain and liver contained high levels of 20:4 in this phosphoglyceride. Except for the early development time of brain, phosphatidylserine was composed of 80-95% C-16 and C-18 acids. Egg contained more oleate, linoleate, and arachidonate than most of the phosphatidylserine fractions.

DISCUSSION

The first article of this series (1) showed that total phospholipids represented ca. two-thirds of the total lipids of brain and heart at all stages of development up to hatching and of liver at the adult stage. The previous data also indicated that the quantity of total phospholipids relative to dry wt remained relatively constant throughout development for heart and liver, but the quantity of brain phospholipids increased as expected. The present class composition data are discussed in terms of relative composition for the sake of brevity, but these data, in conjunction with the earlier data (1), can be used to calculate the absolute amount of any phospholipid/quantity of dry wt.

Class Composition

The literature on the phospholipid composition of the developing embryo has been obtained primarily on the whole embryo (4). Quantitative analysis of individual phospholipids from specific organs, as they develop, have been limited primarily to brain and liver, and heart apparently has not been examined previously. Comparison of the class compositions of the three tissues (Tables I-III and Fig. 2) revealed that each tissue exhibited a characteristic phospholipid distribution which differed dramatically from the composition of the egg. The large difference in the composition between the egg and the embryo tissues rules

TABLE VII

Fatty Acid Composition of Phosphatidylinositol Derived from Brain, Heart, and Liver at Various Stages of Development[a]

Days after incubation initiated	Fatty acid percentages[b]										
	14:0	16:0	16:1	18:0	18:1	18:2	20:3 + 22:0	20:4 + 22:1	22:4 + 24:1	22:5	22:6
10	1.7	15.7	2.8	41.7	12.5	1.4	1.5	15.1	0.8	0.8	2.3
13	1.8	15.1	2.6	41.8	13.4	1.5	0.7	16.1	0.7	0.5	1.5
16	2.1	17.3	2.7	41.3	14.0	1.7	0.4	14.0	0.6	0.4	1.7
19	1.2	12.9	1.8	42.8	13.7	1.6	0.6	16.4	0.8	0.6	2.9
22	1.6	14.6	1.8	39.6	13.6	1.5	0.9	14.8	0.8	0.6	1.6
27	0.8	15.0	1.5	48.5	15.0	1.1	1.0	7.8	0.3	0.2	0.3
53	1.2	16.2	—	51.0	16.7	0.5	0.9	2.8	0.5	—	—
					Liver						
10	T[c]	15.9	—	67.3	3.2	1.4	—	8.5	—	—	—
13	T	15.8	—	49.7	3.4	2.3	0.4	22.1	0.6	0.8	4.1
16	T	13.3	0.5	49.1	3.5	3.0	0.4	22.4	0.5	0.6	3.2
19	T	5.9	—	45.2	1.9	1.8	0.5	37.1	0.6	1.1	4.8
22	T	14.2	0.8	56.4	4.3	4.4	0.3	12.3	0.7	0.7	1.1
27	T	22.8	1.0	50.8	8.9	5.4	1.7	6.5	0.6	—	—
53	T	17.4	0.8	66.3	3.9	6.5	1.0	3.3	—	—	—
Egg	—	13.7	—	43.5	9.0	11.7	—	16.9	—	—	—

[a]Heart phosphatidylinositol is found in the table of phosphatidylserine values (Table VIII).

[b]Percentages represent the mean of duplicate analyses. Agreement between percentages for the major components was ± 5% and ± 10% for minor components.

[c]T = detectable levels that represent less than 0.25%.

against the possibility that egg yolk phospholipids might be transported intact and deposited in a random fashion in the tissues of the embryo. This conclusion is supported further by the fatty acid data discussed later.

Brain contained ca. twice the level of phosphatidylethanolamine and half the concentration of phosphatidylcholine reported previously for the developing chick brain (5) and cerebrum (6). The discrepancy between our data and that reported previously is not readily apparent, but improved analytical techniques since the earlier results were obtained and differences associated with the strain of chicks used could be responsible. The high levels of phosphatidylethanolamine and phosphatidylserine found in brain easily distinguished it from heart and liver phospholipid profiles. Heart, unlike brain, contained the highest level of phosphatidylcholine until after hatching. Liver phospholipids proved to be the most dynamic during development: phosphatidylcholine levels nearly doubled during development, while phosphatidylethanolamine showed the inverse relationship. Feldman and Grantham (7) have shown that the quantity of phosphatidylethanolamine/g liver decreased as development progressed; however, the relative percentages were not given. Miyamoto, et al., (6) examined the phospholipid composition of liver at 8, 13, and 20 days of incubation but failed to observe a significant decrease in phosphatidylethanolamine. Although their values for liver phosphatidylcholine and phosphatidylethanolamine, especially at the early time periods, differ from the data reported here, they did detect the high levels of phosphatidylinositol that characterize the phospholipid profile of this tissue (Table III, Fig. 2). The solvent front material, primarily diphosphatidylglycerol, also containing other phosphorous compounds, such as phosphatidic acid, occurred at high concentrations in liver before hatching. Heart, except just before and after hatching, also contained relatively high levels of the phosphorous containing material in the solvent front. A high level of diphosphatidylglycerol, a phosphoglyceride found in high amounts in mitochondria, correlates with the presence of functioning mitochondria in these tissues early in development (8).

The data demonstrate that high and low levels of some phospholipids, which are characteristic of some mature tissues, also exist in embryonic tissue early in development (see phosphatidylinositol and phosphatidylserine, Fig. 2). They also show that some tissue phospholipid classes change in concentrations as development progresses. This point will be discussed in more detail in the next section.

TABLE VIII

Fatty Acid Composition of Phosphatidylserine Derived from Brain, Heart, and Liver at Various Stages of Development

Days after incubation initiated	Fatty acid percentages[a]										
	14:0	16:0	16:1	18:0	18:1	18:2	20:3 + 22:0	20:4 + 22:1	22:4 + 24:1	22:5	22:6
						Brain					
10	0.6	16.8	1.1	43.2	8.4	1.2	1.0	2.8	3.4	4.0	11.5
13	0.7	14.9	1.2	40.4	7.3	1.0	0.8	2.9	3.5	4.8	15.0
16	0.6	12.1	1.0	44.3	7.2	1.2	1.1	3.0	3.4	3.8	16.9
19	0.4	10.0	0.8	46.5	7.7	1.2	0.8	2.8	2.6	3.5	17.6
22	0.3	10.0	—	55.6	9.6	0.7	1.0	1.9	2.4	2.2	8.4
27	0.3	8.7	0.8	55.6	12.1	0.7	0.9	2.0	3.3	2.0	5.4
53	0.9	9.6	—	61.6	13.9	1.0	0.3	0.4	1.4	1.1	1.5
						Heart (phosphatidylinositol + phosphatidylserine)					
10	0.5	17.2	—	56.0	9.2	1.8	—	11.2	0.4	—	1.1
13	0.8	17.9	—	54.2	9.2	2.6	—	11.1	0.6	0.3	1.1
16	0.7	13.9	—	43.5	6.8	4.0	0.3	20.5	0.9	0.7	1.7
19	0.6	13.0	—	45.7	7.3	5.9	—	19.9	0.5	0.6	1.1
22	0.8	15.8	—	49.0	9.1	5.3	0.2	12.1	0.4	0.5	—
27	0.8	10.6	—	58.5	6.7	5.5	0.2	9.4	0.3	0.1	—
53	0.6	13.1	—	45.2	7.7	11.2	2.2	13.2	0.7	—	—
						Liver					
10	2.4	24.7	0.8	56.0	6.3	1.1	—	3.2	—	—	—
13	1.7	13.9	—	48.9	4.7	2.6	—	11.6	2.0	2.1	4.7
16	0.7	13.2	—	55.9	5.2	4.0	—	8.5	0.4	1.3	1.8
19	1.4	16.2	—	57.4	5.9	2.9	—	6.0	—	—	—
22	1.6	16.4	2.7	48.1	5.4	3.6	—	6.6	0.8	1.5	1.5
27	1.8	25.2	—	50.2	7.2	3.0	—	2.4	0.7	—	—
53	0.6	22.0	0.8	51.1	5.5	10.0	0.5	3.8	0.4	0.3	—
Egg	—	20.4	—	34.2	20.7	9.0	—	9.4	—	1.8	3.9

[a]Percentages represent the mean of duplicate analyses. Agreement between percentages for the major components was ± 5% and ± 10% for minor components.

Fatty Acid Composition of Phospholipid Classes

Brain phospholipids, except sphingomyelin, showed only minor changes in their fatty acid composition until after hatching. This was not unexpected, since the turnover of lipids in this tissue is slow and maximum growth rate occurs around the fifth day of incubation (4), well in advance of the first collection time period. The changes that occurred in the fatty acid percentages of brain after hatching were the most prominent in 22:6, particularly phosphatidylethanolamine and phosphatidylserine which contained the highest level of this acid of all the tissue phospholipid classes. This abrupt change in the level of 22:6 after hatching suggests that a significant turnover of this acid occurred between day 19-22, because the percentage drop was too large to be attributed to a dilution effect due to increased quantities of other lipids. Except for 22:6 at the adult stages, the fatty acid percentages reported previously for phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine of the developing chick cerebrum (6) show very good agreement with the values of this study.

Saturated and monoenoic fatty acids of heart phospholipid classes showed little change in percentage composition as development progressed. However, the level of 22:6 in phosphatidylcholine and phosphatidylethanolamine decreased with increased development. The decrease in the level of 22:6 correlates very well with the decrease in heart cell mitotic index as hatching time approaches. After hatching, the heart increases in size primarily by the enlargement of cells (8). The apparent correlation between the level of 22:6 and mitotic index does not appear to be an isolated incident but also applies to liver.

The fatty acids of all the phospholipid classes, except phosphatidylethanolamine, exhibited significant change in liver before hatching, and all phospholipid classes showed change after hatching. The level of 22:6 decreased in all the phospholipid classes, however, the available data on the mitotic index of embryonic chick liver for comparison is not as good as for heart (8). It has, however, been established that

the mitotic index of both epithelial and endothelial cells decreases sharply as hatching time approaches (8). The previously reported fatty acid composition of phosphatidylethanolamine and phosphatidylcholine of the developing embryo liver (6) agreed reasonably well with the present values, but the composition reported by Feldman and Grantham (7) contained considerably less arachidonic acid and did not show the presence of other C-20 and C-22 acids.

Long chain aldehydes, products of plasmalogens, were found in all three tissues, but only in significant amounts in phosphatidylethanolamine (Table V). The quantities in brain increased as development increased, which agrees with that reported previously by Yanamoto (9). Miyamoto, et al., (6) previously has shown the presence of aldehydes in brain and liver phosphatidylethanolamine. A more detailed study of the alkyl and alk-1-enyl glyceryl ethers of developing rat and chick tissues will appear in a separate publication.

The data demonstrate that each phospholipid class of each tissue exhibits a characteristic fatty acid composition in the developing chick embryo within 10 days and probably much earlier. With few exceptions, the fatty acid composition of all egg phosphatides differed from the tissue phosphatides at all stages of development. From the data reported here, it would appear that the phospholipid classes of the three tissues examined do not incorporate intact preformed phospholipids directly from the yolk. This conclusion agrees with the data reported by Budowski, et al., (10) from a study on the distribution of labeled glycerol and fatty acid moieties in the total phospholipids of several tissues. Hevesy, et al., (11) concluded some 35 years ago from some of the earliest work with radioactive phosphorous and phosphorous labeled hexosemonophosphate that practically all of the phosphorous in the chick embryo had passed through the inorganic stage. However, more recently, Siek and Newburgh (12) concluded from experiments, where several labeled lipids and precursors were used, that some yolk phospholipids were incorporated into embryonic brain without the hydrolysis of the phosphodiester bonds. These data do not rule out the possibility that select molecular species of some phosphatides might be incorporated into the embryonic brain without hydrolysis, but, because of the differences in fatty acid composition between the phosphatides of egg and embryonic brain, only a small percentage would meet the molecular specifications without hydrolysis and reesterification. The fact that the fatty acid composition of yolk phospholipids does not change during incubation (6) also rules against very much selective incorporation of specific yolk phospholipids into embryonic tissue without hydrolysis.

If hydrolysis of the yolk lipids occurs, as would appear to be the case, where does synthesis take place in the embryo? According to Siek and Newburgh (12), who failed to give a suitable reference, Davison suggested that phospholipids might be synthesized in the liver and transported to other organs. That might seem logical, since it is known that the liver becomes ladened with fat; however, the lipid was found not to be phospholipids or triglycerides but sterol esters (1, 7, 13). The first article of this series (1) showed that the quantity of total phospholipid/unit of liver dry wt remained relatively constant throughout development. The uniqueness of the molecular species found in the individual phospholipid class of each tissue would not allow the phospholipids to be synthesized in an organ and transported to other tissues without a unique absorption system in each tissue with specificity for only certain molecular species of each class. The data of this study suggest, at least for the three tissues examined, that the biosynthesis of the complex phospholipids occurs in situ. There is considerable evidence already in the literature to support this hypothesis. Miyamoto, et al., (14) has shown that labeled acetate and a number of long chain fatty acids injected into the yolk at the tenth day of incubation gave rise to phosphoglycerides 48 hr later with specific activities in the brain equal to, in most cases and some times greater than, those of the liver. It also has been suggested by Goodridge (15) that the liver fatty acid synthesizing enzymes are present in the new chick in a latent form and are stimulated by dietary glucose. Petzold and Agranoff (16) have demonstrated that a particulate fraction from embryonic chick brain catalyzes the synthesis of cytidine diphosphate diglyceride, a key intermediate in the synthesis of several phosphoglycerides. Although the evidence cited is not complete enough to establish that all phosphatides can be synthesized in all tissues, it does rule against the lipid synthesis in a specific organ, particularly the liver, and transport to other tissues during embryonic development.

Embryonic vs Tumor Lipids

One objective in examining the lipids of embryonic tissue is to determine whether abnormalities observed in the lipids of neoplasms are associated with neoplasia or rapid cell proliferation. The results from this study shed

some light on the stated objective.

Generally, phosphatidylcholine and sphingomyelin of hepatomas show the greatest percentage change relative to liver: decreased levels of phosphatidylcholine and elevated levels of sphingomyelin (17-20). Examination of liver phosphatidylcholine values during embryonic development (Tables I-III and Fig. 2) shows that the levels were lowest when cell proliferation was the greatest and the highest at maturity. These data suggest that the lower levels of phosphatidylcholine found in most hepatomas are associated with the rapid cell proliferation process. On the other hand, the elevated levels of sphingomyelin in hepatomas probably are not associated with rapid cell proliferation, since all three of the embryonic tissues exhibited the most change in composition and concentration after cell proliferation had ceased or had become very slow. The increase in sphingomyelin may result from an increased availability of precursors due to altered glycosphingolipid metabolism in neoplastic cells (21-23).

The phospholipids of most hepatomas (17, 20, 24, 25), some hepatoma plasma membranes (26), and a number of other neoplasms (27-29) have been shown to contain decreased levels of either and many times both C-20 and C-22 polyunsaturated fatty acids. The phospholipid classes of all three embryonic tissues (Tables IV, V, VII, VIII) contained the highest levels of these acids when cell proliferation was the most rapid, indicating that decreased level of polyunsaturated acids found in neoplasms is not associated with the rapid growth process. Most tumors (17, 24-26, 28) show an increased level of oleic acid, particularly in phosphatidylcholine. The level of 18:1 in the chick embryonic liver remained low in phosphatidylcholine, as well as the other phosphatides throughout development. Comparison of the present data with that of phospholipids from neoplasms suggests the increased levels of 18:1 in the phospholipids of neoplasms are likewise not related to the growth process.

Usually only saturated and monoenic fatty acids of 16-26 carbon-atoms are found in sphingomyelin; however, the analyses of sphingomyelin from Ehrlich Ascites cells (30) and from hepatoma cells cultured on media containing free linoleic acid (17) revealed a high level of 24:2 acid. Sphingomyelin fatty acids from all three embryonic tissues did not contain detectable levels of 24:2 acid, suggesting that such an acid is not associated with the rapid cell proliferation process.

Comparison of differences in lipid structure between normal mature tissue and neoplastic tissue with embryonic tissues provides a means of separating the apparent abnormalities in lipid metabolism into those related to rapid growth and those related directly or indirectly with neoplasia. The exploration of the difference in lipid metabolism between embryonic and normal mature tissues possibly could be just as important as the pursuance of those abnormalities which appear more directly related to neoplasia.

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SHORT COMMUNICATION

Effect of Phospholipase A upon Brain Cholesterol Ester Formation

ABSTRACT

On addition of snake venom phospholipase A to homogenates of rat brain we have been able to show increased ester formation. Within certain limits, the amount of ester formed was dependent upon the amount of phospholipase added. The fatty acid patterns of free fatty acid released and of cholesteryl ester fatty acid formed during 18 hr incubation were compared. Although fatty acid patterns were different, the data were still consistent with the hypothesis that, in demyelination, a neural phospholipase A may play a role in cholesteryl ester deposition.

INTRODUCTION

For many years, neuropathologists have been aware that cholesteryl esters were the major component of the sudanophilic material found in developing and demyelinating nervous tissue (1). The origin of the steryl ester has, however, been a matter of speculation. In degenerating tissue, it has been proposed that the ester is made by infiltrating macrophages, but Werb and Cohn (2) have shown that macrophages can absorb and hydrolyze, but not synthesize, steryl ester. It seems likely, therefore, that cholesteryl ester synthesis occurs endogenously in the developing and degenerating nervous tissue. Two mechanisms have been proposed: cholesteryl esters may be formed directly from free fatty acid and cholesterol or by acylation from phosphatidyl choline mediated by a lecithin-cholesterol acyltransferase comparable to that found in plasma (3). Eto and Suzuki (4) have suggested that the latter system might be operational but, unlike previous authors, were unable to find any such activity in the normal adult rat brain (5).

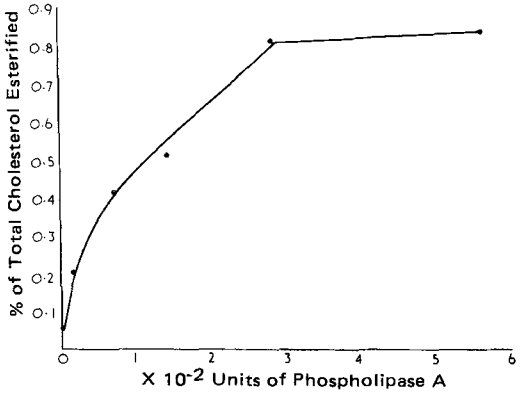

FIG. 1. Stimulation of cholesteryl ester formation by homogenates of adult rat brain by addition of snake venom phospholipase A.

In demyelinating brain, there is increased activity of lysosomal enzymes, such as acid proteinase (6), acid phosphatase (7), and glycosidases (8). Phospholipase A (EC 3.1.1.4) (9) is localized in cerebral lysosomes, and lysolecithin does accumulate in demyelinating nervous tissue (10-12). This enzyme could liberate free fatty acid and could be responsible for cholesteryl ester formation, if the free fatty acid pathway were operational. Further evidence for the possible involvement of phospholipase A is recent work by Webster (13) which has shown increased phospholipase A activity in sciatic nerve undergoing Wallerian degeneration.

EXPERIMENTAL PROCEDURES

Phospholipase A (activity 4 units/mg protein) derived from *Vipera russelli* was purchased from Sigma Chemical Co., St. Louis, Mo. Rats, Norwegian hooded variety, weighed at least 250 g. Homogenates of rat brain were prepared in a medium of 0.15 M phosphate buffer at pH 5.5, which is optimal for rat brain ester synthesis (5). Each incubation contained 205

mg wet wt of rat brain in 1 ml. The phospholipase A was added in a suspension of aqueous glycerol 50:50 (v/v). In controls, a corresponding volume of glycerol-water was added to give a final volume of each incubation of 2.0 ml. Since the reaction rates were likely to be slow (5), the incubations were carried out for 18 hr at 37 C in a shaking incubator. The incubations were terminated by addition of chloroform-methanol (2:1, v/v) and the lipids thereby extracted. Extracts were taken down to dryness, dissolved in chloroform, applied to a silicic acid column, and the neutral lipids were eluted with more chloroform.

The cholesterol and cholesteryl ester contents of each incubation were determined simultaneously by gas liquid chromatography, as described by Ikekawa, et al. (14). Chromatography was carried out on a Perkin-Elmer F-11 gas chromatograph with an Infotronics CRS-208 digital integrator using a 70 cm x 4 mm inside diameter glass column packed with 3% OV-17 on Chromosorb W HP 80-100 mesh (Phase Separations, Ltd., Queensferry, England). The column was programed from 105-280 C at 5 C/min with a carrier gas flow of 60 ml nitrogen/min. Free fatty acids were isolated from the neutral fraction by thin layer chromatography (TLC) utilizing a solvent system of petroleum ether:ethyl ether:glacial acetic acid (82:18:1, by volume). The R_f of free fatty acid was ca. 0.40. The fatty acid was eluted and methylated using 14% (w/v) boron trifluoride in methanol (Phase Separations, Ltd.) following the procedures of Morrison and Smith (15). Methylated fatty acids were resolved on a 2 m x 4 mm inside diameter column packed with 10% (w/w) diethyleneglycol succinate on Celite (80-100 mesh). All assays were carried out in duplicate.

RESULTS

As shown in Figure 1, less than 0.1% of cholesterol in rat brain homogenate is esterified. However, incubation of brain homogenate in the presence of phospholipase A leads to a 10-fold increase in the percentage of cholesterol esterified. When the tissue was incubated in the absence of phospholipase A, the ester present after incubation was ca. 20% less than the zero time level. No esterification was found when cholesterol was incubated only with phospholipase A.

Presumably, the steryl ester formation is related to the amount of free fatty acid being liberated by the phospholipase. Eto and Suzuki (5) already have shown this to be true on adding exogenous free fatty acid to brain suspensions. Qualitative examination by TLC showed that increasing amounts of free fatty acid were present with increasing addition of enzyme. Very little phospholipase A needed to be added to show increased cholesteryl ester. The smallest quantity of enzyme used, shown in Figure 1, was 1.4×10^{-3} units of activity (1 unit will hydrolyze 1 μmole of lecithin/min at pH 6.5 and 37 C). In this study, the pH is not optimal for the phospholipase, but, if it were, this amount of enzyme would be hydrolyzing lecithin at the rate of 1.4 nmoles/min (or 1.5 μmoles/18 hr). This rate of hydrolysis is ca. that described by Cooper and Webster (16) for endogenous brain phospholipase A at pH 5.5. In the present incubations, however, brain phospholipase A_1 and A_2 should be relatively inactive, because taurocholate is required for their in vitro activation (16).

The fatty acid composition of the steryl ester and the free fatty acid present after incubation was examined in pooled samples. There was no apparent relationship between the ester and free fatty acid components. The percent fatty acid composition of the free fatty acid was as follows: C_{16}, 23.6; $C_{16:1}$, 0.4; C_{18}, 8.2; $C_{18:1}$, 48.2; $C_{18:2}$, 0.3; $C_{20:1}$, 4.1; and $C_{20:4}$, 15.1. The percent fatty acid composition of the steryl ester fraction was as follows: C_{16}, 34.7; $C_{16:1}$, 12.0; C_{18}, 6.6; $C_{18:1}$, 27.5; $C_{18:2}$, 4.8; $C_{20:1}$, trace; and $C_{20:4}$, 14.4. At least a portion of the ester being examined, however, was present in the tissue prior to incubation. The fatty acid content of the ester was similar to that described by others for young adult rat brain steryl esters (17). However, the fatty acid profile of the free fatty acids more resembled the fatty acids esterified to cholesterol as a result of demyelination (18). Since the esterifying enzyme preferentially utilizes oleic acid as substrate (5), a free fatty acid pool rich in oleic acid would allow the formation of steryl ester enriched in oleic acid. If such a pool were made available in demyelinating tissue through the action of phospholipase A, it would account for the higher amounts of oleic acid in steryl ester in demyelinating tissue as compared to normal tissue (18). The source of the palmitoleic acid found in steryl esters is not clear, for nervous tissue contains very little of this unsaturated fatty acid (19). Palmitic acid may be desaturated and esterified to cholesterol (18).

Our work shows that cholesteryl ester synthesis is stimulated by release of fatty acid in the brain. It also is suggested that in demyelination increased activity of hydrolytic enzymes, e.g. phospholipase A, may be the ultimate driving force for steryl ester formation.

ROBERT B. RAMSEY
Department of Neurology
St. Louis University
St. Louis, Missouri 63104
A.N. DAVISON
Department of Neurochemistry
Institute of Neurology
The National Hospital
Queen Square
London, WC1N 3BG England

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Comparison of Pyrrolidides with Other Amides for Mass Spectral Determination of Structure of Unsaturated Fatty Acids

B.Å. ANDERSSON[1], **W.H. HEIMERMANN**, and **R.T. HOLMAN**, The Hormel Institute, University of Minnesota, Austin, Minnesota 55912

ABSTRACT

Amides of unsaturated fatty acids give mass spectra which indicate the locations of the double bonds. A survey of several amides of oleic acid was made to evaluate which amide might be most suitable for routine use in elucidation of structure. Methods of preparation of several amides of oleic acid are presented with mass spectral and gas chromatographic data. Tertiary amides have most easily interpretable mass spectra which indicate the position of the double bond in the fatty acid chain. The pyrrolidide is advantageous for fatty acid gas liquid chromatography and mass spectrometry analyses because it can be prepared easily in high yield on a microscale, it is volatile enough for gas liquid chromatographic separations, and it has a simple and easily interpretable mass spectrum which indicates the structure.

INTRODUCTION

Mass spectrometric fragmentation patterns of unsaturated straight chain fatty acids are influenced by the nature of the carboxyl group substituent. Amides have been found to exert a charge stabilizing effect upon the carboxyl containing fragments of the fatty acid derivative produced through electron impact (1-3). These derivatives, therefore, give very simple mass spectrometric cleavage patterns from which the position of the double bond can be deduced (3). In contrast to the commonly used derivatizations of the double bond (4), appropriate modification of the carboxyl group has advantages of speed, simplicity, and direct applicability to mono- and polyunsaturated acids.

The present study was undertaken to determine which amide is most suitable for structural analysis of fatty acids. Different methods of preparation of amides and data obtained from low resolution mass spectrometry (MS) and gas chromatography (GLC) were evaluated and compared. Oleic acid was chosen as the model compound.

EXPERIMENTAL PROCEDURES

Methyl oleate, oleoyl chloride, and triolein were supplied by the Lipids Preparation Laboratory, Hormel Institute, Austin, Minn. Azetidine was purchased from Eastman Kodak Co., Rochester, N.Y., carbodiimides from Pierce Chemical Co., Rockford, Ill.; dimethylamine from Fisher Scientific Co., Fairlawn, N.J.; and the other amines from Aldrich Chemical Co., Milwaukee, Wisc.

Low resolution mass spectra were recorded on a Hitachi Perkin-Elmer RMU-6D single focusing instrument at an ionization potential of 70 eV. The amides were volatilized in an all glass heated inlet system kept at temperatures varying from 170-190 C.

The gas chromatograms were obtained with an F&M model 810 instrument furnished with columns 8 ft x 1/8 in. aluminum packed with 10% diethyleneglycol succinate (DEGS-PS) on 80-100 Supelcoport (Supelco Co., Bellefonte, Pa.), 10% SILAR 10C on 100-120 Gas Chrom Q (Applied Science Laboratories, State College, Pa.), 10% ethylene glycol succinate siloxane (EGSS-X) on 100-120 Gas Chrom Q (Applied Science Laboratories), or 15% ethylene glycol succinate (EGS) (Analabs, North Haven, Conn.) on 100-120 Gas Chrom P (Applied Science). Column temperatures were kept isothermal at 170 C and 220 C (DEGS-PS), 170 C and 240 C (SILAR 10C), or 170 C (EGSS-X and EGS), and the flow rate was 15 ml argon/min.

A GLC-MS combination also was used in which the GLC conditions were the same as mentioned above. Thin layer chromatography (TLC) for check of purity was done on silver nitrate impregnated silica gel plates with diethyl ether-Skelly F mixtures as developing solvents.

RESULTS

Mass spectra of amides: Because the mass spectral characteristics are the overriding requirement, the mass spectra of a series of amides were evaluated prior to preparative methods and GLC characteristics.

Primary amide: The low mass region of the spectrum of octadec-9-enamide (Fig. 1, Table I) shows the very typical ions m/e 59 and 72

TABLE I

Relative Retention Times (RRT) and Key Fragments in Mass Spectra of Amides of Oleic Acid

Amide	RRT[a]		Base peak		McLafferty Rearr. peak		Peaks indicating the double bond position								Molecular peak		
							C_7		C_8		C_9		C_{10}				
	SILAR 10C 240 C	DEGS-PS 220 C	m/e	Relative intensity	m/e	Relative intensity	m/e	Relative intensity	m/e	Relative intensity	m/e	Relative intensity	m/e	Relative intensity	m/e	Relative intensity	Other prominent peaks (m/e)
Primary																	
Octadec-9-enamide	4.4	4.7	41	100	59	71.0	128	2.9	140 142	2.6 1.4	154 156	2.1 1.1	168	1.2	281	4.5	83,263
Secondary																	
N-n-Propyl octadec-9-enamide	5.1	2.9	43	100	101	78.0	170	9.5	182 184	3.0 3.2	196 198	4.5 2.0	210	3.5	323	21.0	114
N-iso-Propyl octadec-9-enamide	3.5	1.8	101	100	101	100	170	12.0	182 184	3.7 3.6	196 198	5.1 1.7	210	4.3	323	30.0	*43,114
N-n-Butyl octadec-9-enamide	6.0	3.7	128	100	115	97.0	184	13.4	196 198	4.0 4.7	210 212	7.8 2.0	224	4.7	337	35.3	
N-Cyclopropyl octadec-9-enamide	9.4	6.1	57	100	99	46.2	168	4.5	180 182	2.0 1.6	194 196	2.5 1.0	208	2.1	321	14.2	112,303
Tertiary																	
N,N-Dimethyl octadec-9-enamide	2.9	1.6	87	100	87	100	156	11.7	168 170	1.7 2.7	182 184	3.0 .9	196	4.1	309	29.2	72,100
N,N-Diethyl octadec-9-enamide	2.6	1.5	115	100	115	100	184	15.3	196 198	1.4 2.8	210 212	3.2 1.0	224	4.7	337	39.8	100
N,N-Di-n-propyl octadec-9-enamide	2.7	1.8	72	100	143	27.0	212	9.8	224 226	.8 1.8	238 240	2.0 .6	252	2.8	365	29.0	114,156
N,N-Di-iso-propyl octadec-9-enamide	2.0	1.3	86	100	143	11	212	4.0	224 226	.9 .7	238 240	1.1 .5	252	1.2	365	16.0	322
N,N-Di-n-butyl octadec-9-enamide	3.3	2.4	86	100	171	2.3	240	8.1	252 254	.8 1.4	266 268	1.7 .5	280	2.4	393	27.2	129,156,184
N-Octadec-9-enoylazetidine	6.9	4.0	99	100	99	100	168	11.0	180 182	1.7 3.0	194 196	2.9 1.0	208	3.6	321	19.0	112
N-Octadec-9-enoylpyrrolidine	8.2	4.8	113	100	113	100	182	11.4	194 196	1.0 2.5	208 210	2.3 .7	222	3.6	335	24.8	
N-Octadec-9-enoylpiperidine	6.8	4.2	127	100	127	100	196	12.8	208 210	1.2 2.7	222 224	2.5 .9	236	4.1	349	32.0	140
N-Octadec-9-enoylhexamethyleneimine	8.8	5.8	141	100	141	100	210	22.7	222 224	2.0 4.4	236 238	4.1 1.7	250	6.9	363	57.5	126,154

[a]Relative to methyl tetracosanoate = 1.

TABLE II

Quantitative Analysis of Corn Oil Fatty Acids Using Pyrrolidides and Methyl Esters and Retention Times of Pyrrolidides Relative to 18:0

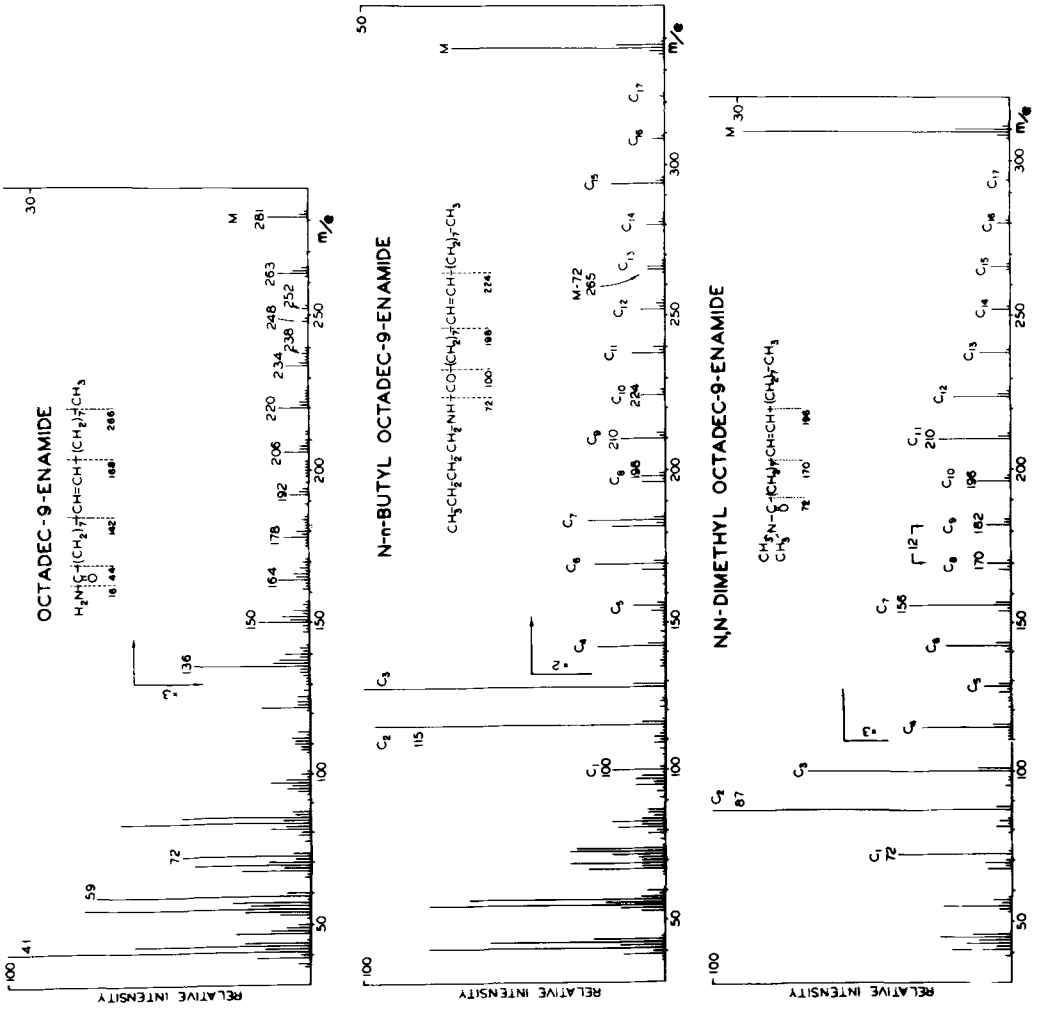
Fatty acids	16:0	18:0	9-18:1	9,12-18:2
		Percent fatty acid		
Pyrrolidides by aminolysis				
SILAR 10C, 240 C	11.4	1.7	25.0	61.9
DEGS-PS, 220 C	11.3	1.7	24.4	62.6
Methyl esters by interesterification ($NaOCH_3$) (12)				
EGS, 170 C	11.0	1.6	24.4	63.0
Methyl esters by interesterification (BF_3) (13)				
SILAR 10C, 170 C	11.0	1.4	23.8	63.8
DEGS-PS, 170 C	11.2	1.7	24.3	62.8
EGSS-X, 170 C	10.9	1.6	24.4	63.1
		Relative retention times, min		
Pyrrolidides				
SILAR 10C, 240 C	.7	1.0	1.2	1.5
DEGS-PS, 220 C	.6	1.0	1.1	1.4

reported by Gilpin (5). The diagnostically useful fragmentation pattern, wherein the molecular ion (m/e 281) yields ions containing the polar part of the molecule (m/e 266, 252, 238, etc.), as is the case with pyrrolidides (3), is diminished by the presence of another series of fragments. The latter series dominates and starts with M-18 (m/e 263), followed by a cleavage of the fatty acid chain at each bond (m/e 248, 234, 220, 206, etc.). The overlapping of these two series of fragments makes it difficult to locate the double bond using the logic applicable to pyrrolidides.

Secondary amides. The spectra of the four secondary amides show pronounced molecular ions with clear fragmentation of the fatty acid chain and charge retention on the amide group. The McLafferty rearrangement ions, for example, m/e 115 in N-n-butyl octadec-9-enamide (Fig. 1, Table I) are dominant. Removal of the amine group occurs (m/e 265) and ions of type $R-C\equiv O^+$ are created. This is more pronounced in the spectrum of the N-cyclopropylamine derivative than in the spectra of the amides of the acyclic amines. The position of the unsaturation still is indicated by the peak cluster, including the first eight carbon atoms from the fatty acid chain. Ions formed by a further migration of the double bond toward the amide group under electron impact (3), together with contribution of ions from the fragment series derived from $R-C\equiv O^+$ (m/e 265) confuses the interpretation by the rule formulated for pyrrolidides.

Tertiary amides: The derivatives formed from alicylic amines give pronounced fragments containing the polar part of the molecule, and the molecular ions vary from 16-57% of the base peak (Table I). The dominant peak in all their spectra is the McLafferty rearrangement ion (m/e 113 for N-octadec-9-enoylpyrrolidine in Fig. 1). The position of the double bond in all these amides can be deduced by the rule formulated for pyrrolidides (3). Ions derived from $R-C\equiv O^+$ (m/e 265), which is formed by elimination of the amine group, make only a minor contribution to the fragmentation pattern. The spectra from tertiary amides of acyclic amines show the same fragmentation pattern as mentioned for the alicylic amides, except N,N-di-iso-propyl octadec-9-enamide, which has a prominent ion at m/e 322, indicating loss of a propyl residue. Additional major peaks in the low mass region, described by others (5,6), are derived from the ion $R-\overset{+}{N}H=CH_2$, where R is one of the alkyl groups of the amide, for instance, m/e 72 ($CH_3-(CH_2)_2-\overset{+}{N}H=CH_2$) in N,N-di-n-propyl octadec-9-enamide.

Methods of preparation of amides: Judging from the literature (7-9), the only method that gives a good yield is aminolysis. Because of their mass spectral advantages, special effort was put on synthesis of tertiary amides. When aminolysis was performed on an analytical scale with acetic acid as catalyst (3), quantitative yields were obtained only with azetidine and pyrrolidine. Preparation of amides according to Jordan and Port (8), using sodium methoxide as catalyst, gave good yields with dimethylamine and the heterocyclic amines. With the acid chloride method involving the formation of the acid chloride of the fatty acid, a quantitative yield is difficult to obtain. Couplings with two


OCTADEC-9-ENAMIDE
N-n-BUTYL OCTADEC-9-ENAMIDE
N,N-DIMETHYL OCTADEC-9-ENAMIDE
RELATIVE INTENSITY
m/e

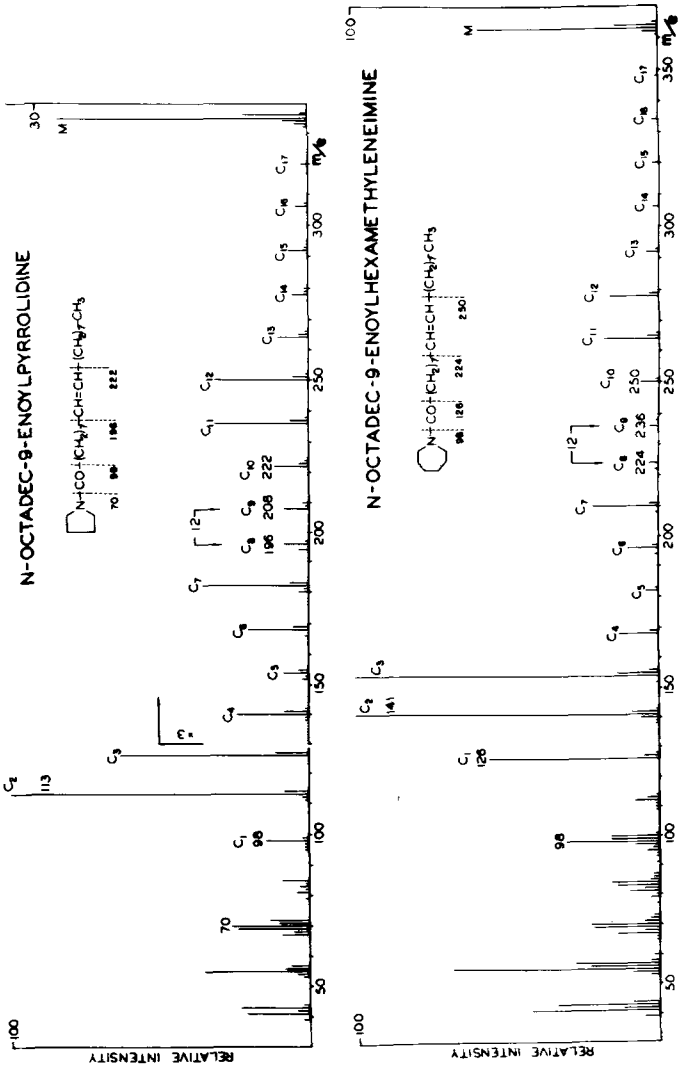


FIG. 1. Mass spectra of primary, secondary, and tertiary amides of oleic acid.

carbodiimides (10,11) were done, but for this method the free fatty acids and the equimolar amounts of the reagents are necessary; and the yields were not quantitative.

A direct conversion of triglycerides to amides was performed on a pure fraction of corn oil triglycerides to give a quantitative yield of fatty acid pyrrolidides. The pyrrolidides of the component 16:0, 18:0, 9-18:1, and 9,12-18:2 acids were separated on DEGS and SILAR 10C, and their retention times relative to 18:0, together with the analytical data obtained, are given in Table II. The data are compared with the analyses of methyl esters by the methods according to Metcalfe, et al., (12) and Morrison, et al. (13).

Gas chromatography: Compared with the corresponding methyl esters, amides of fatty acids have much longer retention times under the same working condtions. DEGS, EGS, and SILAR 10C have been investigated as stationary phases for the amides. SILAR 10C was the most suitable, because it can be used at a higher temperature than can DEGS and EGS. A DEGS that could be used up to 225 C and SILAR 10C which can be used up to 275 C were tested, and relative retention times for different amides with these phases are given in Table I. Under the conditions described above, the retention times for N-octadec-9-enoylpyrrolidine were 24 min on SILAR 10C at 240 C and 47 min on DEGS-PS at 220 C.

DISCUSSION

Of the several amides of oleic acid studied, tertiary amides have the advantage of revealing most clearly in their mass spectra the position of double bonds in a fatty acid, because contributions from competing fragmentation pathways are least.

Fragmentations of amides occur at each bond of the fatty acid chain, with the positive charge located on the amide containing fragment (3). In the competing fragmentation, initial loss of the amine groups creates charge retaining fragments $R-C\equiv O^+$ which are cleaved secondarily at another bond of the chain to produce a series of ions. These latter fragmentations are more prominent with the primary and secondary amides than with tertiary amides. The double bond seems to be mobile in all derivatives after electron impact, and little difference between the amides in this regard was observed. However, the peaks from the competitive fragmentation mentioned above increase the intensities of the satellite peaks 2 amu before or after the main peak in each cluster, so that the localization of the double bond is made more difficult without high resolution MS. The hexamethyleneimide derivative (Fig. 1) gave a spectrum which had the highest intensities of the diagnostic and molecular ions, the latter being (m/e 363) 57% of the base peak. The N,N-dimethylamine derivative had the least complicated spectrum (Fig. 1) with a relative intensity of the molecular peak (m/e 309) of 29% and almost no interference from secondary fragmentation pathways. From point of view of their mass spectra, the tertiary amides are preferred for diagnosis of double bond positions.

The lower acyclic amides had the shortest retention times in GLC and might, therefore, be preferred because of the relatively low temperature limits for stability of these stationary phases which are able to separate by degree of unsaturation, such as DEGS and EGS. However, methods of preparation of the amide derivatives also must be considered.

Azetidine and pyrrolidine are the only amines that we have found to give quantitative yield of amide with fatty acid methyl esters using acetic acid as catalyst (3). The former presently is ruled out as a routine reagent, because it is more volatile and more difficult to handle than pyrrolidine. The reactivity of these two is probably also due to steric factors by which hindrance is minimized because of the ring size (14). GLC behavior of the amides supports this theory when DEGS or SILAR 10C is used as stationary phase. The pyrrolidide has a longer relative retention time than the piperidide (Table I), although the latter has the higher mol wt. The smaller ring size of the pyrrolidide probably makes it easier for the polar groups in the stationary phases to interact with the amide group, wheras the methylene groups close to the nitrogen in the larger size ring of the piperidide shield the amide group. Jordan and Port (8) came to a similar conclusion when sodium methoxide was used as catalyst. Here, the heterocyclic amines and dimethylamine reacted rapidly with good yields, probably because of minimized steric hindrance at the nitrogen atom of the amines.

Dimethylamide could be a useful derivative for fatty acids of simple structure because of its volatility, simple mass spectrum (Fig. 1), and quantitative preparation. However, from the mass spectrometric point of view, it is desirable that the amine part of the molecule be as large as possible. If fatty acids having hydroxy, epoxy, or other charge retaining groups are to be analyzed, these groups induce low mass competitive secondary fragmentations which obscure the diagnostic ions. Therefore, the latter should be moved as far as possible into

the high mass region. A similar argument has been used by Bricas, et al., (15) where long chain fatty acids were used instead of small groups for N-acylation of peptides. Of course, this will cause volatility problems, but, with appropriate selection of stationary phase for the GLC analysis (SILAR 10C), pyrrolidine is the amine of choice for mass spectrometric analysis of structure of fatty acids in general. The introduction of fluorine atoms in the heterocyclic ring to increase volatility and mass (16) may be of even more advantage. The only known limitations upon the method proposed here are the difficulties in the preparation of α- and β-unsaturated and α-branched amides of fatty acids in a quantitative yield by the method described (3). The α- and β-unsaturated amides might better be prepared via the acid chlorides.

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Induction of Essential Fatty Acid Deficiency in Mouse Brain: Effects of Fat Deficient Diet upon Acyl Group Composition of Myelin and Synaptosome-Rich Fractions during Development and Maturation

G.Y. SUN, J. GO and **A.Y. SUN,** Laboratory of Neurochemistry,
Ohio Mental Health and Mental Retardation Research Center, Cleveland, Ohio 44109.

ABSTRACT

A fat-deficient diet was initiated in mice before birth and at different ages during development and maturation. The induction of an essential fatty acid deficiency in brain was most effective when the deficient diet was initiated prenatally. With increasing time on the deficient diet, there was an increase in 20:3(n-9) and a decrease in 20:4(n-6) in the phosphoglycerides of subcellular brain fractions. The highest ratio of 20:3(n-9) to 20:4(n-6) observed was 1.5 for both diacyl and alkenylacyl glycerophosphorylethanolamines in the synaptosome-rich fraction from mice on the deficient diet from before birth to 7 months of age. The acyl groups of brain ethanolamine plasmalogens are quite susceptible to alteration by the fat-deficient diet. Elongated products of 20:3(n-9), tentatively identified as 22:3(n-9) and 22:5(n-9), also were present in brain during essential fatty acid deficiency. These fatty acids were preferentially linked to the alkenylacyl glycerophosphorylethanolamines. It further was observed that, even when the deficient diet was initiated after maturation, similar changes in fatty acid composition occurred in both myelin and synaptosome-rich fractions, but at a slower rate. For mice on the deficient diet from 12-18 months of age, the highest ratio of 20:3(n-9) to 20:4(n-6) was 0.6 for the alkenylacyl glycerophosphorylethanolamines from the myelin.

INTRODUCTION

Alteration of the nonpolar side chains of membrane phospholipids during essential fatty acid (EFA) deficiency has been well recognized in different body organs (1-4), as well as in brain tissue (5-9). In the past, different methods have been employed by individual investigators for the induction of EFA deficiency in brain (6,10). Although it generally is known that EFA deficiency in brain may be most effectively induced during the prenatal period, initiation of the fat-deficient diet during this period may not be desirable due to the high prenatal mortality rate (10). On the other hand, brain fatty acids may not be vulnerable to dietary changes when the deficient diet is initiated after the critical period for brain development (11). The purpose of the present study is to define the degree of EFA deficiency in selected brain membranes during different stages of development and maturation and after different periods of dietary deficiency. We have selected the acyl group compositions of alkenylacyl and diacyl glycerophosphorylethanolamines (GPE) for detailed examination during EFA deficiency, since these two types of ethanolamine phosphoglycerides are rich in long chain polyunsaturated fatty acids (12) and are thought to be metabolized by different pathways in brain (13,14).

MATERIALS AND METHODS

Pregnant C57BL/10J mice were purchased from Jackson laboratory (Bar Harbor, Ma.). Ca. 1 week prior to delivery, the experimental animals were given a fat-deficient diet (General Biochemicals, Chagrin Falls, Ohio). Control animals received the same diet supplemented with 2% corn oil (8). Similar experiments were initiated in other groups of mice at 1, 4 and 12 months of age.

At the end of each experimental period, three control and three experimental animals were decapitated, and the brains were dissected for subcellular fractionation and lipid analysis. The brains were homogenized individually in 20 volume of 0.32 M sucrose containing 1 mM of ethylenediaminetetraacetic acid, 1 mM $MgCl_2$, and 15 mM Tris buffered at pH 7.4. Myelin and synaptosome-rich and microsomal fractions were isolated by differential and sucrose gradient centrifugations, as described previously (12), except that a discontinuous sucrose gradient was used for isolation of the synaptosome-rich fractions. The synaptosome-rich fraction was isolated from the 0.8-1.2 M sucrose interface. The crude myelin fractions were purified further by osmotic shock, reflotation, and

TABLE I

Acyl Group Composition of Diacyl Glycerophosphorylethanolamines in Synaptosome-Rich Fraction of Mouse Brain during Essential Fatty Acid-Deficiency[a]

Acyl groups	Control	Prenatal			1 month	12 months	
	4 months	3 weeks	4 months	7 months	4 months	15 months	18 months
				Percentage area			
16:0	8.1	8.0	6.9	7.5	6.4	8.9	8.4
18:0	34.6	32.2	31.1	30.4	32.9	32.8	34.2
18:1	12.6	12.5	17.7	13.6	13.2	15.7	15.7
20:1	-	0.5	1.2	-	-	0.9	0.8
20:3(n-9)	-	3.3	9.9	12.5	4.5	1.1	3.1
20:4(n-6)	13.5	19.2	11.9	8.2	11.0	13.5	12.8
22:3(n-9)[b]	-	-	0.9	2.2	-	0.3	0.7
22:4(n-6)	4.5	3.3	1.1	2.0	3.7	3.4	2.3
22:5(n-6)[c]	3.0	2.6	2.0	3.7	2.3	3.8	3.5
22:6(n-3)	23.8	17.4	17.3	19.9	26.4	19.8	18.6
	(3)	(3)	(3)	(2)	(2)	(2)	(2)
				Ratios of 20:3(n-9)/20:4(n-6)			
		0.17	0.83	1.52	0.41	0.08	0.24

[a]The ages were: (top) ages at which the deficient diet was initiated and (bottom) ages at which the mice were sacrificed for subcellular fractionation and lipid analysis. Results are mean percentage area from 2-3 determinations (indicated in parentheses) of individual subcellular fractions isolated from each of 3 mouse brains.

[b]Chain length and unsaturation tentatively identified.

[c]Included a small amount of 22:5(n-9) in the essential fatty-acid deficient groups.

further centrifugation. The purity of the synaptosome-rich and purified myelin fractions has been assessed previously by electron microscopic examination, assays of marker enzymes, and determination of the lipid compositions (12,15).

The procedure for extraction of lipids from the membrane pellets has been described (16). Phospholipids were separated by separation-reaction-separation two-dimensional thin layer chromatography (TLC) (16) with a modified solvent system (17). The phospholipids spots were visualized by spraying the TLC plate with 2′,7′-dicholorofluorescein reagent. Acyl groups of individual phosphoglycerides were converted to methyl esters by alkaline methanolysis (18). The conditions for analysis of methyl esters by gas liquid chromatography (GLC) have been described (19). The variation in percentage areas due to GLC analysis usually did not exceed 5% of the peak area and the variation in percentage areas from different brain preparations was usually less than 10% of the peak area.

RESULTS

The acyl group profiles for the two types of ethanolamine phosphoglycerides from brain myelin and synaptosomal-rich fractions are distinctively different (Tables I-III). The diacyl GPE from the synaptosome-rich fraction were rich in 18:0 and 22:6(n-3) whereas the diacyl GPE from the myelin fraction contained high proportions of 18:0 and 18:1 instead. The acyl groups of alkenylacyl GPE in the synaptosome-rich fraction were rich in 22:6(n-3) and contained ca. equal amounts of 18:1, 20:4(n-6), and 22:4(n-6). In the myelin fraction, the alkenylacyl GPE were rich in 18:1 and 20:1 which together comprised over 50% of the total acyl groups of this phosphoglyceride. Ca. 20% of the acyl groups of alkenylacyl GPE were 20:1 as compared to only 4-5% found in the myelin diacyl GPE and 3-7% found in the synaptosomal alkenylacyl GPE.

In the present experiment, we have used 4 month old mice as controls, because results from a previous investigation have indicated little age related change in acyl group composition in mouse brain fractions after maturation (20). We also have analyzed acyl group composition of phosphoglycerides from the microsomal fractions. Results of the analysis indicated that changes in microsomal lipids during EFA deficiency were similar to the changes in the synaptosome-rich fraction. After feeding with the fat deficient diet, increasing proportions of 20:3(n-9) and decreasing proportions of 20:4(n-6) were found in the brain phosphoglyc-

TABLE II

Acyl Group Composition of Alkenylacyl Glycerophosphorylethanolamies in Synaptosome-Rich Fraction of Mouse Brain during Essential Fatty Acid-Deficiency[a]

Acyl groups	Control	Prenatal			1 month	12 months	
	4 months	3 weeks	4 months	7 months	4 months	15 months	18 months
				Percentage area			
16:0	2.4	3.4	-	5.6	3.0	2.2	2.9
18:0	2.8	3.7	3.3	5.2	2.7	2.9	1.8
18:1	15.0	12.6	21.1	16.5	16.9	19.0	18.5
20:1	5.5	3.3	7.2	3.9	6.2	6.4	6.5
20:3(n-9)	-	3.8	14.1	13.0	5.9	2.3	5.1
20:4(n-6)	18.4	20.0	12.3	8.7	12.4	15.0	13.0
22:3(n-9)[b]	-	3.3	6.1	9.6	2.3	1.3	3.1
22:4(n-6)	12.6	9.8	6.3	7.2	7.7	11.2	10.3
22:5(n-6)[c]	3.1	4.3	3.9	6.3	1.7	4.9	8.5
22:6(n-3)	40.2	35.9	30.0	24.1	41.4	31.6	30.3
	(3)	(1)	(3)	(2)	(3)	(2)	(2)
				Ratios of 20:3(n-9)/20:4(n-6)			
	-	0.19	1.15	1.49	0.48	0.15	0.39

[a]Details same as in Table I

[b]Chain length and unsaturation tentatively identified.

[c]Included a small amount of 22:5(n-9) in the essential fatty acid-deficient groups.

erides from the experimental animals. Changes in acyl group composition were observed in both types of ethanolamine phosphoglycerides in the myelin and synaptosome-rich fractions. In addition to the 20:3(n-9), increasing proportions of fatty acids, tentatively identified as 22:3(n-9) and 22:5(n-9), also were present. The proportions of 22:5(n-9) could not be assessed accurately, because it was not resolved from the GLC separation and had retention time similar to the 22:5(n-6) which was present in the controls. There may be a small increase in the proportions of 18:1; however, the increase was not consistent in all experiments.

When pregnant mice were placed on a fat-deficient diet ca. 1 week prior to delivery and then were confined to the diet while nursing, there were ca. 3% of 20:3(n-9) in the acyl groups of diacyl GPE from the synaptosome-rich fraction in the offsprings by 3 weeks. This proportion increased to 12% after 7 months on the fat-deficient diet (Table I). The ratio of 20:3(n-9)/20:4(n-6) in this phosphoglyceride increased from 0.17 at 3 weeks to 1.52 after 7 months. With the deficient diet initiated after weaning (1 month of age) and then maintained for a period of 3 months, the ratio obtained for the synaptosomal diacyl GPE was 0.41. When the deficient diet was initiated in adult mice at 12 months of age, the same ratio was only 0.24 after 6 months on the deficient diet. The proportion of 22:3(n-9) was generally higher in alkenylacyl GPE than in the diacyl GPE (Table II). When the new born mice were reared with a deficient diet for 7 months, 23% of the fatty acids in the acyl groups of synaptosomal alkenylacyl GPE were the (n-9), whereas (n-9) polyunsaturated fatty acids accounted for only 15% of the acyl groups from the synaptosomal diacyl GPE. The ratio of 20:3(n-9)/20:4(n-6) in alkenylacyl GPE in the synaptosome-rich fraction also increased from 0.19 to 1.49 during this period of EFA deficiency. When the deficient diet was initiated after weaning and maintained for a period of 3 months, the ratio for the synaptosomal alkenylacyl GPE was 0.48. However, with 12 month old mice, the ratios obtained after 3 and 6 months of fat-deficient diet were only 0.15 and 0.39, respectively.

Although the proportions of 20:4(n-6) in diacyl GPE and alkenylacyl GPE in the myelin fraction were similar, acyl group changes in myelin during the deficient state were more extensive in the alkenylacyl GPE than in the diacyl GPE (Table III). The ratio of 20:3(n-9)/20:4(n-6) for myelin alkenyl-acyl GPE was 0.81 after feeding a deficient diet to the weanlings for 3 months. This ratio was ca. twice that (0.40) for myelin diacyl GPE from the same mice. The ratio for myelin alkenylacyl GPE, 0.64, was more than 2 times the ratio for myelin diacyl GPE, 0.25, for mice on the deficient diet between 12-18 months of age.

DISCUSSION

During fatty acid deficiency, there was an

TABLE III

Acyl Group Composition of Ethanolamine Phosphoglycerides in Purified Myelin Fractions during Essential Fatty Acid-Deficiency[a]

Acyl groups	Diacyl GPE[b]			Alkenylacyl GPE		
	Control 4 months	1 month 4 months	12 months 18 months	Control 4 months	1 month 4 months	12 months 18 months
16:0	7.8	7.4	7.2	2.7	2.0	1.9
18:0	31.3	28.1	28.4	1.2	0.8	1.3
18:1	25.0	26.1	28.5	37.1	37.8	37.4
20:1	5.0	4.7	4.8	20.1	19.3	20.5
20:3(n-9)	-	4.4	2.9	-	7.1	5.5
20:4(n-6)	14.8	10.9	11.4	15.4	8.8	8.6
22:3(n-9)[c]	-	6.3	1.3	-	6.3	3.5
22:4(n-6)	4.7	2.1	3.2	12.5	6.0	8.9
22:5(n-6)[d]	1.2	0.7	4.4	2.5	2.1	5.9
22:6(n-3)	10.3	9.2	8.2	7.6	6.7	6.5
	(3)	(3)	(2)	(3)	(3)	(2)
		Ratios of 20:3(n-9)/20:4(n-6)				
		0.40	0.25		0.81	0.64

[a]Details same as in Table I.

[b]GPE = glycerophosphorylethanolamines

[c]Chain length and unsaturation tentatively identified.

[d]Included a small amount of 22:5(n-9) in the essential fatty acid-deficient groups.

increase in (n-9) polyunsaturated fatty acids and a corresponding decrease in (n-6) polyunsaturated fatty acids in the phosphoglycerides of mouse brain subcellular fractions. These diet related changes in the mouse brain are in general agreement with previous reports for rats (7-9). We also found similar diet related changes in the acyl groups of phosphoglycerides from the synaptosomal plasma membranes isolated from mouse brains (17). The increase in (n-9) polyunsaturated fatty acids in EFA-deficient brain pertains, not only to 20:3(n-9), but also to its elongated products, such as 22:3(n-9) and 22:5(n-9). The increase in 22:3(n-9) in the EFA-deficient brain mainly was associated with the alkenylacyl GPE. The elongated products may arise from a recycling process (14). During the recycling, 20:3(n-9) could be elongated, desaturated, and further incorporated into the membrane lipids. This hypothesis is similar to the results observed after intracerebral injection of labeled long chain fatty acids in brain (14).

Various degrees of brain EFA deficiency may be attained with the fat deficient-diet, depending upon the age at initiation and duration of the feeding. The degree of deficiency can be indicated by the ratios of 20:3(n-9) to 20:4(n-6) in individual phosphoglycerides (21). When the deficient diet was initiated in pregnant mice ca. 1 week prior to delivery, the ratio of 20:3(n-9) to 20:4(n-6) for synaptosomal diacyl GPE in the offspring increased steadily with age, reaching a maximum of 1.5 after 7 months (Table I). However, after maturation (at 12 months of age), the same ratio after a 6 month period on the fat-deficient diet was only 0.24. The slow rate of increase in ratio indicates that a ratio of 1.5 in brain may not be possible when mature mice are placed on a fat-deficient diet. Apparently, the degree of metabolism of polyunsaturated fatty acids decreases with increasing age.

Fatty acids of myelin phosphoglycerides also were altered, even when the deficient diet was initiated well after maturation. In fact, for 18 month old mice on the deficient diet for 6 months and for 4 month old mice on the deficient diet for 3 months, the ratio of 20:3(n-9) to 20:4(n-6) was greater in the alkenylacyl GPE of the myelin fraction than in the synaptosomal alkenylacyl GPE and in the diacyl GPE. For the diacyl GPE, the 20:3(n-9) to 20:4(n-6) ratio was the same in synaptosomal and myelin fractions. The metabolism of polyunsaturated fatty acids in oligodendroglia and central nervous system myelin appears to be at least as rapid as in the nerve endings of neurons.

Within the same deficient mice, the ratio of 20:3(n-9) to 20:4(n-6) for alkenylacyl GPE was generally higher than the ratio for diacyl GPE. Previous comparisons of the metabolic activity of these two types of ethanolamine phosphoglycerides always have shown the diacyl GPE to be more active (13), but these comparisons were made with precursors that were less direct for the plasmalogen than for the diacyl GPE.

Since (n-9) polyunsaturated fatty acids could label these two types of ethanolamine phosphoglycerides equally well, the alkenylacyl GPE actually may be as metabolically active as the diacyl GPE. Blank, et al., (4) also reported extensive changes in plasmalogen acyl groups in the rat testis during EFA deficiency. Since ethanolamine plasmalogens account for one-third of the myelin phosphoglycerides, an alteration of its acyl groups by the fat-deficient diet may affect the function of the myelin membrane and could have accounted for the higher susceptibility of EFA-deficient rats to allergic encephalomyelitis (22). In a recent study, we also have demonstrated a higher (Na^+,K^+)-ATPase activity in synaptosomal plasma membranes isolated from mice fed a fat-deficient diet (17). Effects upon membrane functions in general may be the consequence of structural alterations due to EFA deficiency.

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Study of Free and Bound Lipids of *Brassica campestris*, var Yellow Sarson[1]

MARY E. MCKILLICAN and **J.A.G. LAROSE**, Food Research Institute, Canada Agriculture, Ottawa, Canada K1A OC6

ABSTRACT

Mature seeds of *Brassica campestris* var. yellow sarson were extracted with hexane to yield free lipid. The residue then was extracted with chloroform-methanol to release bound lipid. Free and bound lipids were separated into polar and nonpolar fractions chromatographically. The nonpolar fraction of both free and bound lipid consisted mainly of triglycerides with small amounts of steryl esters, free sterols, mono- and di-glycerides, and free fatty acids. The principal components of polar bound lipid were phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, and steryl glycoside. In the free polar lipid, there was more phosphatidyl inositol and less phosphatidyl choline and phosphatidyl ethanolamine. Erucic acid content was much greater in the nonpolar fractions and in the polar free lipid than in the polar bound lipid.

INTRODUCTION

Cruciferous oilseeds have received considerable study. A survey of the fatty acids of many *Cruciferae* species has been carried out (1). More recently, Appelqvist (2) studied the fatty acid composition of *Cruciferae* species. Downey (3) has changed the chemical composition of oil and meal of *Brassica* varieties genetically. Tremazi (4) has evaluated some *Brassica* species for use as edible oils. An International Society for Fat Research-AOCS symposium (5,6) included genetic potentials and nutritive value of *Brassica* oils, as well as composition of the seeds and the uses of rapeseed as an edible oil. The production of high erucic oil for industrial use also has been considered (7). In this laboratory, we have studied the lipids of maturing seed of two varieties of *Brassica napus* and of *Crambe abyssinica* (8) and the residual lipids of meal from *Brassica campestris*, echo (9).

The present study of free and bound lipids of *B. campestris* var. yellow sarson was undertaken to provide information for comparison of yellow sarson lipids with those of other *Cruciferae*.

EXPERIMENTAL PROCEDURES

Materials

The yellow sarson seed was grown at the Canada Agriculture Research Station, Saskatoon, Canada. Samples used in this study were hand-picked free of damaged and discolored seeds.

The free lipid was extracted by grinding the seed with cold deoxygenated hexane (20 volume followed twice by 10 volume) in a Virtis homogenizer (10). The bound lipids were removed by a similar extraction of the residue with chloroform-methanol (2:1). Nonlipid material was removed by passage through columns of Sephadex G25 coarse (11-13). The sample was applied in chloroform-methanol-water (190:10:1) and the lipids removed with 20 column volumes of the same solvent. Aliquots of the free lipid and of the bound lipid were dried in vacuo to determine the yield of lipid.

Column Chromatography

Columns 2 cm outside diameter, containing 20 g 325 mesh silicic acid (Bio Sil HA, Bio Rad Labs., Richmond, Calif.) were used to separate groups of lipid components. Nonpolar lipids were removed with chloroform. A fraction containing the most readily eluted polar lipids, including steryl glycoside, was removed from the column with chloroform-methanol (99:1). Chloroform-methanol (1:1) then eluted the remaining polar lipids. When no subdivision of polar lipids was required, the polar lipids were eluted with chloroform-methanol (1:1).

Thin Layer Chromatography (TLC)

Chromatoplates 20 x 20 cm, spread with a 0.25 mm layer of Silica Gel G (Research Specialties, Co., Richmond, Calif., and later Brinkmann Instruments, Rexdale, Canada) were used for all separations. Nonpolar lipid classes were separated with hexane-diethyl ether-acetic acid (90:10:1) (14). Hexane-diethyl ether (70:30) was used with silver nitrate plates to separate fatty acid methyl esters on the basis of their unsaturation (Silica Gel G plates sprayed with 12.5% silver nitrate then reactivated).

The polar components of both free and

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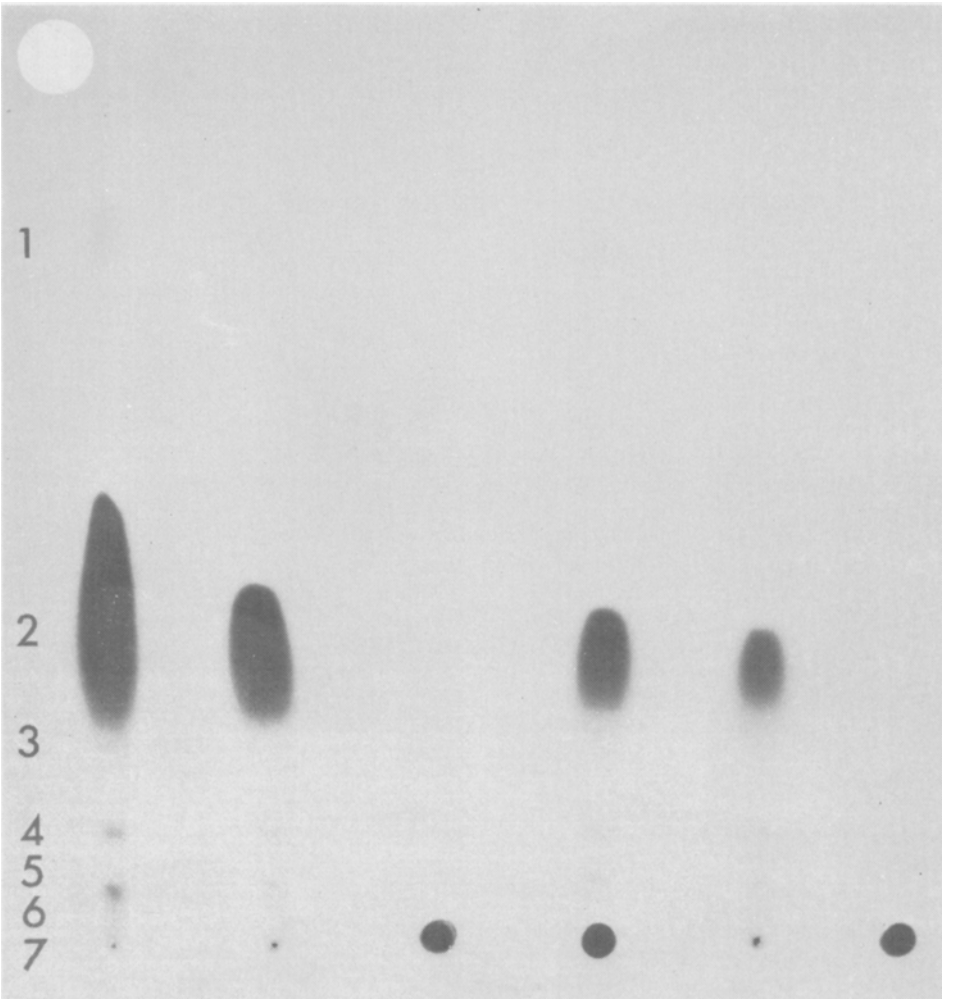

FIG. 1. Lipid Classes: free lipids–(A) total, (B) nonpolar, and (C) polar; Bound lipids–(D) total, (E) nonpolar, and (F) polar. Solvent: hexane-diethyl ether-acetic acid (90:10:1 v/v/v). Components: 1 steryl esters, 2 triglycerides, 3 free fatty acids, 4 diglycerides, 5 free sterols, 6 monoglycerides, and 7 polar lipids.

bound lipid were separated by two dimensional TLC and identified by their R_f in the four two dimensional systems described, by the specific sprays, and by cochromatography with authentic lipids (Applied Science Laboratories, State College, Pa.; Supelco, Bellefonte, Pa.; and General Biochemicals, Chagrin Falls, Ohio). The first three of the systems differed in the addition of ammonia or acetone to the chloroform-methanol-acetic acid-water solvents. The systems are: (A) Nichols system (15), chloroform-methanol-ammonia (7N) (65:30:4) in the X direction, followed by chloroform-methanol-acetic acid-water (170:25:25:6) in the Y direction; (B) Lepage system (16), chloroform-methanol-water (65:15:2) in the X direction, followed by chloroform-acetone-methanol-acetic acid-water (65:20:10:10:3) in the Y direction; (C) Rouser system (17), chloroform-methanol-14N ammonia (65:35:5) in the X direction, followed by chloroform-acetone-methanol-acetic acid-water (100:40:20:20:10) in the Y direction: and (D) System D for separating high R_f components: chloroform-methanol (90:10) in the X direction, followed in the Y direction by Marinetti's diisobutyl ketone-acetic acid-water (40:25:5) (18).

Spots were detected by means of iodine vapor (19), and, on the silver nitrate plates, a guide strip was sprayed with 2′7′ dichlorofluorescein (20). More specific sprays were: molybdate for phospholipids (21), Dragendorff for choline containing lipids (22), alpha naphthol for glycolipids (11), ninhydrin for free amino

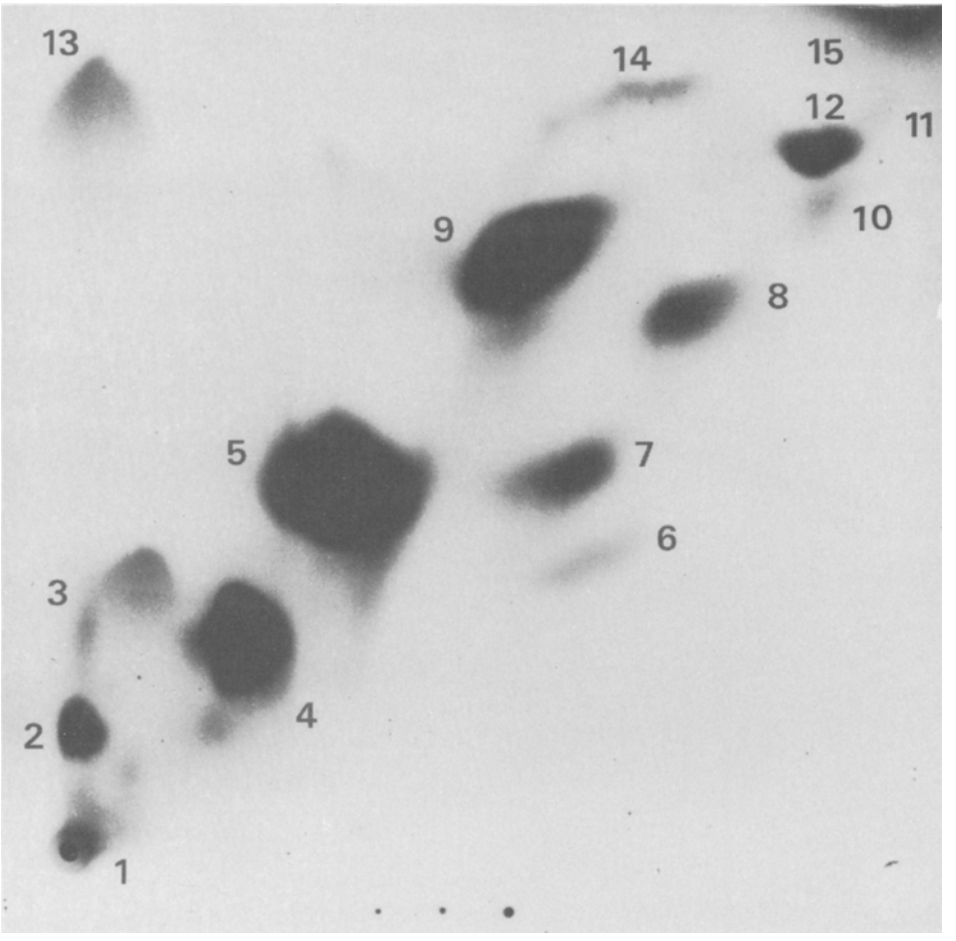

FIG. 2. Polar Bound Lipids, Nichols system. Chloroform-methanol-ammonia (7N) (65:30:4 v/v/v). Chloroform-methanol-acetic acid-water (170:25:25:6 v/v/v/v). 1. Includes lyso phosphatidyl choline, lyso phosphatidyl inositol and glycolipid. 2. Unidentified artifact. 3. Phosphatidyl serine, lower part glycolipid. 4. Phosphatidyl inositol plus glycolipid in lower part. 5. Phosphatidyl choline. 6. Sulfolipid. 7. Digalactosyl diglyceride. 8. Phosphatidyl glycerol. 9. Phosphatidyl ethanolamine. 10,11. Cerebrosides. 12. Steryl glycoside. 13. Phosphatidic acid. 14. Diphosphatidyl glycerol. 15. Monogalactosyl diglyceride, esterified steryl glycoside.

groups, and 20% aqueous perchloric acid (18) for sterol containing lipids.

Gas Chromatography (GLC)

For GLC, the methyl esters were prepared with boron trifluoride reagent (Applied Science Laboratories) according to Morrison's method (23) using the conditions given for triglycerides. The methyl esters were separated from other reactants by TLC (16).

A gas chromatograph with flame ionization detector (HCL Scientific model 1662) was used. Separations were carried out on a stainless steel 1.83 m x 3.18 mm outside diameter column with 5% diethylene glycol succinate on Gas Chrom Q 80-100 mesh at a column temperature of 175 C. The peaks were identified by retention times, cochromatography with authentic methyl esters (Applied Science Laboratories; Supelco), and preliminary silver nitrate-TLC separation of methyl esters. Quantitation of the component fatty acid methyl esters was by means of an internal standard (methyl heptadecanoate).

RESULTS

Lipid Classes

The free lipid represented 41.6% dry matter basis (DMB) of the seed wt and the bound lipid 3.7% (DMB). The ratio of nonpolar to polar was more than 95:5 in the free lipid, while in the bound lipid this ratio was ca. 1:1.

The nonpolar lipid in both free and bound

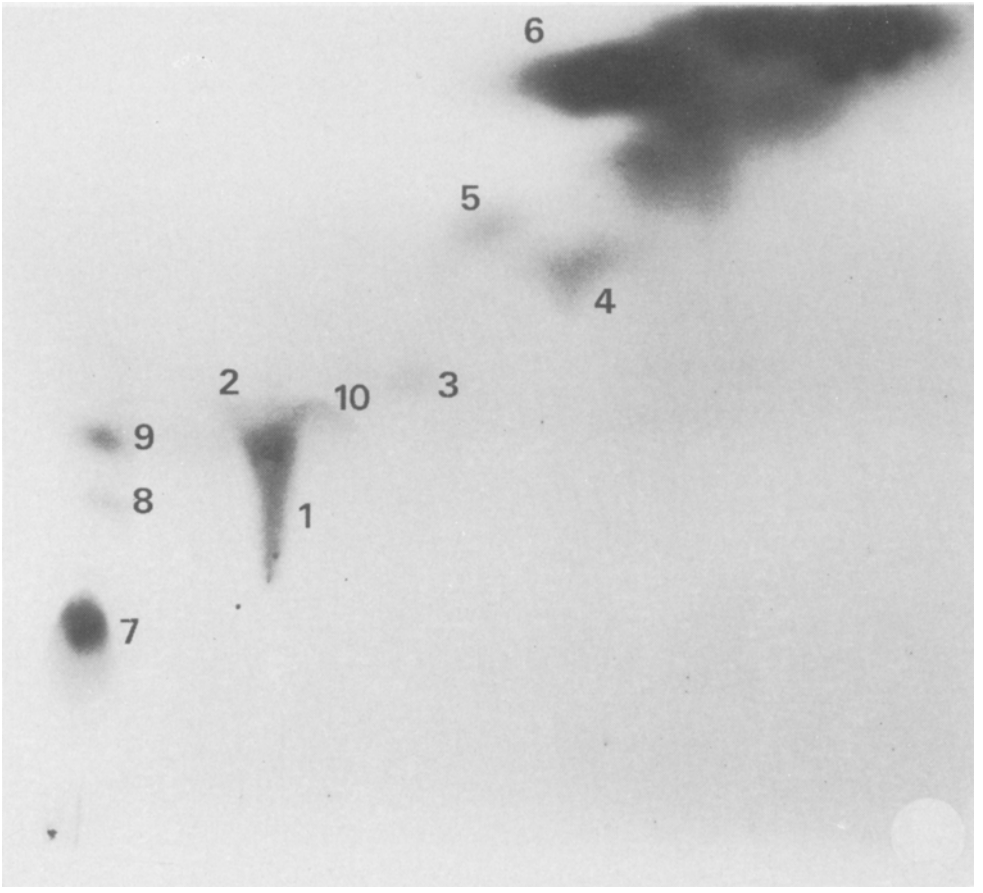

FIG. 3. Polar Bound Lipids, High R_f system. Chloroform-methanol (90:10 v/v). Diisobutyl ketone-acetic acid-water (40:25:5 v/v/v). 1. Steryl glycoside. 2. Cerebroside. 3. Monogalactosyl diglyceride. 4. Esterified steryl glycoside. 5. Unidentified, reinforced by impurity in authentic monogalactosyl diglyceride. 6. Nonpolar. 7-10. Phospholipids.

fractions was mainly triglyceride with some steryl ester, sterol, mono- and di-glyceride, and free fatty acid (Fig. 1). The small amounts of free fatty acids overlapped the triglycerides in the solvent system used (hexane-diethyl ether-acetic acid, 90:10:1). The nonpolar and polar fractions, as separated for fatty acid determinations, also are shown in Fig. 1. The separation of polar bound lipid in the Nichols system is shown in Figure 2. The main components were phosphatidyl choline (no. 5), phosphatidyl ethanolamine (no. 9), phosphatidyl inositol (no. 4) and steryl glycoside (no. 12), with phosphatidyl choline the largest single component. Unidentified glycolipids were present with phosphatidyl serine, as part of, or close to, the phosphatidyl inositol spot and as part of no. 1 near the origin. Trigalactosyl diglyceride (24) and digalactosyl monoglyceride (25) have been reported in plants, and it is possible that these lipids may be present in the unidentified low R_f glycolipids. Spot no. 2 showed without any reagents, with iodine, and with strong acid charring but not with any specific reagents. It appears to be an artifact but has not been identified.

The polar free lipid showed a somewhat similar pattern, but the proportions of the components were different. More phosphatidyl inositol and less phosphatidyl choline and phosphatidyl ethanolamine were found. More diphosphatidyl glycerol than phosphatidyl glycerol and more phosphatidic acid were found. The artifact, no. 2, in the bound was not found in the free. Figure 3 shows the separation in System D of the high R_f bound polar fraction, previously separated on a column with chloroform-methanol (99:1) from most of the nonpolar and lower R_f polar. However, some nonpolar (upper right) and phospholipids (lower

left) remained. Steryl glycoside was the largest high R_f component; esterified steryl glycoside, monogalactosyl diglyceride, and cerebroside were present. The same components were present in the free polar fraction but the proportion of sterol to glyceride glycolipids was lower. Spot no. 5, present in both free and bound polar also was present as a contaminant in the monogalactosyl diglyceride standard and may be a degradation product of monogalactosyl diglyceride.

Fatty Acid Composition

There was great similarity in the fatty acid distribution in the nonpolar lipids of the free and bound (Table I), whereas the polar of the bound lipid was quite different. The nonpolar lipids were very high in erucic, low in palmitic. The polar bound was low in erucic and higher in palmitic and linoleic. The polar free was high in erucic and moderately high in palmitic; the proportion of 18-carbon acids was similar to that in the nonpolar lipids. A shift from erucic to linoleic was found in the polar bound as compared to the other fractions, and the 24-carbon acids were found only in the nonpolar lipid.

TABLE I

Fatty Acid Composition[a]

Fatty acid	Free lipid		Bound lipid	
	Nonpolar	Polar	Nonpolar	Polar
14:0	*[b]	0.7	0.2	*
15:0	0	*	0	0
16:0	2.5	14.3	3.1	18.1
16:1	0.4	3.9	0.9	3.0
17:0	0	*	*	*
18:0	1.1	2.2	1.4	1.3
18:1	11.9	12.6	11.2	16.7
18:2	18.1	20.7	18.3	39.5
18:3	15.6	10.1	14.6	13.9
20:0	0.9	*	1.0	0
20:1	4.6	3.9	6.3	1.7
20:2	0.2	0	0.4	0
22:1	41.5	31.5	38.5	5.7
22:2	0.8	*	0.8	0
24:0	*	0	*	0
24:1	2.5	0	3.2	0

[a]Given in percentage.

[b]* = less than 0.2% and not included in percentage calculations.

DISCUSSION

The high proportion of free lipid and of triglyceride is characteristic of oil seeds, and the polar components of the bound lipid closely resembled those found in the free lipid of the *B. napus* varieties golden and oro and *C. abyssinica* (8), as well as those of the polar bound of the *B. campestris* variety echo (9). The polar free lipid contained different proportions of the same components. The polar bound of yellow sarson and echo contained more steryl glycoside and esterified steryl glycoside than the polar free lipid of yellow sarson or the *napus* varieties, whereas the *napus* varieties and the yellow sarson polar free contained nearly as much of the galactosyl glycerides as of the sterol containing glycolipids.

The fatty acid composition of the nonpolar of both free and bound lipid of yellow sarson was quite similar to that of other erucic acid containing *Cruciferae* previously studied, *C. abyssinica* and *B. napus* var. golden. In the polar, both free and bound, yellow sarson had a larger proportion of linoleic than either *Crambe* or golden rape. The 24-carbon acids were found only in nonpolar yellow sarson, the 20 and 22-carbon dienoic only in yellow sarson and the 22-carbon saturated only in *Crambe*. The yellow sarson is a high erucic species however the membrane lipids (polar bound) showed a low erucic acid content.

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Unusual Lipids II: Head Oil of the North Atlantic Pilot Whale, *Globicephala melaena melaena*

JONAS BLOMBERG, Institution of Medical Biochemistry, University of Göteborg, Göteborg, Sweden

ABSTRACT

Pilot whale head oil (blackfish head oil, raw) was analyzed by means of IR spectroscopy, NMR, thin layer chromatography, and gas liquid chromatography-mass spectrometry. The oil consisted of hydrocarbons (mainly pristane) (3%); waxes and cholesterol esters (9%); triglycerides (87%) (i.e. non-11%, mono-19% and di-57% isovalero triglycerides) and cholesterol and diglycerides (1%). By mass spectrometry, the diisovalero triglycerides were shown to be mainly symmetrical. Fatty acids were iso-branched or normal (only traces of ante-iso acids were found), saturated, or monounsaturated. Isovaleric acid predominated (54 mole % fatty acids), the rest having 10-18 carbon atoms. A 5-carbon fatty acid was the only acid found in the waxes. The alcohol composition qualitatively resembled that of the fatty acids, but major quantitative differences were present. This rules out direct interconversion of all fatty acids and alcohols. The possible role of these lipids in ultrasound transmission is discussed.

INTRODUCTION

The pilot whales (genus *Globicephala*) are distributed widely and show such small inter-species differences that only one (1) or a few (2,3) morphological species differences seem to be recognized. They are ranged in the subfamily Orcinae (ca. 8 species) of the large dolphin family (Delphinidae, ca. 43 species) (4).

The first chemical investigation of an oil from a pilot whale (*Delphinus globiceps = G. m. melaena*) (1,5) was reported by Chevreul in 1817 (6). He discovered that the saponified oil contains a volatile, strongly smelling acid, which he first called acide delphinique (7,8), and later acide phocenique (9). This acid, now known to be 3-methyl-butanoic (isovaleric) acid (10), was the first branched chain acid to be isolated from a lipid. Its occurrence in fat from many toothed whales has been confirmed by several authors (9-17).

The amounts found differ widely between species. An isomer, 2-methylbutanoic acid, has been found in body oil from the common dolphin (*Delphinus delphis*) (12).

The present investigation describes the general lipid composition of head oil of the pilot whale, and the results are discussed in the light of the possible role of these lipids in ultrasound transmission (18,19).

EXPERIMENTAL PROCEDURES

The sample of pilot whale head oil (William F. Nye and Co., New Bedford, Mass., blackfish head oil, raw) was a slightly yellow, free flowing oil with no detectable rancid odor.

TABLE I

Column Chromatography of Pilot Whale Head Oil (448.5 mg) on Silica Gel

Lipid class	Wt mg	Solvent	Volume ml
–	---	0% Diethyl ether in light petroleum[a]	100
Hydrocarbons	15.1 mg	0%	200
–	---	1% Diethyl ether in light petroleum[a]	50
Wax and cholesterol esters	39.8	2% Diethyl ether in light petroleum[a]	200
		5% Diethyl ether in light petroleum[a]	250
Triglycerides	388.4	7% Diethyl ether in light petroleum[a]	100
Triglycerides		10% Diethyl ether in light petroleum[a]	100
Triglycerides		15% Diethyl ether in light petroleum[a]	100
Cholesterol and	4.5	20% Diethyl ether in light petroleum[a]	100
Diglycerides		30% Diethyl ether in light petroleum[a]	100
–		100% Diethyl ether in light petroleum[a]	100
Total	447.9 mg		

[a]This solvent was used for all listed below.

TABLE II

Column Chromatography of Pilot Whale Head Oil Unsaponifiables (10.1 mg) on Aluminium Oxide

Lipid class	Wt mg	Solvent	Solvent ml
Hydrocarbons	2.8	1% Diethyl ether in light petroleum	200
Alcohols	6.0	10% Diethyl ether in light petroleum	300
Fatty acids	0.9	2% Acetic acid in diethyl ether	200
Total	9.7		

Most probably, it originated from animals caught in Newfoundland. It was stored at 4 C in the dark. The physical constants determined were d^{25}_4:0.92, n^{21}_d:1.452, α^{d}_{21}:÷0.06 ± 0.02 C (neat). Saponification value: 289.5, iodine value (Wijs): 22.5.

Chromatography

The oil was analyzed by column chromatography on silica gel and aluminium oxide, and by thin layer chromatography (TLC) in silica gel.

For a typical silica gel column chromatogram, a column (120 x 27 mm) of Mallinckrodt CC-4 200-325 mesh silica gel, activated at 150 C overnight was packed in light petroleum (40-60 C). The elutions were performed as described in Table I.

For preparation of alcohols from unsaponifiables, aluminium oxide (standardisiert zur chromatographische adsorptionsanalyse nach Brockman, Merck, Darmstadt, Germany) was packed into a 100 x 27 mm column, in light petroleum. Chromatography was performed as described in Table II.

For subfractionation of triglyceride subclasses, columns (250 x 10 mm) of Mallinckrodt CC-4 200-325 mesh silica gel activated at 150 C overnight was packed. Crude triglyceride (50-400 mg) subfractions from the first silica column was placed on the column. Elution was performed in 0.5% steps of diethyl ether in light petroleum, starting from 0%, using 200 ml solvent in each step.

The fractions were monitored by TLC, and fractions containing most of each subclass were pooled and subjected to another column chromatogram, as described above. The selection of fractions to be pooled together was made wide to prevent selective losses of material as much as possible.

By this procedure, the non-, mono-, and diisovalero triglycerides were obtained in a pure state, i.e. TLC gave one spot under the conditions described. For TLC, glass plates covered with 0.25 mm Silica Gel H (Fluka) were used. The plates were not activated before use. The eluting solvent was varied according to the separation desired. Light petroleum (40-60 C)-diethyl ether-acetic acid 80:20:1 gave better resolution of the triglycerides; 90:10:1 was better for hydrocarbon, cholesterol esters, and wax. Detection was made by iodine vapors, if not otherwise indicated.

Synthesis of Derivatives and Reference Triglyceride

Methyl esters were made by dissolving the oil in (A) 0.5% sodium methoxide in methanol for 1 hr at room temperature, or (B) 1-3% hydrochloric acid (HCl) in methanol produced by dissolving an appropriate amount of acetyl chloride in methanol, after which the oil was added and the mixture heated on a boiling water bath for 1 hr.

These transesterifications were carried out in closed glass tubes with teflon-faced screw caps to minimize losses of the volatile isovaleric acid esters. The reported amount of isovaleric acid, 54 mole % of fatty acids is derived from the room temperature tubes, as the heated tubes gave ca. 5% lower values, probably through minor leaks.

Transesterifications were complete, as judged by TLC.

Isopropyl esters were prepared by dissolving the oil in isopropanol containing 1-3% HCl and heating for 2-3 hr.

Acetates were obtained by dissolving the alcohols in acetyl chloride and heating on a boiling water bath for 15 min. The acetyl chloride was evaporated, leaving nonvolatile acetates in the tube. Trimethylsilyl ethers were prepared by a previously published procedure (20). Hydrocarbons were obtained from the purified alcohols by first making tosylates in pyridine, then reducing the reaction mixture directly with lithium aluminium hydride, without isolation of the tosylates. A violent reaction occurred once, probably because the pyridine was not dry enough. The fat-soluble products from this reaction were purified by column chromatography. The asymmetrical di-n-pentano myristin was synthesized by the trifluoroacetic anhydride procedure (21) from isopropylidene glycerol.

Saponification

This was done as suggested earlier (22). The

TABLE III

Fatty Acid Composition of Pilot Whale Head Oil, in Mole %[a]

Type of triglyceride	Percentage including C5 acids	Percentage excluding C5 acids			
	All (the whole oil)	0-i	1-i	2-i	All (the whole oil)
No. of measurements	7	1	1	1	5
ai 5:0 appr.	0.2				
i 5:0	54				
i, monounsaturated, and normal 10-13	3	Tr	Tr	1	1
i 14:0	4	5	3	13	10
14:1	0.8	1	1	6	2
14:0	6	12	11	15	13
i 15:0	9	7	12	20	19
15:0	0.2	1	1	0.5	0.5
i 16:0	3	4	7	3	6
16:1	11	24	24	33	24
16:0	3	17	19	2	9
i 17:0 + i 18:0	0.6	2	2	0.4	3
18:1	5	24	19	5	11
18:0	0.6	2	1	1	1
Higher than 18:0	0.2	1	Tr	Tr	Tr

[a]0-i, 1-i and 2-i denote non-, mono-, and diisovalero triglycerides, respectively. Tr = trace, ai = ante-iso, and i = iso. Runs with detection of isovaleric acid were done separately.

oil (3.0 g), 1.5 g sodium hydroxide, 1.5 ml distilled water, and 10 ml ethanol were heated at reflux for 1.5 hr. The saponification mixture then was partitioned between water, light petroleum, and a little ethanol. The partition gave a rough separation of acids and alcohols, and, to obtain a pure product, column chromatography on aluminium oxide was necessary (*vide supra*) (Table II). In the partition, the light petroleum phase contained 10% wt of the oil. The water phase contained glycerol, which was identified by gas liquid chromatography (GLC)-mass spectrometry (MS) and IR spectroscopy). No other polyhydroxy water-soluble compounds were detected in this phase.

Apparatus

Physical constants, NMR (60 MHz), and IR spectra were obtained on standard commercial instruments. GLC was performed on two instruments. Fine separations were made on a Perkin-Elmer 900 gas chromatograph, equipped with glass capillary columns made at this institute. The stationary phase was SE-30 silicone. For triglycerides and wax, a glass column 2 m long, packed with 1% SE-30 on 80-100 mesh Chromosorb W AW-DMCS (Hewlett-Packard, Avondale, Pa.) was used.

A GLC-MS combination instrument designed at this institute (23) was used, when mass spectra were desired. Briefly, the mass spectrometer was single-focusing, equipped with a 60° magnetic deflection system and a jet separator. GLC columns were made from 4 mm inside diameter aluminium tubing, usually 2 m long. Stationary phases were SE-30, OV-1, and Reoplex 400.

Quantitative determinations were made by triangulation of GLC peaks. Mass spectra were counted manually and compared with an internal reference (perfluorokerosene) in some cases. Percentage calculations and drawings of the spectra were done by a computer.

RESULTS

Spectroscopy

The oil gave a proton magnetic resonance spectrum with unusual features in the methyl region (0.9-1.1 ppm). This consisted of several separate resonance lines. Two ca. equal peaks at 0.9 and 1.1 ppm predominated, resembling the isopropyl peaks from isobranched fatty acids (24). Assignments are not evident from the present work.

The IR spectrum was rather similar to that of coconut oil (25), a triglyceride oil with few double bonds and roughly 14 carbon atoms in the average fatty acid. However, the *Globicephala* oil had stronger bands near 8.0 μ and 7.3 μ and additional bands near 7.75, 8.4, 9.2, and 9.75 μ. A shoulder near 3.35 μ and a strong complex absorption around 7.3 μ was indicative of the presence of branching methyl groups.

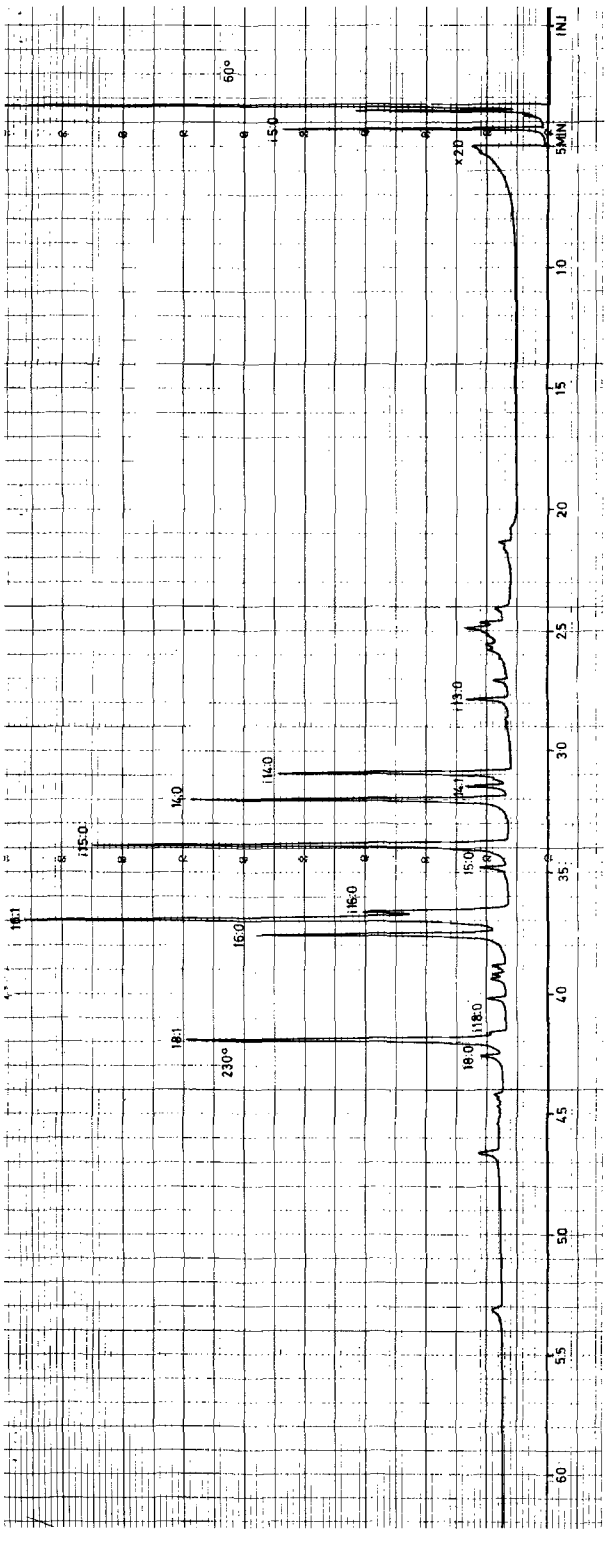

FIG. 1. Gas chromatogram of methyl esters from blackfish head oil (raw), i.e. pilot whale head oil. OV-1 on a 58 m glass capillary column. 60-230 C, 4 C/min.

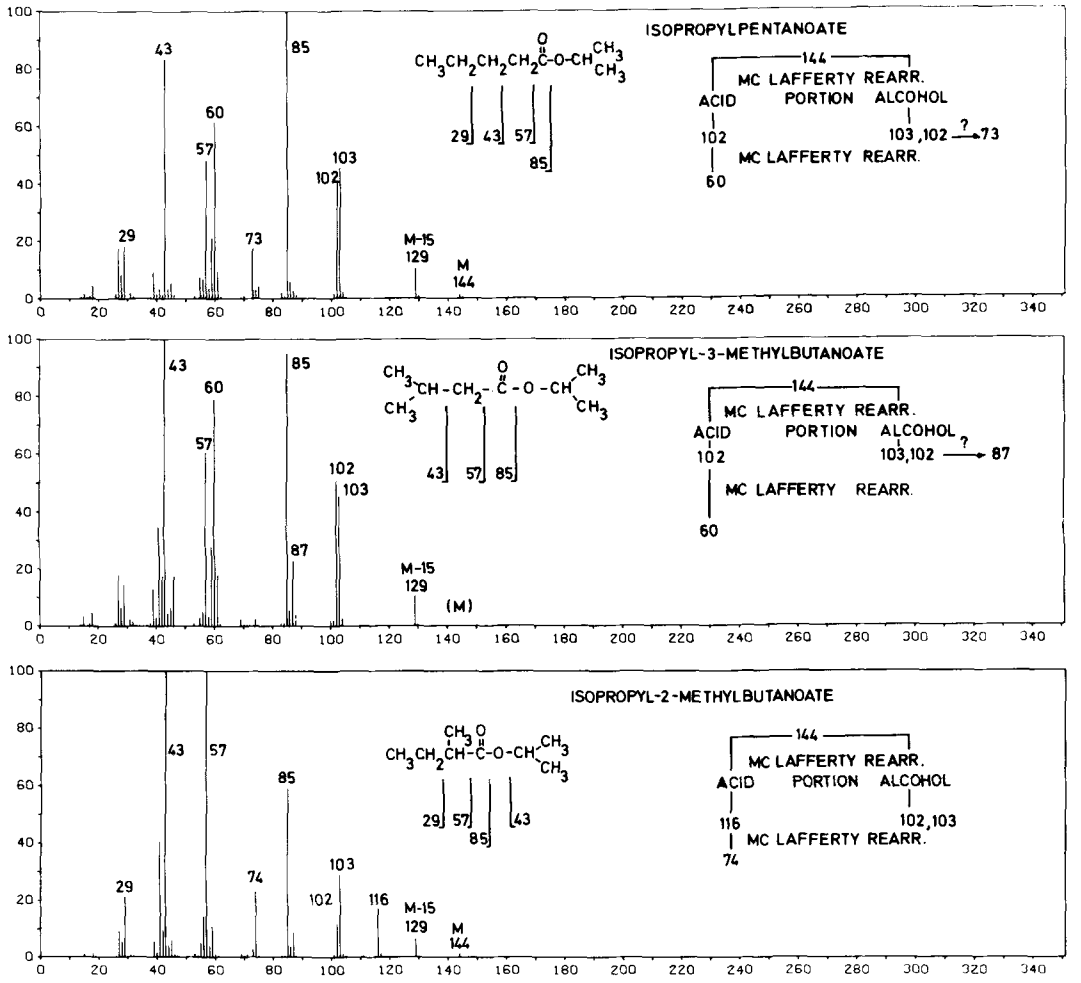

FIG. 2. Mass spectra of pentanoic, 3- and 2-methylbutanoic acids as their isopropyl esters. Electron energy: 20 eV. Source temperature: 100 C.

These spectra are obtainable on request from the author.

Fatty Acid Composition of Entire Oil (Table III and Fig. 1)

The fatty acid composition of the oil was investigated by means of GLC-MS of methyl and isopropyl esters.

The shorter fatty acids were analyzed as the isopropyl esters on a 5 m Reoplex column at 60 C. By comparison of retention times and mass spectra with that of the authentic compounds, 3-methylbutanoic (isovaleric) and 2-methylbutanoic acids were identified (Fig. 2). The longer fatty acids were analyzed as free acids, isopropyl esters, and methyl esters. The best separations were obtained with the methyl esters. The longer fatty acids proved to be either isobranched saturated or normal saturated and monounsaturated. On capillary columns, trace amounts of anteiso acids could be tentatively identified by retention time data. The isopropyl esters gave characteristic mass spectra with M,M-42,M-59, and M-74. The 3 biggest peaks were generally m/e 43,102 (i.e. 74 + 28), and M-42. Unsaturated esters gave a pattern differing mainly in the absence of the strong peaks at m/e 102, analogous to unsaturated methyl esters, which generally lack the base peak at m/e 74.

In the case of the longer fatty acids, the isopropyl derivatives were not as good as methyl esters for the detection of isobranching. This was detected by comparison with spectra of reference normal, iso, and anteiso branched esters obtained from Applied Science, State College, Pa., (BC Mix L) and by the well known fragmentation rules for branched hydrocarbon chains.

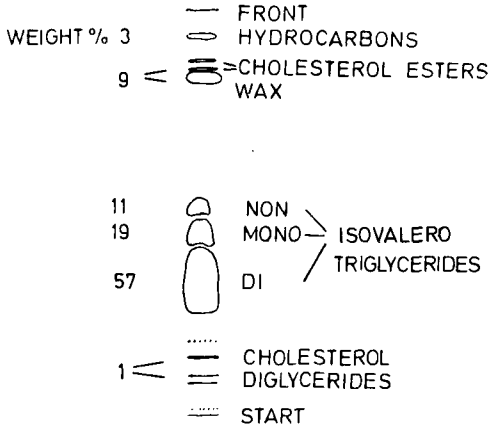

FIG. 3. Thin layer chromatogram of blackfish head oil, raw (pilot whale head oil). 0.25 mm Silica Gel H (Fluka). Solvent: Light petroleum-diethyl ether-acetic acid 80:20:1. Plate not activated before use.

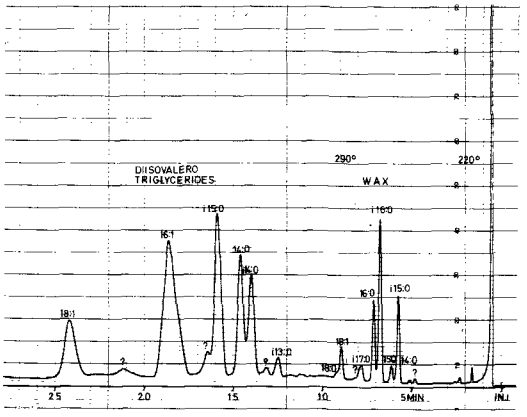

FIG. 4. Gas chromatogram of blackfish head oil, raw (pilot whale head oil). 1% SE-30. Glass column, 2 mx long, 3 mm inside diameter, 220-290 C, 10 C/min.

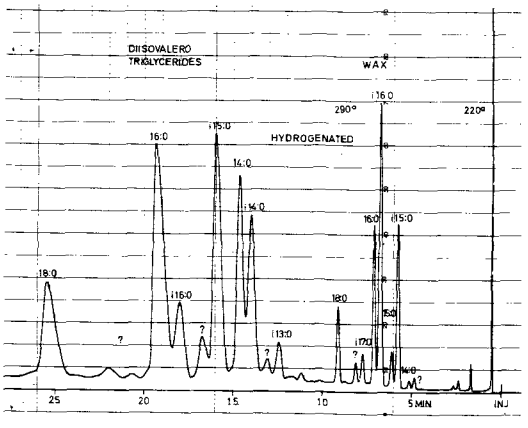

FIG. 5. Gas chromatogram of hydrogenated blackfish head oil, raw (pilot whale head oil). Same conditions as in Figure 4.

Alcohol Composition (Table IV)

Alcohols were prepared from the unsaponifiables by column chromatography on aluminium oxide (*vide supra*). They then were analyzed by GLC-MS as such, and as acetates, trimethylsilyl (TMS)-ethers, and as alcohol-derived hydrocarbons. These derivatives all gave similar percentage compositions of the alcohols. The best means of identifying the alcohols proved to be by MS of the alcohol-derived hydrocarbons. The acetates also showed the branches, but not as well.

The alcohol pattern differs from the acid pattern, but no new structural elements were found. Iso saturated, normal saturated and monounsaturated alcohols occur. With the acetates and TMS-ethers, cholesterol and some unidentified peaks were detected. The latter gave mass spectra too weak for interpretation, but they could be diols like batyl alcohol. The TMS derivative of batyl alcohol gave a mass spectrum which had some resemblance to these spectra. Due to degradation during saponification and GLC, cholesterol probably was underestimated.

Analysis of Lipid Classes (Fig. 3)

The fractions from the silica gel column were further analyzed as described below.

Hydrocarbon fraction: This was injected directly into the GLC-MS instrument. A programed temperature run from 150-250 C was performed on 2% OV-1. Only one major component was detected. Judging from the chromatogram, this component makes up at least 95% of the hydrocarbons. The component was not coincident with any normal aliphatic hydrocarbon. The mass spectrum was very similar to that of pristane, which also coincided with the unknown on GLC. The hydrocarbon fraction, therefore, seems to be predominantly pristane.

Some very small peaks, difficult to distinguish from the background, possibly made up a homologous series, but their mass spectra were too weak to permit detailed examination.

Wax and cholesterol ester fraction: This fraction was analyzed by GLC-MS (Figs. 4, 5). The wax fraction gave a chromatogram which agreed closely with that of the alcohols (Table IV). Only one acid was found, a C-5 acid (Fig. 6). Most probably this was the isovaleric acid detected as the major acid of the oil. The occurrence of 2-methylbutanoic acid in this fraction was not investigated. The GLC conditions would have detected wax with up to 35 carbon atoms, i.e. most of the possible combinations of the alcohols and acids found in the oil. This makes it improbable that there were significant quantities of higher waxes in the oil.

TABLE IV

Alcohol Composition of Pilot Whale Head Oil, in Mole %

Type of alcohol	Wax	Acetates	Trimethylsilyl ethers	Hydrocarbon	Average
14:0	1	2	2	1	2
iso-15:0	22	18	16	23	20
15:0	4	3	3	2	3
iso-16:0	39	37	32	42	37
16:0	20	19	24	19	20
iso-17:0	5	6	5	3	5
Branched 19:0 (?)[a]		2	1	1	1
18:1	8	9	14	7	9
18:0	1	3	1	1	2
Cholesterol, diols (?)	?	1	2	1 (?)	1

[a]? = Tentatively identified.

The cholesterol esters were too unvolatile to be detected at the GLC conditions chosen. They were detected by the Liebermann-Burchard color spray reagent on the TLC plate and by comparison of R_f values with those of reference cholesterol esters. Two closely situated spots gave a positive reaction with the spray reagent, and it is tempting to suggest that these represent cholesterol esters of isovaleric and longer chain fatty acids, respectively.

Triglyceride fraction: This further was separated into non-, mono-, and diisovalero triglyceride subclasses by two additional column chromatograms. To study the possible differences in the pattern of longer fatty acids between these subclasses, methyl esters from each subclass were analyzed by GLC (Table III). A limited number of determinations were made. This and the possible selective losses of triglycerides during the purification procedure make the figures somewhat uncertain. The differences in composition between diisovalero triglycerides and the other two classes are, however, conspicuous, and it is considered unlikely that such differences could be produced by selection of triglyceride species.

It is probable that small amounts of material other than triglycerides were present in these fractions. On GC-MS, the diisovalero triglycerides proved to contain other substances, because, between the big peaks, material giving different mass spectral patterns occurred. These could not be interpreted, however, but one might guess that these are ether lipids, which could also explain the occurrence of diols as detected in the TLC of the unsaponifiables by periodate-benzidine spray.

The specific rotation of the diisovalero subclass, the only subfraction containing enough material for such measurements, was +0.2 ± 0.2 C (21 C, sodium D-line, 10% in methanol). An asymmetrical diisovalero myristin has been studied by other authors (12) and was found to have a specific rotation of +0.75° in methanol. Our value, close to zero, thus provides some support for the suggestion of a symmetric configuration of the diisovalero triglycerides, i.e. the isovaleric acid residues are placed at the 1 and 3 positions in the molecule. Asymmetric components contributing to the specific rotation that we have found might be 2-methylbutanoic acid, which contributes to

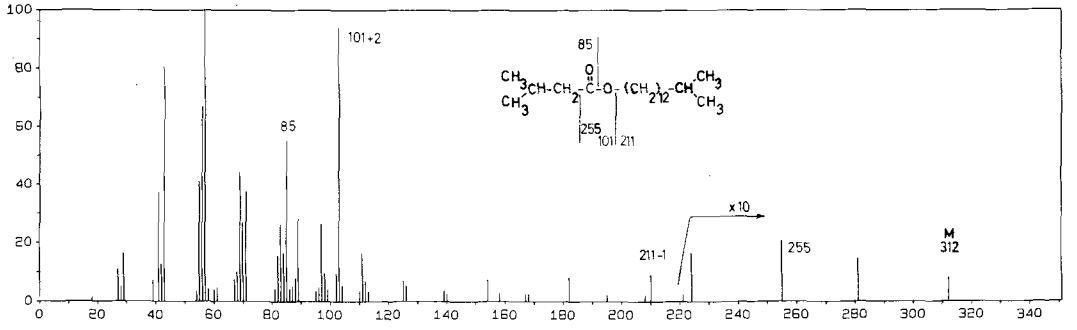

FIG. 6. Mass spectrum of isopentadecanyl isovalerate obtained on gas chromatography of blackfish head oil, raw (pilot whale head oil). Electron energy: 70 eV. Source temperature: 250 C.

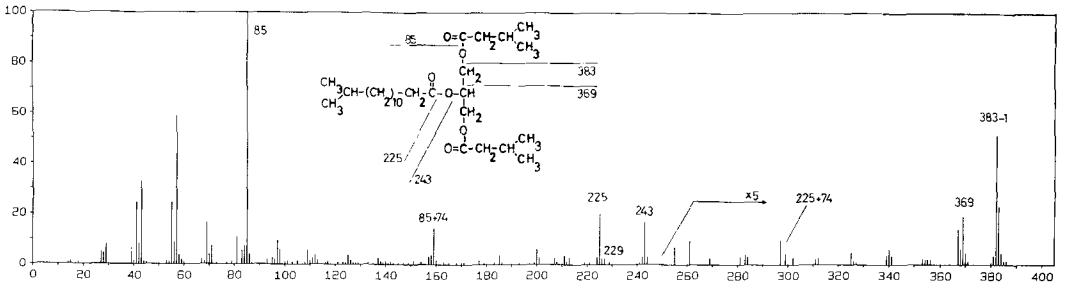

FIG. 7. Mass spectrum of diisovalero isopentadecanoin obtained on gas chromatography of blackfish head oil, raw (pilot whale head oil). Electron energy: 70 eV. Source temperature: 250 C.

the positive rotation of *Delphinus delphis* oil (12) and the minor contaminating substances referred to above.

Due to the relatively high volatility of the diisovalero triglyceride subfraction, it was analyzed easily by GLC-MS (Figs. 4, 5). A representative mass spectrum is shown in Figure 7. The mass spectra were easily interpretable in accordance with the known fragmentation pattern of triglycerides (26). The M or M-18 peaks were not obtained. This was tested both on the mass spectrometer mentioned earlier and an LKB 9000 instrument with the same result.

The peak at m/e 229 is of special interest. It represents the molecule ion minus an acyloxymethylene fragment from the longer fatty acid, attached to one of the primary glycerol carbon atoms. All diisovalero triglycerides give this fragment, but its abundance is highly dependent upon the position of the longer fatty acid. A comparatively high peak at m/e 229 means that the molecule is asymmetrical. If the fragments resulting from losses involving longer fatty acids are compared with those resulting from losses of isovaleric acid containing fragments a clear difference is seen (Figs. 7, 8). The ratio of the loss of a long acyloxy chain (m/e 243) to that of a long acyloxy chain + methylene (m/e 229) is ca. 15-20 in the spectra of the diisovalero triglycerides. The ratio of the loss of isovaleroyloxy to the loss of isovaleroyloxy + methylene is ca. 2-3 in the same spectra.

To check this, an asymmetric di-n-pentano myristin was synthesized. The ratios were 4 and 3, respectively (Fig. 8), which is a strong indication that the diisovalero triglycerides of pilot whale head oil are mainly symmetrical.

Cholesterol and diglyceride fraction: This was analyzed by TLC only. Identification of the cholesterol spot was made by Liebermann-Burchard spray. Appropriate reference substances were run together with the samples.

DISCUSSION

The results of earlier investigations on pilot whale head oil (11,27) agree closely with those of the present one. The observed value for isovaleric acid, 54 mole %, is still open to question, because of the high volatility of its esters. If the amount of isovaleric acid is calculated from the amounts found for triglycerides and wax, a value of 52% is obtained, showing that the above result is ca. correct. Relative response factors have not been calculated, since it is considered likely that the error in triangulating the peaks exceeds this source of error.

It is not certain that this oil is representative of all pilot whales, but preliminary investigations on head oil obtained fresh from a pilot whale caught off the Faroe Islands show a close similarity of the GLC and TLC patterns.

The majority of the diisovalero triglycerides seem to be symmetrical, on the basis of optical rotation and MS. One can, thus, postulate an enzyme specifically esterifying mono- and diglycerides containing longer fatty acids at the 1 and 3 positions with isovaleric acid in the fat cells of melon and jaw tissue of pilot whale.

This distribution of chains: short-medium-short contrasts with that found for triglycerides from pilot whale blubber (28), long-medium-long. This difference at first suggests that long acyls actually are replaced by isovaleroyls by some mechanism in the melon and jaw tissue. Evidence against this view is the occurrence of isobranched medium length acyls in non-, mono-, and diisovalero triglycerides in the head oil and the absence of the highly unsaturated long fatty acids typical of blubber fat in non- and monoisovalero triglycerides. Thus, the data presented speak in favor of a separate pathway of synthesis for all triglycerides in the melon and jaw tissues.

The ether lipids mentioned in this article may very well be similar to those found in other dolphins (13-16). However, judging from TLC of the unsaponifiables, they must be present in small amounts only.

The patterns of alcohols and fatty acids are

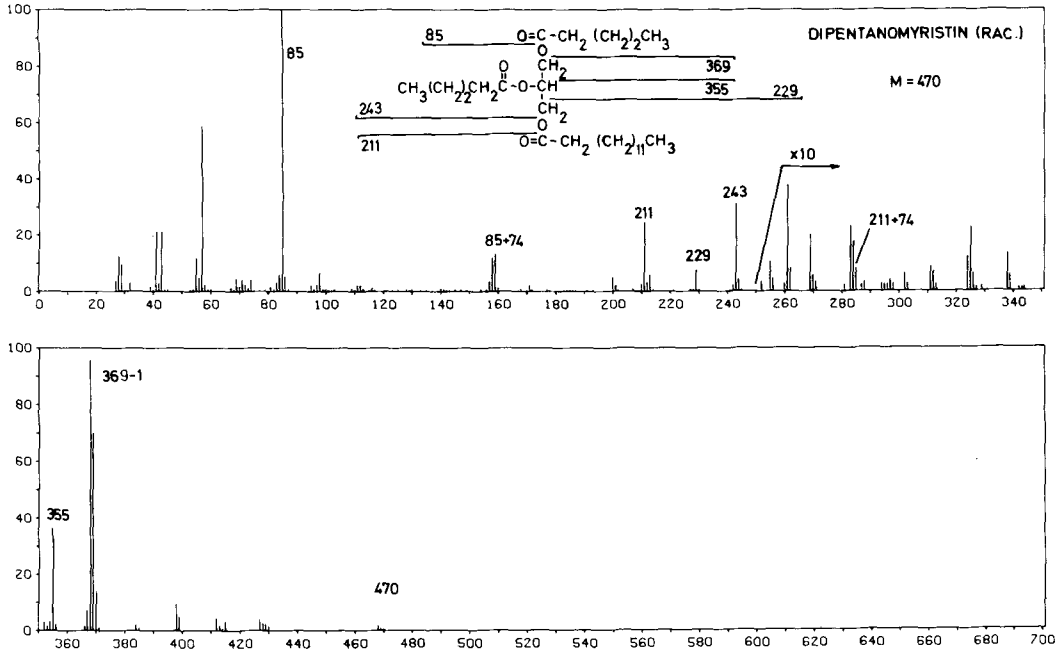

FIG. 8. Mass spectrum of synthetic, racemic, asymmetrical di-n-pentano myristin. Electron energy: 70 eV. Source temperature: 250 C.

similar in a qualitative way. Both contain isobranched saturated, normal saturated and normal monounsaturated components. Chain lengths are ca. the same. Some differences are, however, evident. Isopentanyl, isotetradecanyl, and hexadecenyl alcohols are present in only trace amounts, or not at all. On the other hand, isohexadecanoic acid is scarce compared with isohexadecanol. These differences make it implausible that alcohols and fatty acids are converted directly into each other by an undiscriminating mechanism in the dolphin melon.

One may speculate about why the pilot whale has such unusual triglycerides. The fatty melon of dolphins and porpoises from which our head oil sample was derived has been postulated to serve as an acoustic lens and to transmit sound directly to the inner ear (18,19). The melon and jaw fat bodies may, thus, be important in the echolocation of toothed whales.

If the melon fat functions as an acoustic lens, sound should have a low velocity in the fat, and it should have a low ultrasonic absorption. We are, at present, carrying out investigations along these lines.

It is known that hydrocarbons and fatty acids with short hydrocarbon chains give a lower sound velocity than those with a longer hydrocarbon chain. Branching also lowers the sound velocity (29). This suggests that the oil should have a rather low sound velocity.

The predominance of the 3-methylbutanoic (isovaleric) acid over 2-methylbutanoic acid may depend upon different ultrasound relaxation characteristics (30) and thereby ultrasound absorption of these positional isomers. An additional possibility may be that the methyl branch next to the carboxyl in 2-methylbutanoic acid hampers enzymatic esterification with this acid. In any case, the unusual composition of the oil is suggestive of specific function.

ACKNOWLEDGMENTS

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Characterization of Two Steroidal Olefins in Nonfat Dry Milk

VINCENT P. FLANAGAN[1] and **ALDO FERRETTI**[1], Dairy Products Laboratory, Eastern Regional Research Center[2], Philadelphia Pennsylvania 19118, and **JOHN M. RUTH**, Agricultural Environmental Quality Institute[2], Beltsville, Maryland 20705

ABSTRACT

Two steroidal olefins were isolated from the hydrocarbon fraction of a n-hexane extract of nonfat dry milk. They were characterized as 24-methyl-Δ^2-cholestene (Δ^2-campestene) and 24-ethyl-Δ^2-cholestene (Δ^2-sitostene) by gas chromatography, mass spectrometry, and synthesis.

INTRODUCTION

Most of the investigations on steroids present in milk have been conducted with anhydrous milk fat (AMF). Cholesterol is by far the major steroid compound in milk. Brewington, et al., (1) identified small amounts of dihydrolanosterol and β-sitosterol and confirmed the presence of lanosterol, first proposed by Morice (2). Campesterol also has been found in milk fat (C.R. Brewington, personal communication). Eisner, et al., (3) reported the presence of the pentacyclic triterpenoid β-amyrin. The only nonsterol identified in milk fat is Δ^7-cholesten-3-one reported by Parks, et al., (4). Little work has been done on steroids present in the lipid phase extracted from skim milk, even though this is known to contain higher concentrations of cholesterol, carotenoids, and vitamin A than the fat extracted from normal milk (5). Accordingly, it seemed reasonable to expect that the lipid phase extracted from nonfat dry milk (NFDM) might contain constituents either not present in milk fat or present in such low concentration as to have escaped detection. With this idea in mind, the present work was undertaken with the purpose of identifying novel steroid compounds in NFDM.

EXPERIMENTAL PROCEDURES

Gas Liquid Chromatography (GLC) and Mass Spectrometry (MS)

The GLC analysis was performed using a Hewlett-Packard 5750B gas chromatograph equipped with a flame ionization detector. The column was 2.4 m in length x 3.2 mm outside diameter, stainless steel, treated with dimethylchlorosilane (DMCS), and packed with 3% JXR on 80-100 mesh Gas Chrom Q (Applied Science Laboratories, State College, Pa.). The effluent was split 10:1 for trapping purposes. The temperature was programed from 90-220 C at a rate of 6 C/min. The carrier gas (He) was supplied at a head pressure of 2.8 kg/cm^2. The GLC-MS system was an LKB-9000, operating at an ionizing energy of 70 eV, source temperature 290 C, accelerating voltage 3.5 kv, separator temperature 240 C; the chromatographic column and conditions were as just described. The high resolution mass spectra were determined with a Dupont CEC 21-110B double focusing mass spectrometer at 70 eV. The sample was introduced via a direct probe at a source temperature of 115 C. The spectra were recorded on photoplates, and accuracte mass measurements were made by means of a comparator, with perfluorokerosene as internal standard.

Synthetic Procedures

All melting points (mp) are uncorrected. 3-Cholestanyl acetate, mp 107-108.5 C, was prepared according to Benveniste, et al. (6). 3-Cholestanyl p-toluenesulfonate, mp 135-136 C, was prepared according to Douglas, et al. (7).

Δ^2-Campestene and Δ^2-sitostene were synthesized starting from a commercial sample of β-sitosterol (Chemical Procurement Laboratories, College Point, N.Y.). This particular sample melted at 136-137 C after 2 crystallizations from ethyl acetate and 1 from ethanol. GLC and MS of the recrystallized product showed that it was a mixture of β-sitosterol (85%) and campesterol (15%). Hydrogenation of this mixture, according to Hershberg, et al., (8) yielded a solid (mp 134-135 C) which gave a negative bromine test (9) after crystallization from ethanol. The assumption of two hydrogen atoms was confirmed by MS. The remaining steps in the preparation of the Δ^2-olefins were those used by Fieser and Dominguez (10) for the preparation of Δ^2-cholestene. The crude olefin mixture melted at 69 C. Thin layer chromatography (TLC) on silicic acid impregnated with $AgNO_3$ and developed with n-hexane (J.T. Baker Chemical Co., Phillipsburg, N.J.) indicated that the crude product contained ca. 10% nonolefinic impurity (R_f = O). Purification over acid alumina (J.T. Baker Chemical Co.), with n-hexane as eluent, yielded a crystalline solid (mp 75-76 C) consisting of a 1:9 (GLC) mix-

[1]Present address: Nutrition Institute, BARC-East, Building 308, Beltsville, Md. 20705.

[2]ARS, USDA.

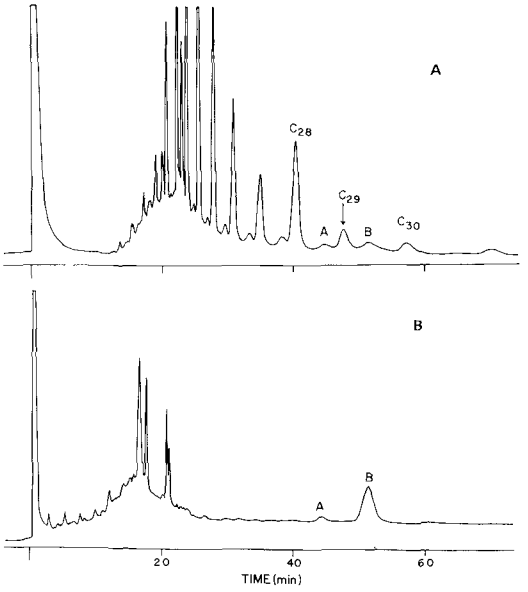

FIG. 1. Flame ionization detector chromatogram of an n-hexane extract of nonfat dry milk: (A) after column chromatography over hydrated alumina (total eluate) and (B) after further purification over acid alumina (Fraction NFDM, AB,3 and 4).

ture of Δ^2-campestene and Δ^2-sitostene (TLC and MS).

Isolation of the Steroidal Olefins from Nonfat Dry Milk

NFDM (3.5 kg), purchased at a local supermarket, was hydrated to a 9% water content, and extracted in a Soxhlet extractor with n-hexane in 350 g batches. The hexane extract was dried over Na_2SO_4 and evaporated to dryness on a steam bath under a stream of nitrogen. The waxy residue was taken up in 2 ml n-hexane and placed on a 50 g column of acid alumina previously hydrated to a 6% water content. The column then was eluted with 200 ml n-hexane and the eluate collected and reduced to dryness as above. The residue was dissolved in 500 µliter n-hexane and placed on a 13.5 cm x 0.7 cm column of acid alumina which then was eluted with n-hexane. Four 5 ml fractions were collected (NFDM, A-D). The first two fractions were combined, taken to dryness, and rechromatographed over acid alumina as above. Again four 5 ml fractions were collected and identified as NFDM, AB,1-4. Each fraction was reduced to 50 µliter and subjected to GLC-MS analysis. More NFDM (2.8 kg) was extracted exactly as above, and the appropriate fractions (NFDM, AB,3 and 4) were used to obtain samples for the high resolution mass spectrometric analysis.

RESULTS AND DISCUSSION

Figure 1A shows the GLC of the n-hexane extract of NFDM after column chromatography over hydrated alumina. This chromatogram is similar to that obtained with the hydrocarbon fraction of AMF (11). Continuous scanning with the GLC-MS system confirmed a hydrocarbon composition with traces of polychlorinated biphenyls. However, the quantitative distribution of the various components is not identical with that observed in AMF (11).

Two mass spectra were recorded for two unknowns, hereafter referred to as A and B, associated with the peaks marked by these same letters (Fig. 1A). These unknowns were investigated further. Purification, accomplished by two passes through columns of acid alumina, afforded a marked enrichment of A and B, which were found in fractions NFDM, AB,3 and 4 (Fig. 1B). The low resolution mass spectra of A (molecular weight 384) and B (molecular weight 398) are shown in Figures 2 and 3, respectively. Both compounds were isolated in a reasonably pure state by trapping off the JXR column and were subjected to high resolution mass spectra, which afforded exact mass measurements and elemental compositions. These are 384.3750 ($C_{28}H_{48}$) for A, and 398.3897 ($C_{29}H_{50}$) for B. The high relative intensity of the molecular ions in the mass spectra of the unknowns (Figs. 2 and 3) and the late elution of the compounds from the JXR column, relative to the C_{28} and C_{29} n-alkane counterparts, are indicative of cyclic or polycyclic molecules. The overall appearance of both spectra (primarily the fragments of m/e 257, m/e 203, m/e 215, m/e 216, and m/e 217, and the presence of M-15 fragments) strongly suggest steroidal structures (12-14). Because the molecules of the unknowns contain each five sites of unsaturation, (if we assume a four-ring steroidal system), these molecules also must contain either a double bond or a fifth cycle. The relatively low intensity of the peaks of m/e 257 excludes the presence of a double bond in the side chain. In fact, in a detailed study, Wyllie and Djerassi (14) established that mass spectra of steroidal olefins with unsaturation in the side chain have a rearrangement ion at m/e 257, which is usually much more intense than the molecular ion and sometimes is the base peak. A prominent feature of the spectrum of A is a strong m/e 330 peak (elemental composition $C_{24}H_{42}$). This corresponds to the loss of a neutral C_4H_6 fragment from the molecular ion. The elimination of butadiene is highly characteristic of Δ^2-steroids (13,15) and results from a retro-Diels-Alder decomposition of ring A following electron impact. The analogous frag-

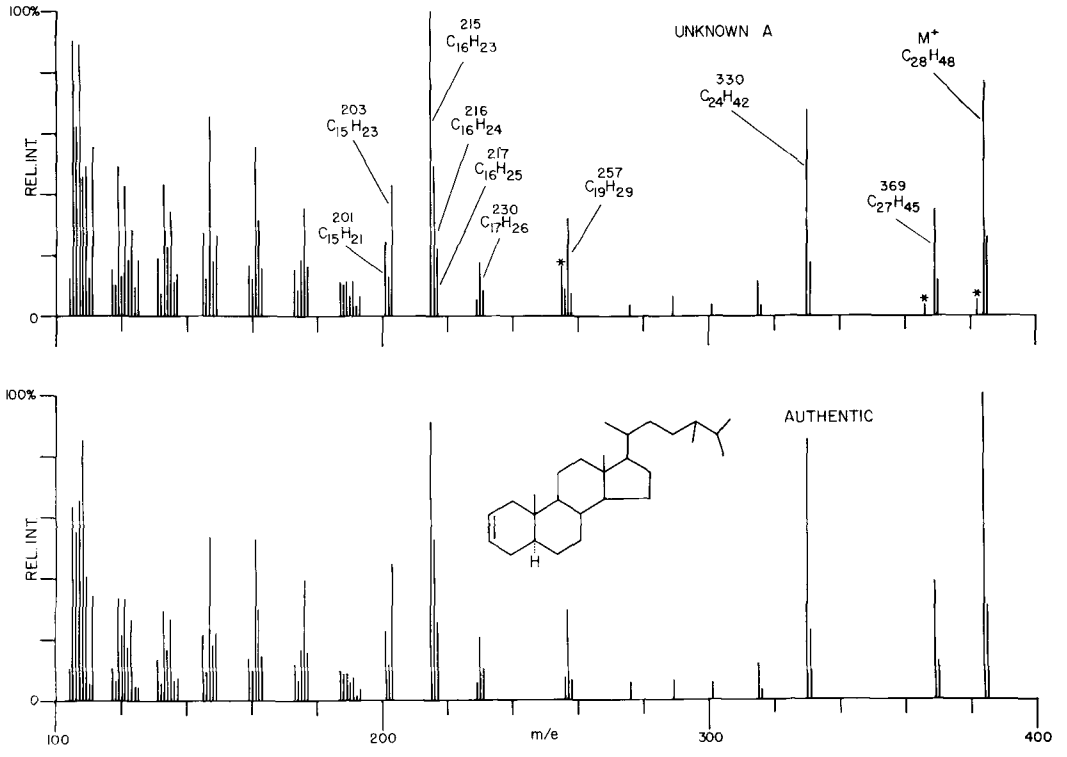

FIG. 2. Mass spectra of compound A and of synthetic Δ^2-campestene. * = peaks from impurity (see "Results and Discussion" section).

ment in the spectrum of compound B occurs at m/e 344 (elemental composition $C_{25}H_{44}$). The fragment of m/e 257, then, must be due to the loss of the side chain at C-17. On the basis of the above evidence, the compounds A and B appear to be Δ^2-steroidal olefins of the ergostane and sitostane series, respectively. In Figure 2, the mass spectrum of A is compared with that of the synthetic Δ^2-campestene. In Figure 3, the spectrum of B is compared with that of synthetic Δ^2-sitostene. The peaks marked with asterisks are due to impurities, possibly steoridal diolefins which are eluted along with A and B. The mol wt of such impurities are 382 and 396 and both have their base peaks at m/e 255. The association of the peaks of m/e 382 and m/e 396 with that of m/e 255 was confirmed by their behavior in multiple fast scanning. The retention times (JXR column) of compounds A and B are identical with those of synthetic Δ^2-campestene and Δ^2-sitostene, respectively. On the basis of the excellent agreement between the mass spectra and chromatographic behavior of the unknowns and those of the synthetic olefins, A is identified as a 24-methyl-Δ^2-cholestene and B as a 24-ethyl-Δ^2-cholestene. Because A and B occur naturally, the location of the methyl and ethyl groups (respectively) is assumed to be at C-24. The stereochemical configuration at C-24 in both compounds remains undetermined.

We estimate that the concentration of A and B in the samples of NFDM used in this study is less than 1 ppm. Their origin, or their precursors, can be objects for speculation. The hypothesis that A and B might be artifacts arising from the corresponding stanol esters, by elimination of a molecule of acid during column chromatography, must be ruled out, because 3-cholestanyl acetate, and even 3-cholestanyl p-toluenesulfonate, did not undergo such elimination by chromatography over acid alumina under the conditions described above. Δ^2-Steroidal olefins have been prepared from tosylate esters with alumina but only after prolonged treatment (7). Thermal or electron impact induced elimination of one molecule of acid from hypothetical stanol ester precursors of the unknowns during MS (16) also must be ruled out, because the column chromatographic procedure employed for the purification of the extract of NFDM completely removes compounds having oxygenated functions.

Phytene, a nonsteroid constituent of the

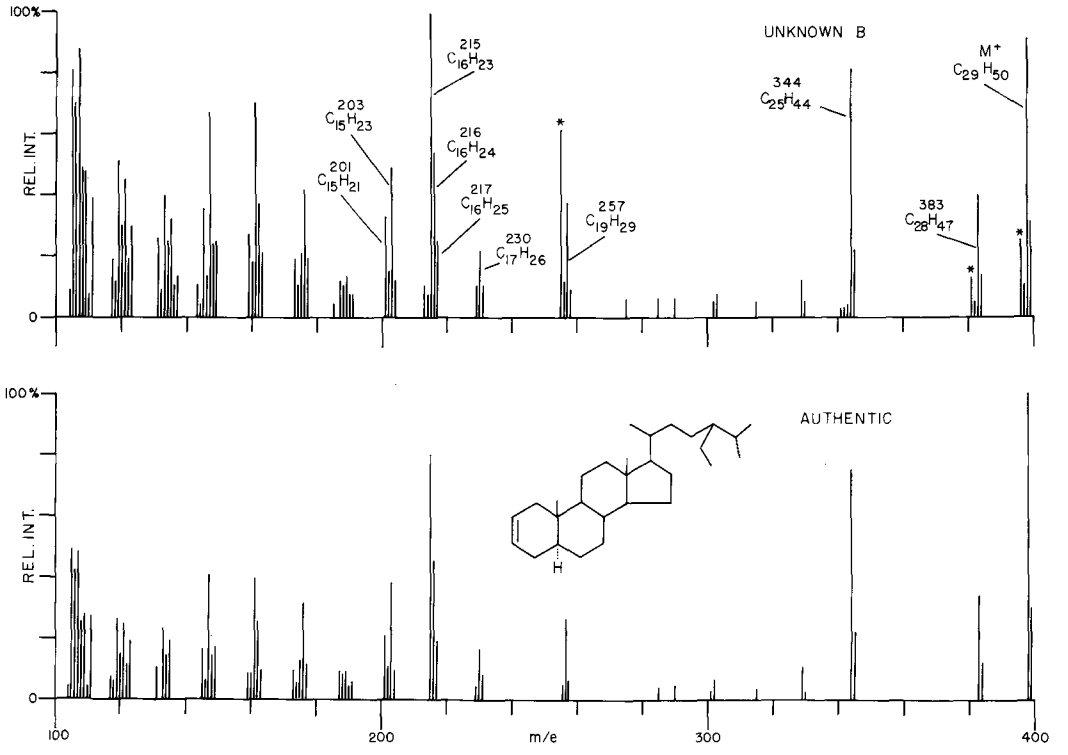

FIG. 3. Mass spectra of compound B and of synthetic Δ^2-sitostene. * = peaks from impurity (see "Results and Discussion" section).

unsaponifiable fraction of AMF (11), was not detected in the present investigation. Conversely, when AMF (prepared from mixed-herd milk, Beltsville, Maryland) was extracted as previously described (11) and the extract worked up in the same manner as the extract of NFDM, no trace of steroid olefin could be detected by GLC-MS. Phytene probably is introduced into milk via feed. The steroidal olefins A and B or their precursors also are almost certainly of plant origin. If this is the case, their occurrence in the lipid fraction of milk would depend upon the nature of the feed. As mentioned in the introductory section, certain lipoid constituents tend to concentrate in the skim milk portion. The lack of uniformity in the occurrence of phytene and of the two steroidal olefins might be another example of preferential distribution or association exhibited by certain constituents. At any rate, given the suspected plant origin of phytene and of A and B and the uncertainty about their precursors, these compounds may not be regarded as normal constituents of milk.

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D.P. Schwartz of this laboratory provided thin layer chromatographic analysis.

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Comparative Studies on the Fatty Acid Composition of Moderately and Extremely Thermophilic Bacteria

MIEKO OSHIMA and **AKIHISA MIYAGAWA**, Department of Biochemistry, Kitasato University School of Medicine, Kanagawa, Japan

ABSTRACT

The fatty acids of three strains of extremely thermophilic bacteria and three strains of moderately thermophilic bacteria were examined by gas liquid chromatography. All the thermophiles contained straight, iso, and ante-iso branched fatty acids. Iso $C_{17:0}$ acid was abundant in both the moderately thermophilic strains (10-33%) and the extremely thermophilic strains (50-61%). The pair of fatty acids iso $C_{15:0}$ and iso $C_{17:0}$ was the predominant pair in both the moderately (34-64%) and extremely (76-87%) thermophilic strains. The pair of fatty acids ante-iso $C_{15:0}$ and ante-iso $C_{17:0}$ was present in larger amount in moderately (25-34%) than in extremely (8.5-15%) thermophilic strains. No hydroxy, cyclopropane, or unsaturated fatty acids were found. One extreme thermophile, *Flavobacterium thermophilum* HB-8 was grown at 6 different culture temperatures from 49-82 C, and the changes of its fatty acid composition were studied. The ratios of iso $C_{17:0}$/iso $C_{15:0}$ and ante-iso $C_{17:0}$/ante-iso $C_{15:0}$ were much greater at higher culture temperatures, indicating chain elongation.

INTRODUCTION

The fatty acids of various microorganisms are known to be affected by the temperature at which the cells are grown. Bacterial cells which contain unsaturated fatty acids have higher proportions of unsaturated acids when grown at below their optimum temperature for growth (1-4). The *cis*-vaccenic acid content of *Escherichia coli* phosphatidyl ethanolamine, phosphatidyl glycerol, and cardiolipin fraction was found to increase with concomitant decrease of palmitic acid, when cultures were transferred from 40 to 20 C (5,6). Similar phenomena were observed with *Bacillus lichenformis* (7) and *Serratia marcescens* (4). Even in *Bacillus megaterium* cells, which contain only saturated fatty acids at 37 C, induction of an enzyme causing desaturation of the C-5 position of the fatty acid chain was observed at 20 C (8). It is believed that the inverse relationship between temperature and the extent of desaturation is due to the effect of temperature upon the induction and stability of the desaturating enzyme system (9,10).

TABLE I

Growth Temperature and Gram-Staining Properties of Thermophilic Bacteria

Organism	Growth temperature C			Gram staining
	Optimum	Maximum	Minimum	
Moderate thermophile				
B. stearothermophilus				
IAM 1035[a]	55	65	40	+
NCR2184[b]	65	68	40	+
Thermophile V-2[c]	60	70	40	–
Extreme thermophile				
Thermus flavis AT-62[d]	70	80	50	–
Thermus aquaticus YT-1[e]	71	80	50	–
Flavobacterium thermophilum HB-8[f]	75	85	49	–

[a]Obtained from the type culture collection of Institute of Applied Microbiology, Tokyo University.

[b]Supplied by Dr. Sone (Jichi Medical College).

[c]From Beppu hot springs, Japan.

[d]From Beppu hot springs, Japan.

[e]From the geyser basin of Yellowstone National Park.

[f]From mine hot spring, Izu Pennisula, Japan.

On the other hand, when grown at high temperature, there is a shift in fatty acid synthesis in the direction of more saturated fatty acids (1). The replacement of linolenic acid by linoleic acid was observed in the thermophilic alga, *Cyanidium caldarium*, when grown at 40 C instead of 20 C (11).

Microorganisms which can grow at over 50 C are called thermophilic bacteria, and a few strains, which can even grow at over 70 C (extreme thermophiles), have been isolated recently from hot springs (12-15). Branched chain fatty acids have been found in the moderate thermophile, *Bacillus stearothermophilus* (16-18) and the extreme thermophile, *Thermus aquaticus* (19,20). However, no comparative studies have been made on the fatty acid compositions of thermophilic bacteria.

This article describes comparative analyses of the fatty acids in several strains of moderately and extremely thermophilic bacteria. The effect of growth temperature upon the fatty acid composition of the extreme thermophile, *Flavobacterium thermophilum*, strain HB-8 (sp.nov.) also is reported.

MATERIALS AND METHODS

All thermophilic strains except *Thermus flavis* AT-62 were grown in neutral medium (pH 6-7.5) containing yeast extract (0.2%), polypeptone (0.8%), and NaCl (0.2%). *T. flavis* was grown in medium (pH 7.0) containing beef-extract (0.4%), polypeptone (0.4%), K_2HPO_3 (0.3%), and KH_2PO_4 (0.1%). The cells were cultured with shaking or bubbled with air at their optimum growth temperatures, listed in Table I.

Cultured cells were harvested in the late log phase and washed thoroughly with 0.9% NaCl solution. Then, the packed cells were mixed with 10 volumes methanol (w/v) with stirring and then with 20 volumes chloroform. The chloroform-methanol extract was partitioned with 0.2% $BaCl_2$ solution, and the lower organic phase was separated and evaporated to dryness. The crude lipid extract was treated with 3% HCl-methanol at 100 C for 4 hr, and the fatty acid methyl esters were extracted with petroleum ether and subjected to gas liquid chromatography (GLC).

F. thermophilum HB-8 was cultured at 49,52,60,65,75, or 82 C in medium of the composition described above, and the cells were washed thoroughly and lyophilized. Lyophilized cells (5-10 mg) were treated directly with 3% hydrochloric acid (HCl) in dry methanol at 100 C for 4 hr. The fatty acid methyl esters extracted with petroleum ether (boiling point [bp]

TABLE II

Percentages of Principal Fatty Acids in Moderate and Extreme Thermophiles

Organism	Percentage fatty acid composition												
	C_{14}	iso C_{14}	iso C_{15}	ante-iso C_{15}	C_{16}	iso C_{16}	iso C_{17}	ante-iso C_{17}	C_{18}	iso C_{19}	ante-iso C_{19}	iso C_{20}	ante-iso C_{20}
B. stearothermophilus													
IAM 1035	T[a]	T	23.1	12.6	3.8	18.2	22.5	18.0	---	2.1	---	---	---
NCR 2184	T	T	31.2	11.7	7.0	2.5	33.1	14.3	---	---	---	---	---
Thermophile V-2	---	---	24.0	11.0	14.0	6.9	10.9	23.3	1.5	---	2.8	T	1.9
T. Flavis AT-62	---	---	26.0	3.3	---	1.0	61.0	7.1	---	---	T	---	---
T. aquaticus YT-1	---	---	33.0	3.7	4.6	3.2	50.1	4.8	---	---	T	---	---
F. thermophilum HB-8	---	---	21.0	5.0	1.2	5.0	55.2	10.2	T	T	2.1	T	T

[a]Contents of less than 1% are designated as T.

TABLE III

Fatty Acid Compositions of the Extreme thermophile, *F. thermophilum* HB-8 at Different Growth Temperatures

Fatty acid	Growth temperature C					
	49	53	65	69	75	82
Iso C_{14}	1.5	2.1	1.1	---	---	---
Iso C_{15}	31.1	34.1	33.9	25.9	21.8	12.2
Ante-iso C_{15}	13.1	7.9	8.7	11.8	4.8	2.4
Iso C_{16}	6.4	9.1	7.7	6.0	5.2	1.8
C_{16}	1.7	T[a]	1.3	T	T	1.1
Iso C_{17}	31.3	35.1	38.8	40.7	56.8	64.2
Ante-iso C_{17}	11.2	6.8	7.9	9.8	9.2	11.8
C_{18}	1.0	2.0	T	T	T	T
Iso C_{19}	---	---	---	T	1.2	5.0
Ante-iso C_{19}	---	---	---	T	T	1.3

[a]Contents of less than 1% are designated as T.

30-60 C) were analyzed in a Shimadzu-GC-5A gas chromatograph.

A column (3mm x 3.5m) of 25% polyethylene glycol succinate on Celite 545 (silanized with hexamethyldisilazane [HMOS], 80-100 mesh) was used to obtain clear separation of iso and ante-iso branched acids. The fatty acid methyl esters were determined by their retention times on GLC and by GLC-mass spectrometry (MS) using a Shimadzu LKB, model 9000, GC-MS spectrometer. Iso $C_{15:0}$ and ante-iso $C_{15:0}$ were identified from the metastable ions of m/e 177 and m/e 155, respectively.

RESULTS AND DISCUSSION

The percentage compositions of fatty acids in the bacteria are listed in Table II. The fatty acids in the moderate and extreme thermophiles were qualitatively similar, consisting mainly of C_{14}-C_{17} iso and ante-iso branched acids. No cyclopropane, hydroxy, or unsaturated fatty acids were detected in any of the thermophilic bacterial strains. Iso and ante-iso branched acids are present in many bacterial strains, such as *Bacillus* (21), *Micrococcus* (22), *Staphylococcus* (22), and *Streptomyces* (23). But these branched fatty acids in most bacteria so far examined are mainly C_{15} acids (22, 24). Iso $C_{17:0}$ acids were the most abundant in both moderate thermophiles (10-33%) and extreme thermophiles (50-61%). The predominance of iso $C_{17:0}$ acids in thermophiles may be attributed to the hot environment of these bacteria.

The branched chain acids of C_{17} in thermophilic organisms also was reported by Bauman and Simmonds (25) and Heinen, et al. (19). The former found C_{21} cyclopropane and C_{20} unsaturated acids, as well as C_{14}-C_{17} branched chain acids in bacterial masses obtained directly from hot springs. Cyclopropane or unsaturated fatty acids were not detected in any of the present cultures. Heinen, et al., (19) analyzed the fatty acids of *T. aquaticus*. Their results differ from ours. Our results indicate that the branched chain fatty acid 15:0 (iso $C_{15:0}$ + ante-iso $C_{15:0}$) constitutes over 37% of the total, while there is scarcely any straight chain C_{15} fatty acid (Table II).

Heinen, et al., (19) also reported that $C_{15:0}$ fatty acid constituted nearly half the total fatty acid content, but they found that C_{15} branched acid was absent. When large amounts of $C_{15:0}$ fatty acids are present, a series of acids, such as $C_{17:0}$ or $C_{13:0}$ acids, usually have been detected, as well in the bacteria so far examined (22). Our finding of iso C_{15} rather than straight chain C_{15} in *T. aquaticus* seems more understandable, since it can be the direct precursor of iso C_{17} (24), and, in this organism, iso $C_{17:0}$ acid constitutes over 50% of the total fatty acid.

The relative abundance of branched chain fatty acids is known to reflect the availability of precursors, since these acids are synthesized from amino acids (26). Therefore, their abundance is affected by the composition of the growth medium. In this work, all strains except *T. flavis* were grown in medium of the same composition and so the availability of amino acids was constant. Since the fatty acid composition also is affected by the age of the culture (27), cells were harvested at the same stage of growth (late log phase).

The effect of temperature upon the fatty acid composition of the extreme thermophile, *F. thermophilum* HB-8 is shown in Table III. The amount of longer chain acids increased on elevating the temperature. The percentage of

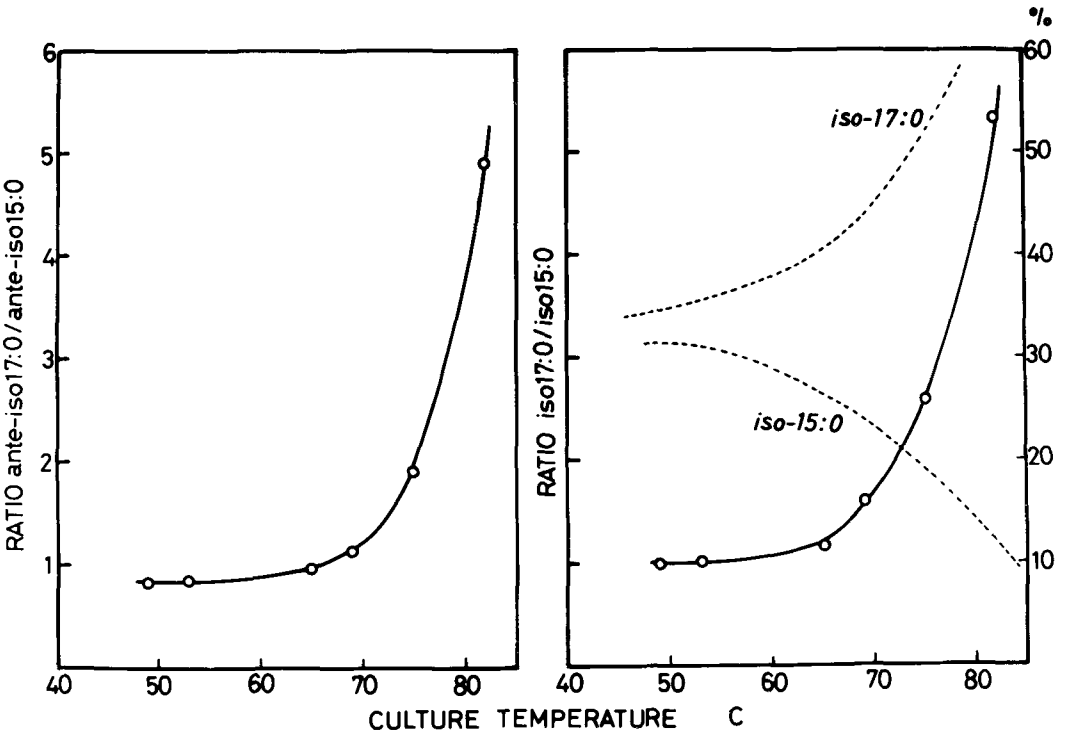

FIG. 1. Effect of temperature upon the iso and ante-iso fatty acid contents of *F. thermophilum*. Ante-iso $C_{17:0}$/ante-iso $C_{15:0}$ ratio (left) and iso $C_{17:0}$/iso $C_{15:0}$ ratio (right). Dotted lines show increase in the percentage of iso $C_{17:0}$ and decrease in that of iso $C_{15:0}$ at high growth temperatures.

iso $C_{17:0}$ increased at high growth temperature while those of iso $C_{15:0}$ and iso $C_{16:0}$ decreased (Table III and Fig. 1). At the optimum temperature of 75-80 C, two-thirds of the total acids were iso $C_{17:0}$. Iso $C_{19:0}$ acid also was detected at over 75 C. The ratios of iso $C_{17:0}$/iso $C_{15:0}$ and ante-iso $C_{17:0}$/ante-iso $C_{15:0}$ increased greatly at high growth temperature, indicating chain elongation of fatty acids at high temperature (Fig. 1).

The melting point of lipid in the biological membranes of microorganisms may determine the minimal growth temperature. Although the melting points of fatty acid are not directly correlated with the liquid crytstalline states of lipid complexes in biomembranes, it is interesting to consider the physical properties of fatty acids. Ante-iso $C_{15:0}$, ante-iso $C_{17:0}$, iso $C_{15:0}$, and iso $C_{17:0}$ have melting points of 25.8, 38.0, 52.2, and 60.5 C, respectively. Table II shows that the pair of fatty acids iso $C_{15:0}$ and iso $C_{17:0}$ constitutes 76-87% of the total fatty acids in extreme thermophiles and 34-64% in moderate thermophiles. Extreme thermophiles contained appreciably more iso branched acids that have high melting points than moderate thermophiles. A difference was also apparent in the amount of ante-iso acids. (Table II) which have much lower melting points than iso acids: moderate thermophiles contained twice as much of the pair of fatty acids ante-iso $C_{15:0}$ and ante-iso $C_{17:0}$ (25-34%) than extreme thermophiles (8.5-15%). In thermophiles, long chain iso branched acids apparently are synthesized for maintaining the physical properties of the membrane lipid at high temperature.

Large amounts of a novel glycolipid (over 70% of the total lipid) have been found in the extreme thermophile, *F. thermophilum* (28). Temperature may affect the glycolipid content which contains fatty acid residues, as well as fatty acid itself. The effect of temperature upon the complex lipid in the membranes of thermophiles requires study.

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Hydrolysis of Synthetic Triacylglycerols by Pancreatic and Lipoprotein Lipase[1]

N.H. MORLEY, A. KUKSIS, and **D. BUCHNEA,** Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario Canada M5G 1L6

ABSTRACT

The stereochemical course of the hydrolysis of synthetic sn-glycerol-1-palmitate-2-oleate-3-linoleate, sn-glycerol-1,2-dipalmitate-3-oleate and their antipodes by pancreatic and milk lipoprotein lipase was investigated by thin layer and gas liquid chromatographies of the diacylglycerol intermediates. The enzymic hydrolyses were made with bile salts or lysolecithin in a 1:1 molar ratio to the substrate as emulsifiers and were limited to short time intervals which minimized isomerization and the reversal of lipolysis. In all instances, the products of hydrolysis by lipoprotein lipase contained a marked preponderance of the 2,3-diacylglycerols, while the composition of the diacylglycerol intermediates in the products of pancreatic lipase varied with the nature of the fatty acid in the 1 and 3 positions of the triacylglycerol molecule. Pancreatic lipase, but not lipoprotein lipase, gave a preferential release of unsaturated fatty acids. The above results are similar to those obtained with radioactive trioleoylglycerol and conventional stereospecific analyses and suggest that lipoprotein lipase may favor attack on the sn-1 position. It is hypothesized that the small amounts of the 1,2-diacylglycerols present may have arisen from a reversal of lipolysis also catalyzed by this enzyme.

INTRODUCTION

Most enzymes, including phospholipases, display a highly specific stereochemical course of action. The acylglycerol lipases appear unusual, since they are believed to hydrolyze enantiomeric acylglycerols at comparable rates. Thus, Tattrie, et al., (1) has shown that the palmitic and oleic acids in the 1 and 3 positions are released at equal rates during hydrolysis of 1,2-dipalmitoyl-3-oleoyl-sn-glycerol by pancreatic lipase. Similarly, Assmann, et al., (2) recently demonstrated that the unlabeled and labeled oleic acids in the 1 and 3 positions, respectively, of 1,2-dioleoyl-3-(9,10-^{3}H) oleoyl-sn-glycerol are released at about the same rates during hydrolysis by the lipases of postheparin plasma. Furthermore, Nilsson-Ehle, et al., (3) has shown that the lipoprotein lipase hydrolyzes the primary positions ahead of the secondary, as demonstrated earlier by Mattson and Beck (4) for pancreatic lipase. In addition, pancreatic lipase has been shown to yield ca. equimolar amounts of the 1,2- and 2,3-diacylglycerols from natural triacylglycerols and has been incorporated as a means of random generation of diacylglycerols in the original Brockerhoff method of stereospecific analysis (5).

On the other hand, stereospecific analyses of the diacylglycerol intermediates of lipoprotein lipase digestion (6) have indicated a significantly higher proportion of 2,3-diacyl-sn-glycerols than would have been anticipated from a purely random degradation. Since the diacylglycerols make up only a small proportion of the total digestion products of lipoprotein lipase, the results of the fatty acid and diacylglycerol intermediate analyses need not be in conflict. However, early studies by Karnovsky and Wolff (7) had shown that ca. 49% of the radioactivity originally present in the 1-^{14}C-glycerol of trioleoylglycerol may be recovered in the hydroxyl carbon of the diacylglycerols released by treatment with a clearing factor. Since this discrepancy could not be explained by isomerization or incomplete recoveries of enantiomers, it was decided to reinvestigate the problem using synthetic triacylglycerols which yield chromatographically distinguishable 1,2- and 2,3-diacylglycerols during enzyme digestion. The present studies with several pairs of mixed acid enantiomers confirmed our earlier claims (6) that the 2,3-diacylglycerols tend to accumulate in the digests of lipoprotein lipase, while the products of pancreatic lipase hydrolysis contained a preponderance of the more saturated diacylglycerols, regardless of their steric configuration.

MATERIALS AND METHODS

Reagents

Lecithin was isolated from total lipid extracts of egg yolk by preparative thin layer chromatography (TLC) (6). Lyso (1-acyl) lecithin was obtained from Sigma Chemical Co., St. Louis, Mo., and glycocholate from Pfaltz and

[1]Presented in part at the AOCS Fall Meeting, Ottawa, September 1972.

Bauer, Acetochemical Co., Flushing, N.Y. Mixed bile salts were purchased from Difco Chemicals, Detroit, Mich. Trisil/Bisacetamide (BSA) was from Pierce Chemical Co., Rockford, Ill. The stigmasterol and tridecanoylglycerol used as internal standards in the gas liquid chromatography (GLC) analyses were, respectively, from Applied Science Laboratories, State College, Pa., and Eastman Organic Chemicals, Rochester, N.Y.

Pancreatic lipase was obtained from General Biochemicals, Chagrin Falls, Ohio, while the bovine milk lipoprotein lipase was saved from an earlier preparation (6) held at -20 C.

Substrates

The synthetic triacylglycerol substrates, sn-glycerol-1-palmitate-2-oleate-3-linoleate (A) and its antipode (B) were prepared by reacting the appropriate C_{16} and C_{18} mixed acid diacylglycerols with the corresponding fatty acid chloride in pyridine (8, 9). The resulting triacylglycerol was isolated by silicic acid column chromatography and the triacylglycerols were resolved further from minor amounts of free fatty acids and mono- and diacylglycerols by TLC in a neutral lipid solvent system (8). The synthetic substrates, sn-glycerol-1-oleate-2,3-dipalmitate (C) and its antipode (D) were obtained from Supelco, Bellefonte, Pa., and (carboxyl-^{14}C) glycerol trioleate (15.2 mC/mM) was purchased from ICN Chemical and Radioisotope Division, Irvine, Calif. The commercial products were checked for purity by chromatography. Furthermore, each enantiomer of the synthetic triacylglycerols was subjected to Grignard degradation, and the 1,2(2,3)- and 1,3-diacylglycerols were resolved by GLC on polar and nonpolar columns (10). In each case, only the anticipated molecular species in the appropriate proportions were found which attested to the success of the syntheses and the probability of the correctness of the stated structures.

Enzyme Hydrolyses

Hydrolyses with lipoprotein lipase were performed using two types of substrate emulsions. In one, 20 mg (20 mM) triacylglycerol was mixed with 12 mg dry glycocholic acid and then emulsified with 2 ml 0.1 M Tris buffer at pH 8.0. Aliquots of the emulsion were activated at 37 C for 30 min with normal rat serum (which contained ca. 1 mg/ml lecithin). In the other, 12 mM triacylglycerol was sonicated in a glass tube with 6.5 mg (12 mM) lysolecithin in either saline or Tris buffer. Aliquots of these emulsions were activated with rat serum which had been delipidated (11) and extracted in the ammonium BSA buffer of the assay system. In some instances, the activation period was reduced to 5 min. In all cases the activated substrate was treated with lipoprotein lipase, as described previously (6). Saline was substituted for the enzyme solution in the blank controls. Total lipids extracted from the hydrolysates between 0-20 min were separated into the component neutral lipids by TLC on borate treated Silica Gel G and the 1,2(2,3)-diacylglycerols recovered free from contamination with the 1,3-isomers. GLC also permitted analyses of aliquots of the total lipid extracts without TLC separation.

Hydrolyses with pancreatic lipase were carried out according to Luddy, et al., (12) with 20 mg aliquots of triacylglycerols in a Vortex mixer for 1.5 min at 40 C, without the addition of organic solvent. The mixed 1,2(2,3)-diacylglycerols released were separated from the total lipid extracts and their proportional composition determined by argentation TLC and GLC.

Analyses of Diacylglycerols

The synthetic triacylglycerol substrates had been selected to yield 1,2- and 2,3-diacylglycerols of differing mol wt and degree of unsaturation. Thus, the 1,2-diacylglycerols from compound A had a carbon number of 34 which readily is resolved from carbon number 36 of the 2,3-diacylglycerol by GLC. Since the 1,2-diacylglycerols released from compound A had only 1 double bond and the 2,3-diacylglycerol had 3 double bonds, the 2 isomeric diacylglycerols could be resolved readily by argentation TLC. For the diacylglycerol end products of compound B, the reverse situation is found, and the same methods are, therefore, applicable. The 1,2- and 2,3-diacylglycerols of compounds C and D are distinguishable chromatographically by separation of the carbon numbers 32 and 34, and saturated and monounsaturated species of diacylglycerols.

GLC

Aliquots of 1,2(2,3)-diacylglycerols in the hydrolysates of either pancreatic or lipoprotein lipase were reduced to dryness and trimethylsilylated with an excess of Trisil/BSA at room temperature overnight. Separations based upon carbon number were made on a Beckman GC-4 gas chromatograph equipped with stainless steel columns (50 cm x 2 mm inside diameter) packed with 3% OV-1 on 100-120 mesh Gas Chrom Q. The temperature was programed from 150-350 C in 16 min. Peak areas were estimated by an Infotronics electronic peak area integrator. The peaks were identified and quantitated in relation to tridecanoylglycerol or

TABLE I

Products of Digestion by Lipoprotein Lipase of Tri-(-^{14}C)-Oleoylglycerol Emulsified with Different Agents

Emulsifier	Molar ratio[a] with triolein[b]	Incubation min	Triglyceride digestion moles %	Products DG moles %	Products MG moles %	Products FFA moles %
Sodium glycocholate	1.2	15	15.9	2.6	11.4	31.5
Egg yolk extract	0.8	10	34.4	3.9	38.4	66.3
		20	46.2	3.6	22.5	108.9
Lecithin[d]	0.2	10	5.9	2.2	3.9	9.5
		20	9.6	2.7	3.7	16.7
	0.8	10	28.0	5.9	21.0	51.3
		20	33.5	6.5	23.1	64.8
Lyso (1-acyl) lecithin[d]	0.8	10	32.9	4.2	24.6	66.0
		20	52.5	4.2	29.7	119.7
Lyso (1-acyl) lecithin, no serum activation	0.8	10	6.2	3.6	2.4	9.3
		20	11.2	5.7	5.4	16.8

[a]Molar ratio for lecithin equivalent except for sodium glycocholate.

[b]The substrate consisted of 12 or 20 mg unlabeled triolein in addition to the tri-(1-^{14}C)-oleoylglycerol.

[c]DG = diglycerides, MG = monoglycerides, and FFA = free fatty acids.

[d]Single determinations. Other values are means of duplicate determinations.

stigmasterol used as internal standard.

The diacylglycerols released from the synthetic triacylglycerols by Grignard degradation were resolved and quantitated by an F&M biomedical gas chromatograph equipped with glass columns (180 cm x 3 mm inside diameter) containing 3% SILAR 5 CP on 100-120 mesh Gas Chrom Q, as described by Myher and Kuksis (10).

Argentation TLC

For argentation TLC suitable aliquots of the mixed 1,2(2,3)-diacylglycerols were acetylated with 5 μC 1-^{14}C-acetic anhydride in pyridine. The labeled diacylglycerol acetates were purified by TLC in a neutral lipid solvent system and the monoene and triene fractions resolved by argentation TLC on Silica Gel G containing 20% silver nitrate using 1.2% methanol in chloroform as the developing solvent. To facilitate the location of the bands of labeled acetates, unlabeled carrier was added in the form of acetylated diacylglycerols isolated from a pancreatic lipase digest of commercial trioleoylglycerol. The radioactivity of the bands was estimated following elution with chloroform in a Mark 1 6894 series Nuclear Chicago liquid scintillation system with external quenching.

RESULTS

Selection of Emulsifier

Since lipoprotein lipase is active only with emulsions of triacylglycerols, a surface active material is required as an emulsifier. In previous work (6), this need was met by egg yolk lipids, which were rich in phosphatidylcholine and phosphatidylethanolamine. To simplify the mass analyses of the intermediates, purified lecithin, lysolecithin, and bile salts were considered as potential emulsifiers of simple chemical structure. Figure 1 shows the effect of the

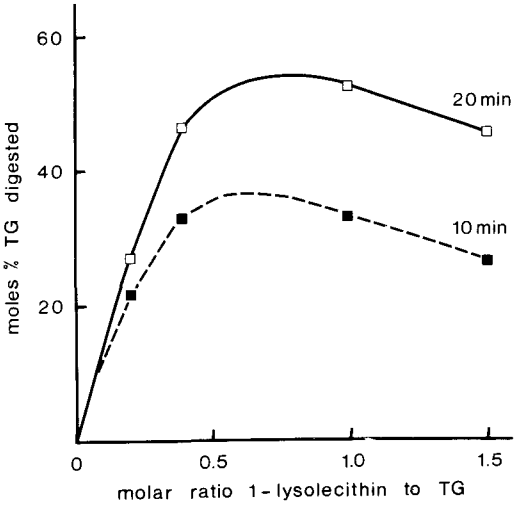

FIG. 1. Effect of 1-lysolecithin upon hydrolysis of trioleoylglycerol. Tri-(1-^{14}C)-oleoylglycerol was added for quantitation. TG = triglycerides.

TABLE II

Products of Digestion by Lipoprotein Lipase of Tri-(-^{14}C)-Oleoylglycerol Activated with Delipidated Serum

Activation[a] min	Incubation min	Triglyceride digestion moles %	Products DG moles %	MG moles %	FFA moles %
5	2	9.9	2.4	9.9	15.2
	10	44.6	4.5	39.3	85.8
10	2	8.5	1.8	8.1	14.1
	10	32.7	3.8	27.9	62.4

[a]The substrate consisted of 12 mg unlabeled triolein in addition to the tri-(1-^{14}C) oleoylglycerol.

[b]DG = diglycerides, MG = monoglycerides, and FFA = free fatty acids.

proportion of lysolecithin to substrate on the rate of hydrolysis by lipoprotein lipase. The optimum ratio was ca. mole to mole, which was higher than that reported by other workers (13, 14). This may have been due to the fact that the lysolecithin in these experiments was added with a delipidated serum. Similar results were obtained with lecithin and total egg yolk lipids. Lecithin in a molar ratio of 0.2, a level considered suitable by others, (13, 14) was not satisfactory under our conditions. Sodium glycocholate was a much less effective emulsifier than either the lecithins or egg yolk lipids.

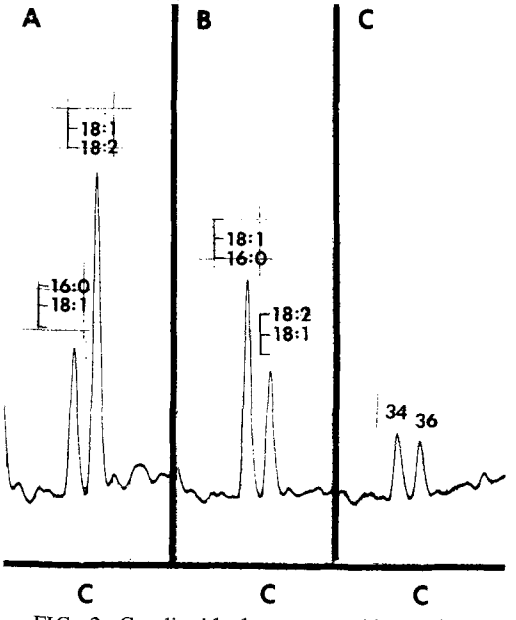

FIG. 2. Gas liquid chromatographic elution patterns of 1,2- and 2,3-diacylglycerols from lipoprotein lipase digestion of triacylglycerols A and B. C represents a pooled control without addition of enzyme. The temperature was programed from 150-350 C in 16 min. Major peaks, representing carbon numbers 34 and 36 were eluted at 290 and 300 C respectively.

Table I lists the various emulsifiers tried and gives the yields of monoacyl- and diacylglycerols and free fatty acids, as well as the estimates of the overall extent of digestion at 10-20 min of incubation, as quantitated by tri- (1-^{14}C)-oleoylglycerol as substrate. With the need for phospholipid filled by lysolecithin, delipidated serum extracted in buffer could be introduced into the reaction medium without loss of capacity for activation. Table I confirms our previous observation that the omission of the activation reduced the triacylglycerol hydrolysis by 80-90%. Table II shows that the period of preactivation of substrate could be shortened to as little as 5 min without reducing the enzyme activity. There was little difference in the levels of diacylglycerol accumulated in the medium with the different emulsifiers, namely an average 5-6 moles % of the total digestion products. Since the proportion of the enantiomers obtained might vary with the composition of the incubation medium, we examined the diacylglycerols collected with both glycocholate and lysolecithin as the emulsifiers.

Lipolysis of Synthetic Triacylglycerols

The structures of the diacylglycerol intermediates were determined on the basis of the known structure of the original triacylglycerols and the GLC and argentation TLC properties of the 1,2(2,3)-diacylglycerols recovered from borate TLC. Figure 2 shows the GLC elution patterns recorded for the 1,2(2,3)-diacylglycerols from the lipoprotein lipase digestion of triacylglycerols A and B, incubated in the presence of sodium glycocholate as the emulsifier. In both instances, major peaks are seen for carbon numbers 34 and 36. The larger peak in each case, however, represents the 2,3-diacylglycerol. A calculation of the peak areas reveals that the enzyme yielded ca. the same ratio of

TABLE III

Diacylglycerol Composition of Hydrolysate during Digestion of Synthetic Triacylglycerols by Lipoprotein Lipase

Substrate	Emulsifier	Argentation chromatography 1,2-[a]	Argentation chromatography 2,3-[a]	Gas liquid chromatography 1,2-[b]	Gas liquid chromatography 2,3-[b]
A		%	%	%	%
sn-glycerol-1-palmitate-2-oleate-3-linoleate	Glycocholate	21.3	78.7	34.9	65.1
		35.5	64.5	29.9	70.1
				31.4[c]	68.6[c]
	Lysolecithin	23.1	76.9	20.3	79.7
		27.2	72.8	23.8	76.2
B					
sn-glycerol-1-linoleate-2-oleate-3-palmitate	Glycocholate	20.7	79.3	23.9	76.1
		25.2	74.8	30.0	70.0
				35.5[c]	64.5[c]
	Lysolecithin	22.7	77.3	17.5	82.5
		22.0	78.0	24.8	75.2
C					
sn-glycerol-1-oleate-2,3-dipalmitate	Lysolecithin			24.1	75.9
D					
sn-glycerol-1,2-dipalmitate-3-oleate	Lysolecithin			34.6	65.4

[a]Means of duplicate determinations of diacylglycerol fractions as monoenes and trienes.

[b]Means of duplicate determinations of diacylglycerol fractions as carbon numbers 32, 34, or 36.

[c]The source of the lipoprotein lipase in these two experiments was rat postheparin plasma. In all other cases, the source was bovine skim milk.

1,2- to 2,3-diacylglycerols in each case. Table III gives the results of analyses of the diacylglycerol composition of the hydrolysates under other conditions of degradation which comprised the effect of different emulsifying agents, as well as the consequences of using delipidated or fresh serum. This table also includes analyses of diacylglycerol products when the source of the lipoprotein lipase was rat post heparin plasma (6) instead of bovine skim milk. In all instances, a preferential accumulation of the 2,3-diacylglycerols was observed with the AB as well as the CD pairs of isomeric triacylglycerols. The average ratio of 2,3- to 1,2-diacylglycerols was 75 to 25. Only small amounts of 1,3-diacylglycerols were recovered from any of the incubation mixtures under the present experimental conditions.

Since the 1,2- and 2,3-diacylglycerols released from the A and B triacylglycerols also differed in the degree of unsaturation, they could be separated by argentation TLC. The results of these analyses are included in Table III. In all instances, the proportions for the 1,2- and 2,3-diacylglycerols agreed closely with those based upon GLC analyses of the corresponding products of lipolysis. Since the analyses by carbon number and by the number of double bonds are not affected by possible isomerization, because the corresponding isomers would overlap in these analytical systems, both sets of results are not only consistent but experimentally sound. As pointed out above, there was very little isomerization observed under the present conditions. It would, therefore, appear that lipoprotein lipase preferentially attacks position 1 of the sn-glycerol, regardless of whether it is occupied by palmitic, linoleic, or oleic acids. These results fully agree with previous findings based upon stereospecific analyses of the 1,2 (2,3)-diacylglycerols released from trioleoylglycerol by lipoprotein lipase under comparable conditions (6).

Figure 3 shows the proportions of the 1,2- and 2,3-diacylglycerols derived from the synthetic A and B triacylglycerols by hydrolysis with pancreatic lipase. It is seen that the proportion of the 1,2-isomer exceeds that of the 2,3-isomer for triacylglycerol A, while the opposite result is obtained for B. This suggests that pancreatic lipase favors the release of linoleate over palmitate, regardless of the type of primary position involved. Table IV gives the results of other analyses of pancreatic digests obtained with triacylglycerols A,B,C, and D. In all cases, the unsaturated fatty acid was prefer-

TABLE IV

Diacylglycerol Composition of Hydrolysate during Digestion of Synthetic Triacylglycerols by Pancreatic Lipase

Substrate	Argentation chromatography		Gas liquid chromatography	
	1,2-[a]	2,3-[a]	1,2-[b]	2,3-[b]
	%	%	%	%
sn-Glycerol-1-palmitate-2-oleate-3-linoleate	59.7	40.3	60.3	39.7
	59.6	40.4	61.5	38.5
sn-Glycerol-1-linoleate-2-oleate-3-palmitate	43.0	57.0	41.8	58.2
	45.9	54.1	45.7	54.4
sn-Glycerol-1-oleate-2,3-dipalmitate			35.1	64.9
sn-Glycerol-1,2-dipalmitate-3-oleate			66.7	33.3

[a]Diacylglycerol fractions were determined as monoenes and trienes.

[b]Diacylglycerol fractions were determined as carbon numbers 32, 34, or 36.

entially released. Furthermore, the GLC findings were reproduced faithfully in the results derived by argentation TLC of the diacylglycerols from the corresponding incubations of triacylglycerols A and B.

DISCUSSION

Hydrolysis with Lipoprotein Lipase

The results of the chromatographic studies of the diacylglycerols released from synthetic triacylglycerols coincide with those obtained previously by stereospecific analyses of the hydrolysis products of radioactive trioleoylglycerol. In contrast to the previous methods, the present assays are more rapid and much more direct, especially the GLC technique, and less likely to be subject to error due to involved analytical manipulations. The accumulation of the 2,3-diacyl-sn-glycerols during lipoprotein lipase hydrolyses must, therefore, be considered real and deserves further discussion.

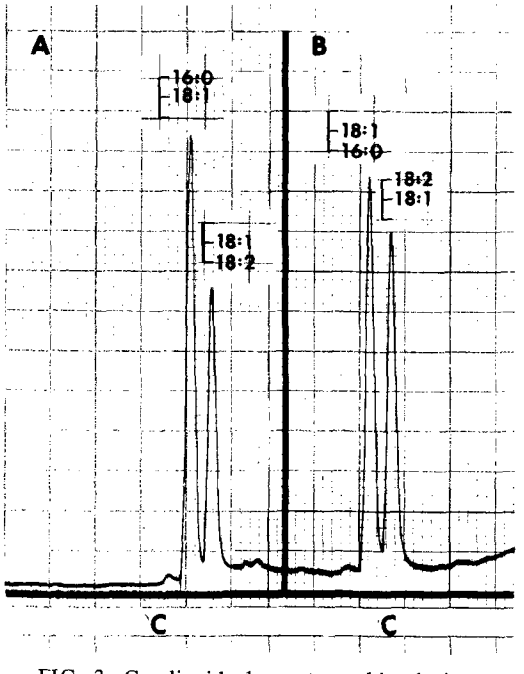

FIG. 3. Gas liquid chromatographic elution patterns of 1,2- and 2,3-diacylglycerols derived from synthetic A and B triacylglycerols by pancreatic lipase hydrolysis. Temperature was programed for 150-350 C in 16 min and major peaks appeared at 290 and 300 C.

It should be emphasized that such a disproportionation of the composition of a relatively minor intermediate is fully compatible with a largely random release of large amounts of fatty acids from the 1 and 3 positions of the triacylglycerol molecule, as observed by Assmann, et al., (2) for postheparin plasma lipase and Jensen (15) for milk lipase. The accumulation of the 2,3-diacylglycerols in a small total population of diacylglycerols also is not excluded by the results of Nilsson-Ehle, et al., (3), who showed that the primary positions of triacylglycerols are attacked by lipoprotein lipase ahead of the secondary position.

The apparent conflict between our results and those obtained by Karnovsky and Wolff (7) may be due to differences in the experimental conditions, although both analyses followed a comparable degree of overall hydrolysis. Karnovsky and Wolff conducted their experiments under the conditions of Borgstrom which had been shown to lead to a significant lipase catalyzed reversal of lipolysis. The present short term digestions would have afforded less opportunity for random reesterification of the 2-monoacylglycerols to the 1,2- and 2,3-diacylglycerols. Nevertheless, the presence of 25%

total X-1,2-diacylglycerol as 1,2-diacylglycerol could be accounted for by partial random reversal of lipolysis from 2-monoacylglycerol, with the 2,3-diacylglycerol the exclusive original diacylglycerol product of hydrolysis. Nilsson-Ehle, et al., (3) demonstrated the acylation of 2-monoacylglycerols to diacylglycerols by milk lipase.

The possibility remains, however, that some 1,2-diacylglycerol could be formed as the result of enzyme attack on the sn-3 position of the triacylglycerol, since Greten, et al., (16) has shown hydrolysis of 1,2-dialkyl-3-acyl-sn-glycerols by lipoprotein lipase. Nilsson-Ehle, et al., (17) claims that such degradation takes place at much lower rates than for triacylglycerols and, therefore, may not adequately indicate specificity. Alternatively, a disproportionation of the intermediate 1,2- and 2,3-diacylglycerol mixture could have occurred as a result of unequal rates of further hydrolysis of diacylglycerols. Using equimolar mixtures of enantiomeric diacylglycerols, we have shown elsewhere (unpublished results) that toward the end of hydrolysis, there is a tendency for the 2,3-diacylglycerols to accumulate in the incubation medium. The observed differences were not sufficient to account for the marked preponderance of the 2,3-isomers seen during triacylglycerol digestion.

Hydrolysis with Pancreatic Lipase

In agreement with previous work (1), the hydrolysis of synthetic triacylglycerols by pancreatic lipase did not show any significant specificity for either of the primary positions. However, on the basis of the composition of the diacylglycerol intermediates, the pancreatic lipase appeared to attack the unsaturated fatty acids in marked preference to the saturated acids, in both 1 and 3 positions of the triacylglycerol molecule. Other studies (18) have shown that the preferential release of unsaturated acids by pancreatic lipase depends upon the interfacial conditions of the digestion medium and the overall structure of the substrate. Saturated and unsaturated acids are released at ca. equal rates in the presence of organic solvents and elevated temperature (12). Reesterification of 2-monoacylglycerols to form diacylglycerols has been demonstrated experimentally for pancreatic lipase in the absence of albumin and calcium (19).

Comparative Studies of Pancreatic and Lipoprotein Lipase

Despite earlier claims to the contrary (2,3,7,15), pancreatic and lipoprotein lipases appear to possess different specificities. It is clear that both enzymes attack the primary positions preferentially, if not exclusively. However, lipoprotein lipase appears to prefer attack on the sn-1 position, while pancreatic lipase does not seem to differentiate between the primary positions of the triacylglycerol molecule. Moreover, the need for bile salts is essential for pancreatic lipase activity, (15) while they tend to inhibit lipoprotein lipase (20) which is activated best by lysolecithin (13). It appears that both enzymes catalyze the reversal of lipolysis but not necessarily to the same extent or under the same conditions. Furthermore, most preparations of lipoprotein lipase appear to contain more than one lipolytic activity (21). Thus, Egelrud and Olivecrona (22) showed that purified bovine lipoprotein lipase had rather low substrate specificity which could be modified by different types of emulsifiers. Greten, et al., (16) showed that the triacylglycerol lipase was distinct from a phospholipase A_1 as seen from different sensitivities to heat and inhibitors. It is, therefore, possible that the presence of these enzyme activities may have obscured the true stereochemical course of the reaction of the triacylglycerol lipase.

In summary, the present results would suggest that a stereospecific attack on acylglycerols by conventional preparations of lipoprotein lipase remains a strong possibility. Further studies with purified enzymes in the presence of appropriately labeled hydrolysis products in combination with stereospecific analyses may be required to clarify this problem.

ACKNOWLEDGMENTS

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In Vitro Activity of Δ6 and Δ9 Desaturases in Hepatomas of Different Growth Rates

ABSTRACT

The in vitro activity of Δ9- and Δ6-desaturases was determined in the microsomal fraction of C3H/S normal mouse liver, SS1K fast growing hepatoma, and SS1H slow growing hepatoma. These tumors are two different sublines of a spontaneous hepatoma transplanted in 1949 by J.W. Wilson, Brown University, Providence, R.I., into C3H/ST W: strain. The activity of the two enzymes showed a parallel decrease in the two studied hepatomas. These alterations could be attributed to a fundamental change in structure or function in the tumors. The fatty acid desaturase activity seems to be independent of cell division in malignant tissues.

INTRODUCTION

Weber and collaborators (1) have developed the molecular correlation concept as a working hypothesis in cancer research. According to this hypothesis, it could be possible to predict the carbohydrate metabolic pattern of cancer cells on the basis of the growth rate of the tumor. In recent experiments (submitted for publication), we found that the capacity of conversion of linoleic acid to γ-linolenic acid was depressed in the Novikoff hepatoma.

In view of these facts, it was of interest to study the relationship between the microsomal fatty acid desaturating activity for stearic acid (Δ9-desaturase) and linoleic acid (Δ6-desaturase) in two hepatomas of different growth rates.

EXPERIMENTAL PROCEDURES

The tumors are the K and H sublines of a spontaneous hepatoma (second S) which was the first (no. 1) transplanted in 1949 by J.W. Wilson, Brown University, Providence, R.I., into

TABLE I

In Vitro Oxidative Desaturation of Stearic Acid to Oleic Acid and Linoleic Acid to γ-Linolenic Acid by Normal Liver, Host Liver, and SS1K Hepatoma

Tissue[a]	Percentage of conversion[b] Stearic 18:0 → 18:1	Linoleic 18:2 → 18:3
Normal liver (5)	34.8 ± 3.0	16.0 ± 0.4
Host liver (4)	26.2 ± 1.5 P<0.01	16.0 ± 0.7 NS
SS1K hepatoma (5)	10.0 ± 1.3 P<0.001	4.4 ± 0.5 P<0.001

[a]Numbers in parentheses indicate the number of individual samples in each group.

[b]Probability (P) values are related to normal liver. Data are the means ± standard error. NS = not significant.

TABLE II

In Vitro Oxidative Desaturation of Stearic Acid to Oleic Acid and Linoleic Acid to γ-Linolenic Acid by Normal Liver, Host Liver, and SS1K Hepatoma

Tissue[a]	Percentage of conversion[b] Stearic 18:0 → 18:1	Linoleic 18:2 → 18:3
Normal liver (5)	51.2 ± 1.8	22.2 ± 0.9
Host liver (4)	54.6 ± 5.0 NS	17.3 ± 1.4 P<0.02
SS1H hepatoma (4)	16.1 ± 0.8 P<0.001	8.0 ± 1.3 P<0.001

[a]Numbers in parentheses indicate the number of individual samples in each group.

[b]Probability (P) values are related to normal liver. Data are the means ± standard error. NS = not significant.

C3H/ST W: (first S) strain. Hepatoma SS1K is a fast growing one and classified as a poorly differentiated hepato cellular carcinoma (2) and SS1H a slow growing and well differentiated one. The hepatomas were maintained by implants in C3H/S male mice and were used for experiments 20 days after implants for the SS1K and 180 days for the SS1H. Animals from the same breed were used as controls.

Animals were killed by decapitation, and liver tissue and hepatomas were homogenized and the microsomes isolated by differential centrifugation, as previously described (3). Microsomal protein was estimated by the biuret method (4). 1-^{14}C Linoleic acid (57.0 mC/mmole, 99% radiochemically pure, Radiochemical Center, Amersham, England) and 1-^{14}C stearic acid (54.0 mC/mmole, 99% radiochemically pure, Radiochemical Center) were diluted to a specific activity of ca. 1.7 mC/mmole with the corresponding unlabeled pure fatty acid.

The assay conditions were as follows: 5 mg microsomal protein was incubated in an open test tube with 100 nmoles of the diluted labeled fatty acid in a Dubnoff Shaker at 37 C for 30 min in a total volume of 1.5 ml 0.15 M KCl, 0.25 M sucrose, containing 2 μmoles adenosine 5'-triphosphate (ATP), 0.1 μmoles coenzyme A (CoA), 1.2 μmoles NaF, 0.5 μmoles nicotinamide, and 62 μmoles phosphate buffer (pH 7.0).

After incubation, the mixture was saponified and the extracted free fatty acids esterified (3). The distribution of radioactivity between linoleic and γ-linolenic methyl esters was determined by gas liquid radiochromatography in a Pye apparatus (3). The conversion of 1-^{14}C stearic acid was measured by thin layer chromatography of the fatty acid methyl esters on $AgNO_3$ impregnated silica gel plates (5).

RESULTS AND DISCUSSION

The activity of Δ9- and Δ6-desaturases in the fast growing SS1K hepatoma is given in Table I. Although a significant decrease in the conversion of stearic acid to oleic acid in the host liver compared to normal liver was determined, ca. 70% reduction in the Δ9- and Δ6-desaturase activity was observed in the hepatoma.

Table II shows the activity of Δ9- and Δ6-desaturases in the slow growing SS1H hepatoma. While the Δ6-desaturase activity was somewhat lower in the host liver, a drastic reduction was observed in the activity of both enzymes in the hepatoma when compared to normal liver.

The decrease in the activity of Δ9- and Δ6-desaturases in the tumor bearing mice could be explained by the existence of a biochemical relationship between the tumor and the host (6).

From the results shown in Tables I and II, it is interesting to note the parallel decrease in the activity of both enzymes in the two studied hepatomas. These results also show a rather similar scope reduction in the specific activity of Δ9- and Δ6-desaturases in both hepatomas.

According to these findings, we can assume that the low level fatty acid desaturase activity found in the hepatomas could be the consequence of an individual enzymatic change produced during carcinogenesis or the manifestation of a single, more fundamental change in structure or function. Since Δ9- and Δ6-desaturases are different enzymes and are controlled by separate mechanisms (7), it seems logical to accept the second explanation as the correct one and to assume that fatty acid desaturation activity is independent of cell division in malignant tissues.

L.E. CHIAPPE,
M.E. DE TOMAS[1]
O. MERCURI[1]
Cátedra de Bioquímica
Instituto de Fisiología
Facultad de Ciencias Médicas
La Plata, Argentina

[1]Member, Carrera del Investigador, Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

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A Simple, Sensitive Method for Lipid Phosphorus[1]

ABSTRACT

A method is described for quantitatively determining lipid phosphorus with a linear range from 0.7-10.0 μg. The method is simple and rapid, requiring one stable reagent and a single extraction with 1-butyl acetate after the phosphorus is converted to inorganic phosphate by means of a perchlorate digestion. The stable complex is read at 310 nm.

INTRODUCTION

Numerous procedures are available for the quantitative determination of lipid phosphorus; the more sensitive methods, however, require either a sequence of involved steps, unstable reagents, long heating periods, or a combination of the three. In addition, a less sensitive method (1,2) we had been using generates sulfur dioxide fumes which produced an allergic reaction in some lab personnel.

Procedures developed recently by Winters (3) for the determination of μg quantities of phosphate in nuclear materials proved to be readily adaptable to lipids and served as the basis for the work described herein.

MATERIALS

All chemicals used were reagent grade with the following exceptions: 1-butyl acetate was obtained from Matheson, Coleman and Bell, Norwood, Ohio, as either BX1730 spectroquality or BX1735; synthetic dipalmitoyl phosphatidyl choline from Schwarz/Mann, Van Nuys, CAlif. Molybdate reagent (3): 29.2 g of ammonium molybdate was dissolved in 100 ml of concentrated (12N) hydrochloric acid. When completely dissolved, 400 ml distilled water was added and the solution stored under refrigeration with aliquots removed for use as needed. It appears that the reagent needs to be aged for 24 hr prior to use. Our experience has been that a shelf-life of 6 months is practical when stored in a glass bottle. Winters (3), however, cautioned that the reagent appears to degrade more rapidly than this.

PROCEDURE

A suitable aliquot of lipid containing 0.7-5.0 μg phosphorus (P) is pipetted into a 10 ml Kjeldahl flask that has been calibrated to contain 15.0 ml. Two glass beads are added, and any solvent is removed using a steam table. After the flasks have cooled, 1.0 ml 70% perchloric acid is added to each sample and to flasks containing suitable standards and blanks. Immediately before digestion add two drops of concentrate nitric acid. Digestion is carried out in two steps: first, a manual one with constant shaking over a microburner to oxidize the bulk of the organic matter and the second for 20 min (2) on an electrically heated Kjeldahl rack. Two additional drops of concentrated nitric acid are added before proceeding with the second digestion step. Samples, standards, and blanks must be digested.

After cooling, the flasks are made to 15 ml using distilled water. Aliquots (10 ml) are pipetted into 16 x 125 mm screw cap culture tubes fitted with Teflon lined caps, and 1 ml reagent is added and mixed well by swirling. 1-Butyl acetate (5.0 ml) is added, the tubes capped, shaken thoroughly, and centrifuged

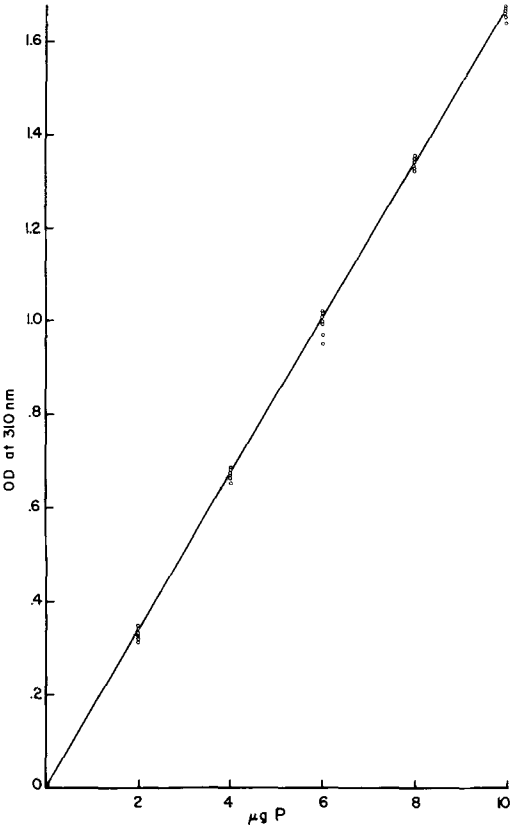

FIG. 1. Calibration curve for P analysis using a KH_2PO_4 standard.

[1]Technical Paper no. 3777, Oregon Argiculture Experiment Station.

sufficiently to separate the layers cleanly. The upper layer is transferred to a 1.0 cm silica cell and read at 310 nm.

RESULTS AND DISCUSSION

Several of the requirements for complex formation and subsequent extraction were examined by Winters (3) and needed to be met as the method was adapted to the perchlorate digestion. The final acid concentration is critical for formation and extraction of the complex with the range of 0.60-0.95 M acid (HCl and $HClO_4$) giving values within 2% of the maximum. Once beyond this range, the values drop rapidly. Without digestion the $HClO_4$ concentration, when diluted to 15 ml, is 0.77 M; therefore, small losses on digestion can be tolerated. Overheating or lack of condensation would give low values; however, the HCl in the reagent would correct for all except large losses, as it brings the final acid concentration to 0.92 M when there is no loss of perchloric acid. Molybdate concentration is far less critical, the curve being essentially linear between 3 and $12x10^{-3}$M.

Winters (3) recommended the washing of the 1-butyl acetate extract with 0.85 M HCl. In our experience, this step proved unnecessary if the phases are centrifuged sufficiently. We have used an International Clinical centrifuge with a 221 head and obtained satisfactory separations with 3 min runs at full speed (1470 G at the tip.) Insufficient centrification will lead to erroneously high results.

The purity of the 1-butyl acetate was found to be quite critical and not directly related to the grade. The best results were obtained from MCB BX 1735 (their fine chemicals grade) with slightly higher blanks being obtained using their spectrograde. Two other sources, including one labeled reagent grade, were totally unsatisfactory even after redistillation. A preliminary check on the suitability of a particular batch of butyl acetate may be made by reading it against wate at 310 nm; values below 0.1 absorption usually indicate that blank values for the final reaction will be in an acceptable range.

The choice of tubes used for the extraction appears to be critical. The use of 27 x 100 mm screw cap centrifuge tubes with Teflon lined caps gave erratic results. This was partially attributable to frequent occurrences of leakage; the other contributing factor may relate to the large interfacial surface area and the small volume of solvent present.

It also was observed that the digestion flasks should not be washed; a thorough rinsing with tap water and then distilled water resulted in considerably lower background levels of phosphorus. New flasks should be cleaned via a perchloric digestion.

The molybdophosphoric acid complex in 1-butyl acetate shows less than 1% change in absorbance from 7-25 min after formation, 6 min being consumed in sample preparation.

Standard curves were made using potassium dihydrogen phosphate (1); the one shown in Figure 1 contains the data points from 3 separate runs made over a span of 10 days. Linearity was observed from 0.0 to 1.6 absorption. The molar absorbancy coefficient was calculated to be 25,400.

Two sets of 10 each of the synthetic phosphatidyl choline, assayed at 4.39% P, were run to check the accuracy and precision of the method. The set a 2.63 μg P had a mean of 2.57 μg P ± 0.11 (SD) while those at 5.27 μg P averaged 5.13 μg P ± 0.15 (SD).

ROBERT R. LOWRY
IAN J. TINSLEY
Department of Agricultural Chemistry
Oregon State University
Corvallis, Oregon 97331

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[Received March 7, 1974]

LETTER TO THE EDITOR

An Improved Method for Thin Layer Chromatography of Plasma Lipids by Single Development

Sir: Details of a thin layer chromatographic procedure for separating mixtures of phospholipids, monoglycerides, nonesterified fatty acid, 1:2 and 1:3 diglycerides, free cholesterol, triglyceride and cholesterol ester by single development previously have been reported from this laboratory (Storry, J.E. and B. Tuckley, *Lipids* 2:501 [1967]). Since this report, modifications to the silica gel layer, solvent system, and spray reagent have been introduced, resulting in better separation of nonesterified fatty acids and replacement of highly toxic benzene in the developing solvent. With the previous method, in the presence of high levels of nonesterified fatty acids, overlap with the cholesterol band could occur. On the other hand, at low levels, the nonesterified fatty acid band was sometimes too diffuse to allow quantitative recovery from the plate. The present method overcomes both disadvantages and, therefore, can be used for samples containing a much wider range of nonesterified fatty acid concentration.

The modified layers are prepared from slurries of Silica Gel H (E. Merck A.G., Darmstadt, Germany) calcium sulphate and 50 m$\underline{\underline{M}}$ sodium bicarbonate (15:2:35 w/w/v). The use of Silica Gel H and calcium sulphate freshly prepared, rather than the proprietary Silica Gel G, produces plates with more tenacious layers which also give more uniform chromatographic results. Impregnation of the layers with sodium bicarbonate suppresses any spreading of the nonesterified fatty acid band which facilitates the detection and recovery of very small quantities and also the separation of large quantities of fatty acids without interference with free cholesterol (Fig. 1). The reduced spreading of nonesterified fatty acids results from reaction of the nonesterified fatty acids with the impregnated sodium bicarbonate to produce a sharp leading edge of very slowly migrating sodium salts. Against this leading edge of fatty acid salts, there is a compressed band of more

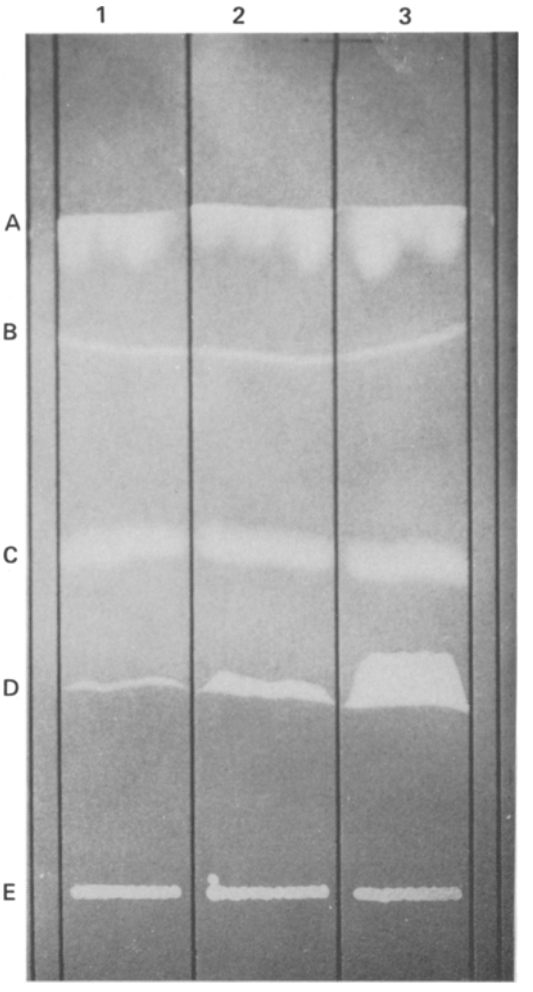

FIG. 1. Separation of lipid mixtures containing increasing proportions of free fatty acids, using thin layers prepared from proprietary Silica Gel H, calcium sulphate and sodium bicarbonate. Lanes 1, 2, and 3 each contain (A) 4.2 mg cholesterol oleate, (B) 0.16 mg mixed triglycerides, (C) 0.72 mg cholesterol, (E) 2.8 mg phospholipid, and (D) 0.1, 0.5, and 2.0 mg respectively, mixed free fatty acids.

mobile free fatty acids, produced from the salts by the ascending acetic acid front of the developing solvent mixture, resulting in a sharp trailing edge.

The original solvent system of benzene, diethyl ether, ethyl acetate, acetic acid (80:10:10:0.2) has been replaced by toluene, diethyl ether, ethyl acetate, and acetic acid (80:10:10:0.75). The greater proportion of acetic acid is necessary with the sodium bicarbonate impregnated silica gel layers to achieve similar R_f values to the original thin layer system.

The positions of separated lipids are identified under UV light after spraying the plates lightly with a methanolic solution containing 0.025% dichlorofluorescein and 0.005% rhodamine B. This spray reagent is a simplification of that proposed by Jones, et al. (*J. Chromatogr.* 23:172 [1966]) and gives good contrast between lipid and background in UV light (366 nm).

Glass troughs originally used for holding the developing solvent have been replaced by ones of stainless steel. These measure 22 cm long, 4 cm wide at the base, tapering to 1.7 cm at the open top, and are made from welded stainless steel sheet (22 gauge, 18/8 quality).

B. TUCKLEY
J.E. STORRY
National Institute for Research in Dairying
Shinfield
Reading, England RG2 9AT

[Received January 16, 1974]

ERRATUM

In "Effects of Different Culture Media and Oxygen upon Lipids of *Escherichia coli* K-12" by William F. Naccarato, John R. Gilbertson, and Rose A. Gelman (*Lipids* 9:322 [1974]), an error occurred in the third sentence in the second paragraph of the "Introduction." The word anaerobically should be changed to aerobically. The sentence should read: "If this were true, 2-alkanols should disappear when *E. coli* are grown aerobically in media with glucose as the only carbon source."

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Influence of Rabbit Milk upon Cholesterolaemic Response of Offspring of Rabbits

D.C.K. ROBERTS and **C.E. WEST**, Department of Experimental Pathology, John Curtin School of Medical Research, The Australian National University, Canberra City, A.C.T., 2601 Australia

ABSTRACT

The milk lipids from the dams of two strains of rabbits differing in their cholesterolaemic response, one hyperresponsive and one hyporesponsive to dietary cholesterol, were analyzed. The hyperresponsive dams had significantly higher ($P < 0.05$) cholesterol and phospholipid concentrations than the hyporesponsive dams but similar triglyceride concentrations. Cross fostering experiments with hyporesponsive and hyperresponsive offspring were carried out. Offspring from hyporesponsive parents suckled on hyperresponsive dams resembled hyperresponsive offspring in their cholesterolaemic response. However, offspring from hyperresponsive parents responded as hyperresponsive whether they were raised on their natural dams or on foster hyporesponsive dams. We conclude that the trait for hyperresponder characteristics is uninfluenced by rabbit milk, while the trait for hyporesponder characteristics is dependent upon the cholesterol and phospholipid concentrations in milk.

INTRODUCTION

The effect of milk lipid constituents upon the plasma cholesterol concentration of the suckling young have been reported in many species (1-3). The hypercholesterolaemia seen in the suckling calf and rat has been attributed to the concentration of triglyceride in the milk (1,4) and in the rabbit to the concentration of cholesterol in the milk (5). The effect of various cholesterol intakes in early development upon the subsequent cholesterolaemic response to dietary cholesterol has been reported in man, pigs, and rats (6-8). In man, the plasma cholesterol concentration at 12 months was uninfluenced by earlier low or moderate cholesterol diets (6). Reiser and Sidelman (8), report that, in male rats after 24 weeks on a cholesterol diet, there is an inverse correlation between the dam milk cholesterol concentration and the plasma cholesterol concentration. They also report a similar situation for male and female pigs (7). In this article we report the effect of milk lipid levels upon the dietary induced cholesterolaemic response of the offspring of two strains of rabbits, one hyperresponsive (HR) and one hyporesponsive (HO) to dietary cholesterol.

MATERIALS AND METHODS

Animals and Diets

THe rabbits used were hyper- and hyporesponding males and females selectively bred, as described previously (9), from a colony maintained at the Animal Breeding Establishment of the Australian National University. The animals were allowed free access to a basic diet containing no added cholesterol, except for a 3 week period from an age of 10 weeks, when they were phenotyped as HR- or HO in their cholesterolaemic response to dietary cholesterol. During this period, each animal, caged individually, received 70 g/day of a cholesterol containing diet of 0.28% (w/w) cholesterol (200 mg cholesterol/day). The detailed composition and preparation of the diet has been described previously (9). At all times, animals had free access to water. At least 10 weeks were allowed to elapse after this period before the animals were mated. At this time, the plasma cholesterol concentration had returned to the precholesterol feeding concentration.

Cross-Fostering of Progeny

Within 2 days of birth, young from matings of HR males and females were cross-fostered with those from matings of HO males and females. The young had access to the basic diet

TABLE I

Analysis of Milk from Hyporesponder (HO) and Hyperresponder (HR) Rabbit Dams[a]

		Dams	
		HO (4)	HR (4)
Total cholesterol	mg/dl	46 ± 5.4	93 ± 11.2[b]
Phospholipid	mg/dl	97 ± 8.1	163 ± 25.3[c]
Triglyceride	g/dl	13.9 ± 0.98	15.9 ± 1.30
Protein	g/dl	9.2 ± 0.53	8.6 ± 1.54

[a]Results are mean ± standard error. Comparisons are by Student's t-test between HO- and HR dams.

[b]$P < 0.01$.

[c]$P < 0.0$[illegible]

TABLE II

Mean Plasma Cholesterolaemic Response of Offspring Suckled on Natural and Foster Rabbit Dams[a]

Dam	Mean cholesterolaemic response[b] mg/dl ± standard error of offspring suckled on	
HO[c]	Natural HO	Foster HR
024	505 ± 73.7 (5)	983 ± 52.0[d] (3)
980	---	1036 ± 57.3 (5)
994	512 ± 62.2 (7)	845 ± 71.0[e] (5)
HR	Natural HR	Foster HO
951	1153 ± 74.6 (6)	1072 ± 49.4 (6)
953	1177 ± 64.2 (6)	1096 ± 103.8 (4)
964	856 ± 88.9 (6)	1043 ± 75.5 (6)

[a]Comparisons by Student's t-test of offspring cholesterolaemic response between natural and foster mother.

[b]Increase observed after 3 weeks on a daily diet of 70 g feed containing 200 mg cholesterol.

[c]HO and HR refer to hypo- and hyper-responder rabbits, respectively.

[d]$P < 0.005$.

[e]$P < 0.01$.

from birth to 10 weeks of age. They were weaned at age 6 weeks and at 10 weeks of age were caged individually and fed the same cholesterol containing diet for 3 weeks, as described above. Plasma cholesterol concentration was determined before and after this period as described previously (10).

Sampling and Analysis of Milk

Ten days post partum, the dams were milked manually. Oxytocin (1 unit/kg) was given intravenously via the marginal ear vein prior to milking. The milk (1 volume) was added to 20 volumes of chloroform:methanol (2:1, v/v) and allowed to stand overnight. The protein was precipitated by centrifugation at 3000 x g for 10 min, and the lipid containing supernatant was aspirated into flasks and the nonlipid contaminants removed by the method of Folch, Lees, and Sloane Stanley (11). Neutral lipids were separated from phospholipids by the method of Zilversmit (12) on columns of silicic acid-kieselguhr (Hyflo supercel, Townson and Mercer Pty, Lane Cove Australia). Total cholesterol was measured, as previously described (10), and lipid phosphorus by the method of Eibl and Lands (13). Glyceride glycerol was determined by the method of Zilversmit (12). The protein precipitates were dissolved in sodium hydroxide (1N) and aliquots taken for estimation of protein nitrogen by direct nesslerization of Kjeldahl digests (14).

RESULTS AND DISCUSSION

The analyses of milk from four HR- and four HO dams are shown in Table I. The cholesterol, phospholipid, triglyceride, and protein concentrations for HR dams agree with the literature values (15,16). The milk of HO dams has significantly lower cholesterol ($P < 0.01$) and phospholipid concentration ($P < 0.05$) but similar protein and triglyceride concentrations to the milk of the HR dams. The mean plasma cholesterolaemic response of litters suckled on foster dams together with the response of litters from separate matings of the same parents suckled on their natural mothers are shown in Table II. Data from male and female offspring are pooled, as we previously have shown that there is no difference in their cholesterolaemic responses (9). We also previously have shown that offspring from repeated matings of the same parents have similar cholesterolaemic responses (9).

It can be seen that the cholesterolaemic response of HR offspring remains the same (HR) whether they are raised on their natural or HO foster dams (Table II). We previously have shown that the cholesterolaemic response has a heritability of 50 ± 4.7% (9), the remaining variation being determined environmentally. The trait for HR appears to be independent of previous exposure to high or low concentrations in milk of cholesterol and phospholipid. However, HO offspring fostered on HR dams have a significantly higher ($P < 0.01$) cholesterolaemic response than HO offspring raised on their natural dams. For HO offspring, the increased cholesterol and phospholipid concentrations in the milk of HR dams appears to influence their subsequent cholesterolaemic response so that they resemble HR offspring. Thus, the trait for HO would seem to

depend, for its expression, upon the milk cholesterol and phospholipid concentrations. These results differ from those reported by Reiser and Sidelman (8) in which an inverse relationship between milk cholesterol and the cholesterolaemic response in male rats after 24 weeks on a cholesterol diet is reported. However, an earlier article by the same authors reports that female rats raised on high cholesterol formula milk, after 9 weeks on a cholesterol diet, had higher serum cholesterol concentration than those raised on a low cholesterol formula (17).

Rabbit milk cholesterol is drived principally from plasma cholesterol (18) and we previously have shown that the plasma cholesterol concentration is heritable in noncholesterol fed female rabbits (19). The plasma cholesterol concentration at age 10 weeks of the male and female offspring suckled on HO or HR natural or foster dams did not show any significant differences. However, it was necessary to analyze the male and female data separately, as we have shown that there is a sex difference in the noncholesterol fed plasma cholesterol concentration (9). This meant that there were few observations/litter and consequently the failure to show any significant differences between litters suckled on the various dams may, in part, be due to this. The plasma cholesterol concentration of the four HR dams at 10 weeks of age (170 ± 18.4 mg/dl, mean ± standard error) was significantly higher ($P < 0.01$) than that of the 4 HO dams (74 ± 5.3). This could account for the difference in milk cholesterol concentration. The plasma cholesterol concentration of the dams, however, was not measured at the time of milk collection. The significance of the increased phospholipid concentration in the milk of HR dams remains obscure. Formula feeding experiments may help to determine whether it is cholesterol or phospholipid which causes the failure of the control of dietary induced cholesterolaemia in the HO rabbit.

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Evidence for Acyl Transfer Reactions between Neutral Glycerolipids in Dogfish *(Squalus acanthias)* Serum

DONALD C. MALINS and **PAUL A. ROBISCH**, Pioneer Research Unit,
Northwest Fisheries Center, National Marine Fisheries Service, NOAA,
U.S. Department of Commerce, Seattle, Washington 98112

ABSTRACT

Radioactively labeled triacylglycerols, 1,3-dioleoyl-2-[1-^{14}C]-palmitoylglycerol and 1,3-[9,10-^{3}H]-dioleoyl-2-palmitoylglycerol, were incubated with dogfish (*Squalus acanthias*) serum for periods of up to 10.0 hr. Changes in the positional distributions of carbon-14 and tritium within the triacylglycerols in 5.0 hr and 10.0 hr indicate that intermolecular and intramolecular shifts occur among the fatty acids. In addition, a maximum of 8.3% of the carbon-14 and 5.9% of the tritium was incorporated into 1-alkyl-2,3-diacylglycerols; essentially all of this incorporated radioactivity was associated with the acyl chains in the 1.5 and 5.0 hr incubations. In the 10.0 hr incubations, however, 25% of the tritium incorporated into the 1-alkyl-2,3-diacylglycerols was associated with the O-alkyl chains. Radioactivity was not incorporated significantly into free fatty acids in the 1.5 and 5.0 hr incubations. These results indicate that acyl transfer reactions take place among molecular species of triacylglycerols, as well as between triacylglycerols and 1-alkyl-2,3-diacylglycerols. In the latter case, the conversions appear to be operative in the virtual absence of net biosynthesis.

INTRODUCTION

Triacylglycerols incorporate a substantial amount of radioactivity from labeled acid in such diverse systems as the liver of dogfish (*S. acanthias*) (1-4), the arteries of human umbilical cords (5), and mouse brain (6). These findings indicate that the triacylglycerols are metabolized actively in a number of different tissues. Yet, little information is available on possible metabolic relations that exist between these glycerides and other lipids.

In the present work, metabolic conversions taking place between triacylglycerols and other lipid classes were investigated using dogfish (*S. acanthias*) serum. The serum of this species of small shark contains large amounts of triacylglycerols and 1-alkyl-2,3-diacylglycerols, together with small amounts of phospholipids, wax esters, and cholesterol esters (7).

We studied the metabolism of lipids in the serum of *S. acanthias* using triacylglycerols containing acids labeled with carbon-14 and tritium on positions 2 and 1,3, respectively. The incorporated radioactivity was largely confined to the triacylglycerols and the 1-alkyl-2,3-diacylglycerols. It was shown that acyl transfer reactions occur among the triacylglycerols and between triacylglycerols and 1-alkyl-2,3-diacylglycerols in the virtual absence of net biosynthesis. The results are of interest with respect to previous work showing that acyl transfer reactions take place between the phospholipids of human serum (8).

MATERIALS AND METHODS

Preparation of Radioactively Labeled Triacylglycerols

The triacylglycerols, radioactively labeled on the acyl chains, were synthesized in our labora-

TABLE I

Distribution of Carbon-14 from 1,3-Dioleoyl-2-[1-^{14}C]-Palmitoylglycerol among Acyl Chains of Triacylglycerols from *Squalus Acanthias* Serum[a]

Location	1.5 hr dpm/mM (x 10^3)	5.0 hr dpm/mM (x 10^3)	10.0 hr dpm/mM (x 10^3)
Positions 1,3	1,560	22,700	36,900
Position 2	130,000	101,000	67,600

[a]Data normalized to administered dose of 572,000 dpm; experimental conditions are given in the text.

TABLE II

Distribution of Tritium from 1,3-[9,10-^{3}H]-Dioleoyl-2-Palmitoylglycerol among Acyl Chains of Triacylglycerols from *Squalus Acanthias* Serum[a]

Location	1.5 hr dpm/mM (x 10^3)	5.0 hr dpm/mM (x 10^3)	10.0 hr dpm/mM (x 10^3)
Positions 1,3	131,000	108,000	106,000
Position 2	3,370	4,330	33,200

[a]Administered dose of 572,000 dpm; experimental conditions are given in the text.

tories by the two step procedure described by Mitchell (9). The triacylglycerols obtained were 1,3-dioleoyl-2-[1-^{14}C]-palmitoylglycerol (0.03mCi/mMole) and 1,3-[9,10-^{3}H]-dioleoyl-2-palmitoylglycerol (28mCi/mMole). The former glyceride was prepared by reacting palmitoyl-[1-^{14}C]-chloride (0.68mCi/mMole) with 1,3-dichloro-2-propanol (Eastman Organic Chemicals, Rochester, N.Y.) in pyridine. The resulting 1,3-dichloro-2-[1-^{14}C]-palmitoylglycerol then was converted to the radioactively labeled triacylglycerol by reaction with sodium oleate in dimethylformamide (10). The tritium labeled triacylglycerol was prepared in the same manner by reacting sodium[9,10-^{3}H]oleate (100mCi/mMole) with 1,3-dichloro-2-palmitoylglycerol. Each radioactively labeled triacylglycerol was chromatographed on thin layer plates to a final purity of greater than 98% (11).

Preparation of Serum

Blood was taken from four mature dogfish (*S. acanthias*) caught in Puget Sound, Wash. during March 1973. An average of 50 ml was obtained from each fish via the caudal vein. The blood was cooled, allowed to clot, and the serum was obtained by centrifugation (1.73×10^4 g) at 10 C. The serum then was fortified with 10mM adenosine triphosphate (ATP), 10 mM $MgCl_2$, 0.1 mM coenzyme A, 2.5 mM glutathione, 200 mM sucrose, and 80 mM Tris-HCl buffered at pH 7.4, as described in a study with a cell-free preparation of *S. acanthias* liver (1). In the previous study with *S. acanthias* liver (1), this medium was favorably employed to test for the incorporation of administered fatty acid into glycerolipids and wax esters. The serum contained 3.4 mg lipid/ml and 28.5 mg protein/ml (12).

Incubation Conditions

Fortified serum (40 ml) was pipetted into 250 ml flasks and placed in a water bath maintained at 14 C. The 1,3-dioleoyl-2-[1-^{14}C]-palmitoylglycerol ($2.16 \times 10^{-2}\mu$Ci) and the 1,3-[9,10-^{3}H]-dioleoyl-2-palmitoylglycerol ($2.57 \times 10^{-1}\mu$Ci) were added simultaneously to each flask as a suspension in 0.6 ml 1% Triton X-100. The incubations were carried out under nitrogen with agitation for periods of 1.5-10.0 hr. After each incubation period, the reactions were terminated by rapidly freezing the flasks in dry ice, followed by immediate isolation of the lipid (13). Control experiments with serum boiled for 15 min at 100 C indicated that no more than 0.005% of the radioactivity in the specifically labeled triacylglycerols was incorporated into other lipid sites under these conditions.

Analyses of Lipids

The triacylglycerols and 1-alkyl-2,3-diacylglycerols were isolated by preparative thin layer chromatography (TLC) as previously described (1-4, 11), and the purity (greater than 99%) was established by chromatographic and spectral analyses of derivatives (11). The triacylglycerols and 1-alkyl-2,3-diacylglycerols were hydrolyzed with pancreatic lipase (14). Saponification of the resulting 2-acylglycerols, followed by TLC (2,4), was employed to obtain the acids occupying position 2 of the triacylglycerols. Hydrolysis of the 1-alkyl-2,3-diacyl-

TABLE III

Incorporation of Carbon-14 from 1,3-Dioleoyl-2-[1-^{14}C]-Palmitoylglycerol into Lipids of *Squalus Acanthias* Serum[a]

Lipid class	1.5 hr		5.0 hr		10.0 hr	
	dpm/mM (x 10^3)	Percent administered dose	dpm/mM (x 10^3)	Percent administered dose	dpm/mM (x 10^3)	Percent administered dose
1-alkyl-2,3-diacylglycerols	640	0.4	2,990	1.7	14,800	8.3[b]
Position 1	n.d.[c]	n.d.	<50	-	<50	-
Position 2	n.d.	n.d.	2,970	1.7	16,950	9.5
Position 3	n.d.	n.d.	271	0.1	786	0.4
Phospholipids	159	0.1	234	0.1	430	0.3
Wax esters	<50	-	305	0.1	197	0.1
Cholesterol esters	<50	-	<50	-	234	0.1
Free fatty acids	<50	-	<50	-	680	0.4

[a]Data normalized to administered dose of 572,000 dpm; experimental conditions are given in the text.

[b]Discrepancies between radioactivity for the whole molecule and the sum of the components is due to experimental error.

[c]n.d. = not determined.

TABLE IV

Incorporation of Tritium from 1,3-[9,10-^{3}H]-Dioleoyl-2-Palmitoylglycerol into Lipids of *Squalus Acanthias* Serum[a]

Lipid class	1.5 hr		5.0 hr		10.0 hr	
	dpm/mM (x 10^3)	Percent administered dose	dpm/mM (x 10^3)	Percent administered dose	dpm/mM (x 10^3)	Percent administered dose
1-alkyl-2,3-diacylglycerols	3,090	1.7[b]	10,600	5.9	8,100	4.5
Position 1	n.d.[c]	n.d.	<50	-	2,170	1.2
Position 2	n.d.	n.d.	n.d.	n.d.	732	0.4
Position 3	3,970	2.2	10,700	6.0	5,190	2.9
Phospholipids	147	0.1	477	0.3	193	0.1
Wax Esters	n.d.	n.d.	n.d.	n.d.	90	<0.1
Cholesterol esters	n.d.	n.d.	n.d.	n.d.	<50	-
Free fatty acids	<50	-	<50	-	1,100	0.6

[a]Administered dose of 572,000 dpm; experimental conditions are given in the text.

[b]Discrepancies between radioactivity for the whole molecule and the sum of the components is due to experimental error.

[c]n.d. = not determined. There was insufficient radioactivity incorporated for accurate measurements.

glycerols with pancreatic lipase yielded fatty acids from position 3 and 1-alkyl-2-acylglycerols. The latter structures were saponified to obtain the acids of position 2 and an 0-alkylglycerol. Both products were purified by TLC (3). Wax esters and cholesterol esters were isolated by TLC in hexane: benzene: acetic acid (60:40:1, v/v/v) (15). Free fatty acids and phospholipids were obtained, as previously described (16). The composition of the lipids in the serum was as follows: triacylglycerols (44.1%); 1-alkyl-2,3-diacylglycerols (30.4%); phospholipids (20.1%); wax esters (0.9%); and cholesterol esters (1.2%).

RESULTS AND DISCUSSION

Only a limited amount of information exists on the metabolism of lipids in serum (17-18). In the present work, an attempt was made to obtain a preliminary understanding of metabolic relations that exist between the lipids of dogfish serum. The metabolic fate of carbon-14 and tritium labeled 1,3-dioleoyl-2-palmitoylglycerol was investigated and the incorporation of radioactivity into the major lipid classes of the serum was monitored.

The findings indicate that almost all of the carbon-14 and tritium was confined to the triacylglycerols and 1-alkyl-2,3-diacylglycerols during the course of the experiments (Tables I-IV). Surprisingly little radioactivity was associated with the phospholipids, wax esters, and cholesterol esters (Tables III and IV). Only trace amounts of radioactively labeled free fatty acids were detected in the serum. With the exception of tritium in the 10.0 hr experiment, the radioactivity associated with these acids was an insignificant fraction of the radioactivity incorporated into the 1-alkyl-2,3-diacylglycerols.

In 1.5 hr, the positional distribution of carbon-14 and tritium in the triacylglycerols remained virtually unaltered; however, in 5.0 and 10.0 hr, substantial changes in these distributions occurred (Tables I and II). In 10.0 hr, 35% of the carbon-14 and 24% of the tritium was found on positions 1,3 and 2, respectively. Thus, rearrangements took place that apparently involved intermolecular and intramolecular shifts in fatty acids.

The carbon-14 and tritium in the 1-alkyl-2,3-diacylglycerols (Tables III and IV) were essentially confined to the acyl chains, particularly in the 1.5 and 5.0 hr experiments. No evidence was found for the incorporation of carbon-14 into the 0-alkyl chains and tritium only appeared to a measurable extent in 10.0 hr. At this time, it comprised 25% of the total tritium in the 1-alkyl-2,3,-diacylglycerols. Because little radioactive free fatty acid was found, the rearrangements of fatty acids between the triacylglycerols and the 1-alkyl-2,3-diacylglycerols are attributed to acyl transfer reactions, rather than to the hydrolysis of ester bonds and the reincorporation of fatty acid.

The very slow accumulation of tritium into the 0-alkyl chains apparently results from the lack of free acid to initiate the biosynthesis of the alcohol precursor of the ether linkage (19). Moreover, lack of free acid or the low concentration of 18:1 acid (<0.5 mole% of the acid fraction) (20) may explain the small degree of

radioactivity incorporated into the wax esters. Previous studies with a cell-free system from the liver of *S. acanthias* showed that radioactively labeled acid is incorporated into acyl chains of wax esters at a rate comparable to the biosynthesis of acyl chains of triacylglycerols (1).

Interestingly, the carbon-14 and tritium showed a remarkable specificity for positions 2 and 3 of the 1-alkyl-2,3-diacylglycerols, respectively (Tables III and IV). This tendency, which cannot be explained fully, because of the molecular rearrangement in the triacylglycerols themselves, may be related to the fact that acyl transferases of high positional specificity are operative in the serum. This possibility is worthy of a more detailed investigation.

The lack of significant radioactivity in the 0-alkyl chains in 1.5 and 5.0 hr indicates that the acyl transfer reactions take place in the virtual absence of the net biosynthesis of ether linkages. The relatively low levels of tritium found in the 0-alkyl chains in 10.0 hr may arise from small pools of free acids gradually released by the enzymic hydrolysis of acyl chains occupying positions 1,3 of the tritium labeled triacylglycerols. These acids ultimately contribute to the very slow biosynthesis of the 1-alkyl-2,3-diacylglycerols in the serum.

The findings indicate that the triacylglycerols are not active precursors of the 1-alkyl-2,3-diacylglycerols in the dogfish serum. The main interconversions appear to involve acyl transfer reactions between neutral glycerolipids which are reminiscent of those occurring among the various phospholipids of human serum (8). The results obtained from the present study imply that acyl transfer reactions may be operative between the neutral glycerolipids of other animal systems. Such conversions may proceed rapidly and extensively in the virtual absence of net biosynthesis.

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On Extraction of Acyl and Alkyl Dihydroxyacetone Phosphate from Incubation Mixtures

AMIYA K. HAJRA, Department of Biological Chemistry and Mental Health Research Institute, The University of Michigan, Ann Arbor, Michigan 48104

ABSTRACT

Chloroform-methanol mixture was shown to extract acyl and alkyl dihydroxyacetone phosphate from enzyme incubation mixtures. However, when the lipid extract was washed with water to remove nonlipid materials, 70-80% of acyl and alkyl dihydroxyacetone phosphate were lost in the aqueous phase. It was shown that, keeping the pH low (< 2.5) during the partition of lipids by the Bligh and Dyer method, most ($> 95\%$) of the acyl and alkyl dihydroxyacetone phosphate were recovered in the chloroform-rich phase. n-Butanol was shown to extract 80-90% of these lipids from incubation mixtures at neutral pH.

INTRODUCTION

Acyl and alkyl dihydroxyacetone phosphate (DHAP) are important intermediates in the biosynthesis of glycerolipids containing ester and ether bonds (1,2). A number of workers were unable to show the formation of these lipids in vitro though the dephosphorylated products (acyl and alkyl dihydroxyacetone) were shown to be formed in different systems (3-5). It seems likely that these phospholipids were formed in the above mentioned experiments but were not recovered by the standard lipid extractions employed. Most of the workers used chloroform-methanol to extract the lipid and then washed the lipid extract to remove nonlipid materials, as described either by Bligh and Dyer (6) or by Folch-Pi, et al. (7). We generally used a modified Bligh and Dyer extraction method at low pH (8,9) to wash these polar lipids. This article provides evidence that most of the acyl or alkyl DHAP is removed from the chloroform layer during the washing procedure at neutral pH and that this loss can be prevented by reducing pH of the aqueous layer during partition of lipid into the organic phase. It also is shown that n-butanol is a fairly efficient solvent for the extraction of these lipids at neutral pH.

MATERIAL AND METHODS

[^{32}]DHAP was prepared by the enzymatic phosphorylation of dihydroxyacetone with [γ-^{32}] adenosine triphosphate (ATP) (10). Palmitoyl [^{32}P]DHAP was prepared by incubating potassium palamitate, ATP, coenzyme A (CoASH), Mg^{++}, and [^{32}P]DHAP with guinea pig liver mitochondria, and the labeled lipid was purified by column chromatography on silicic acid (10). [1-^{14}C]Hexadecyl DHAP was prepared by incubating [1-^{14}C]hexadecanol and palmitoyl DHAP with guinea pig liver mitochondria, and the labeled phospholipid, after treatment with alkali, was purified by column

TABLE I

Comparison of Different Extraction Methods of Radioactive Acyl and Alkyl Dihydroxyacetone Phosphate from Simulated Incubation Mixtures[a]

	Radioactive lipid extracted by solvents			
	Palmitoyl ^{32}P DHAP[b]		[1-^{14}C]Hexadecyl DHAP	
Extraction method	cpm x 10^{-4}	(Percent)	cpm x 10^{-4}	(Percent)
Acidic Bligh and Dyer	10.23	(95.6)	4.27	(99.4)
Bligh and Dyer	2.03	(18.9)	1.24	(28.8)
Folch-Pi extraction	1.11	(10.3)	0.93	(21.0)
Butanol extraction	7.82	(73.1)	3.52	(81.8)

[a]The incubation mixture contained Tris-hydrochloric acid (HCl) buffer (75 mM, pH 7.4); NaF (8 mM); adenosine triphospahte (ATP) (8 mM); coenzyme A (CoASH) (80 μM) $MgCl_2$ (4 mM); glutathione (4 mM); bovine serum albumin (1.0 mg); heat denatured (100 C, 10 min) guinea pig liver mitochondria (0.65 mg protein); and either palmitoyl [^{32}P]DHAP (4.1 nmoles), 1.07 x 10^5 cpm or [1-^{14}C]hexadecyl DHAP (2.3 nmoles, 4.3 x 10^4 cpm) in a total volume of 1.2 ml. The radioactive lipids were extracted by different methods, as described in the text.

[b]DHAP = dihydroxyacetone phosphate.

and thin layer chromatography (TLC) (2). Other methods and materials were the same as described previously (9,10).

Four different methods were compared to check the efficiency of extraction of acyl and alkyl DHAP.

Acidic Bligh and Dyer method: In this modified Bligh and Dyer method (9), 4.5 ml chloroform-methanol (1:2) was added to 1.2 ml incubation mixture. After mixing some time the precipitated protein was removed by centrifugation. To this solution (with or without protein) 1.5 ml aqueous mixture of KCl-H_3PO_4 (2 M-0.2 M) was added followed by 1.5 ml chloroform. After mixing (Vortex mixer, Scientific Products, Evanston, Ill.) and centrifugation (500 x g for 10 min), the upper layer (pH 2.3) was removed, and an aliquot of the lower organic layer was used to determine the amount of lipid extracted from the incubation mixture.

Bligh and Dyer method: The method is the same as described above, but, instead of the KCl-H_3PO_4 solution, water was used to remove the nonlipid material as described in the original method (6).

Folch-Pi extraction method (7): Nineteen volumes (22.8 ml) of chloroform-methanol (2:1) were added to the incubation mixture and the precipitated protein was removed by filtration. Water (0.2 volumne, 4.6 ml) was added to the extract, and, after mixing and centrifugation, the upper aqueous layer was removed. The amount of lipid present in the lower layer was determined.

Butanol extraction: The method was the same as described by Daae and Bremer (11). To 1.2 ml incubation mixture, 2.0 ml butanol and 2.0 ml water were added. After thorough mixing, the phases were separated by centrifugation at low speed (500 g for 10 min) and lipid present in the upper butanol layer was determined.

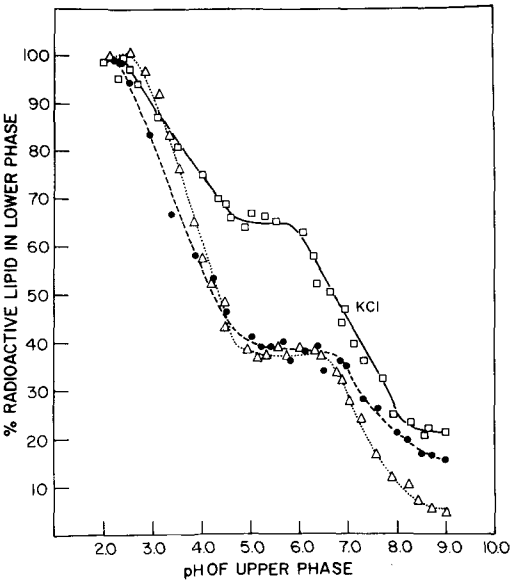

FIG. 1. Effect of pH upon the partition of palmitoyl dihydroxyacetone phosphate (DHAP) and hexadecyl DHAP in a chloroform-methanol-water system. The Tris salt of either palmitoyl [^{32}P] DHAP (4.1 nmoles, 9.0-6.0 x 10^4 cpm) [Δ···Δ] or [1-^{14}C] hexadecyl DHAP (2.3 nmoles, 4.3 x 10^4 cpm) [●—●] were dispersed in 1.2 ml 0.1 M buffers of different pH (phosphoric acid-NaH_2PO_4 buffer between pH 2.0-4.0, acetate buffer between pH 3.5-5.5 and NaH_2PO_4-Na_2HPO_4 buffer between pH 5-8 and Tris-HCl buffer between pH 8.0-9.0). To the solution, 4.5 ml chloroform-methanol [1:2] was added, mixed, and then 1.5 ml water and 1.5 ml additional chloroform were added. After thorough mixing the phases were separated by centrifugation (500 x g, 10 min), and the pH of the upper layer was determined by a pH meter as reported above. An aliquot of the lower layer was used to determine the fraction of radioactive lipid present. The curve marked KCl [□—□] showed the partition of palmitoyl [^{32}P] DHAP in the same system, when 1.5 ml, 2 M KCl, instead of water, was used to separate the phases.

RESULTS

A simulated incubation mixture containing heat-denatured mitochondria, radioactive acyl or alkyl DHAP and different cofactors was used to compare the different extraction methods (Table I). The modified Bligh and Dyer procedure, using KCl-H_3PO_4 instead of water, is seen to be most efficient in extracting the radioactive acyl and alkyl DHAP from the incubation mixture. The usual lipid extraction methods, as described by Bligh and Dyer (6) or by Folch-Pi, et al., (7) (Folch-Pi extraction method), extract little of the acyl and alkyl DHAP into the chloroform-rich phase (only 10-30%). The butanol extraction of these polar lipids is fairly efficient but not nearly as complete as the acidic Bligh and Dyer method (Table I).

It was found that chloroform-methanol can extract quantitatively acyl or alkyl DHAP from aqueous systems. For example, after adding chloroform-methanol (either by Bligh and Dyer [6] or by the method of Folch-Pi et al. [7]), the precipitated protein was removed from the single phase by filtration; all the radioactive acyl or alkyl DHAP was found to be present in the filtrate. Only during the washing of the chloroform-methanol extract with water at neutral pH were most of these polar lipids partitioned into the aqueous phase. When 2 M aqueous potassium chloride (KCl) was used instead of water for the phase separation, a larger fraction of the labeled acyl DHAP was found to be present in the lower chloroform phase (32%

TABLE II

Comparison of Methods for the Extraction of *Biosynthetic Palmitoyl Dihydroxyacetone* [^{32}P] Phosphate from Incubation Mixtures[a]

Extraction method	[^{32}P] Labeled lipid formed cpm x 10^{-5}
Acidic Bligh and Dyer	2.48
Bligh and Dyer	0.42
Folch-Pi extraction	0.28
Butanol extraction	1.85

[a]The incubation mixtures contained Tris-hydrochloric acid (HCl) buffer (75 mM, pH 7.4) NaF (8 mM); adenosine triphosphate (ATP) (8 mM); coenzyme A (CoASH) (80 μM) $MgCl_2$ (4 mM); glutathione (4 mM); bovine serum albumin (1.0 mg); potassium palmitate (0.4 mM); guinea pig liver mitochondria (0.45 mg protein); and [^{32}P] DHAP (0.25 mM, 6 x 10^6 cpm) in a total volume of 1.2 ml. The lipids were extracted by different methods (see text) and then dried under nitrogen. The dry lipid extracts were washed by the same way under acidic conditions to remove the water-soluble radioactive compounds (9). Radioactivitiy of an aliquot of the washed lipid extract was determined. Another aliquot was used for thin layer chromatography, and acyl DHAP was found to be the only radioactive lipid present.

instead of 9% by the Folch-Pi method and 35% instead of 19% by Bligh and Dyer Method).

The effect of pH upon the partition of acyl and alkyl DHAP into a chloroform-methanol-water system (Bligh and Dyer extraction) is shown in Figure 1. It is seen that the pH of the upper methanol-water phase determines the extractability of acyl and alkyl DHAP into the lower organic phase. Below pH 3, most of these lipids are present in the chloroform phase, but at neutral pH, 70-80% of the lipids are not recovered in the lower layer. KCl was seen to increase the efficiency of extraction at high pH by salting out the lipid from the aqueous to the organic phase but had no effect below pH 3.

The extraction methods also were compared by checking the efficiency of extraction of biosynthetically formed acyl [^{32}P] DHAP. Table II shows the comparison of the different methods of extraction of labeled acyl DHAP formed in guinea pig liver mitochondria. The lipid was extracted by different methods and then further washed under acidic conditions to remove the contaminating [^{32}P] DHAP (8). As found with the simulated incubation mixture, the modified Bligh and Dyer method (KCl-H_3PO_4) extracts the labeled lipid most efficiently. The usual extraction methods, useful for other lipids, are found to extract only a small fraction of acyl DHAP. Similar results also have been found for the extraction of alkyl DHAP from incubation mixtures.

DISCUSSION

Acyl DHAP was first discovered in guinea pig liver mitochondria as a rapidly labeled lipid formed from γ-^{32}P ATP by using the acidic Bligh and Dyer extraction (12). We have used this modified Bligh and Dyer extraction method to study the formation of acyl and alkyl DHAP (1,2). The results presented here show that these lipids are extracted very efficiently by this extraction method. Apparently, in a two phase system, the solubility of acyl and alkyl DHAP depends upon the pH of the medium. The data in Figure 1 show that the partition of these lipids into the chloroform phase depends upon the dissociation of the phosphate group. The pK_1 of these lipid phosphates is ca. 2.0 and pK_2 is 7.2. The observed titration curve (A.K. Hajra, unpublished results) of acyl DHAP closely follows the partition curve in Figure 1. These results show that, when one or both the acidic groups are dissociated, acyl and alkyl DHAP partition largely into the water-methanol phase. This phenomenon is not unusual because similar results have been known for a long time, as in the isolation of fatty acid from a saponification reaction. The sodium or potassium salts of fatty acids partition into water phase, whereas the free acids partition into the organic phase. Folch-Pi and LeBaron (13) showed the advantage of acid extraction for the isolation of polyphosphoinositides, and Long, et al., (14) reported that lysophosphatidic acid, like acyl DHAP, can be extracted into chloroform layer only at low pH.

Daae and Bremer (11) and Monroy, et al., (15) showed that lysophosphatidate can be extracted efficiently from incubation mixture at neutral pH by n-butanol. While butanol was fairly effective in extracting acyl and alkyl DHAP at neutral pH (Table I and II), it also extracted a large amount of nonlipid material which must be removed by washing or by chromatographic methods. Though butanol extraction is not as efficient as the acidic Bligh and Dyer method, this procedure may be preferable for the extraction of acid-labile lipids. Acyl and alkyl DHAP are found to be stable at room temperature under the acidic extraction conditions described here (acidic Bligh and Dyer Method) and also were found to be stable when the acidic lipid extracts were stored overnight at 2 C. However, some lipids may undergo chemical transformation when stored at low pH. We observed the formation of a lipid which has properties similar to 1-0-alkyl, glycerol-2,3 cyclic phosphate when 1-0-alkyl, glycerol-3-phosphate was stored overnight at 2 C in acidic (pH $\simeq$ 2.5) $CHCl_3$-methanol. It also should be pointed out here that the lipids, which are cationic at low pH, e.g. lecithin, may

not be extracted completely into the $CHCl_3$-rich layer with the acidic Bligh and Dyer extraction, because a part of these lipids may partition into the aqueous layer for the reason discussed above.

These results give a probable explanation of the inability of some workers to show the in vitro formation of acyl and alkyl DHAP in various systems (3-5). As shown here, the usual lipid extraction methods are not suitable for extraction of these polar lipids into chloroform-rich phase at neutral pH. The dephosphorylated products, acyl or alkyl dihydroxyacetone are nonionic and can be extracted with solvents at any pH. Data reported by other workers, who apparently used a neutral Bligh-Dyer extraction, on the biosynthesis of acyl and alkyl DHAP (16-18) would, therefore, appear from our finding to have obtained low values for these phosphorylated compounds.

These results also raise the possibility that other acidic lipids may not be extractable by chloroform-methanol. It has been shown that phosphatidyl inositol cannot be extracted completely by the Bligh and Dyer method (19). We found that phosphatidiic acid containing two long chain acyl groups was extractable by this method at neutral pH but lysophosphatidic was like acyl DHAP, i.e. partitions in chlorform phase only at low pH. Recently Daae also reported that lysophosphatidate is not extracted by the regular Bligh and Dyer extraction procedure (20). We successfully have employed the difference in partition of acyl or alkyl DHAP or lysophosphatidate in chloroform phase with pH to assay or purify these lipids from incubation mixtures.

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9,12,15-Octadecatrien-6-ynoic Acid, New Acetylenic Acid from Mosses

BENGT ÅNDERSSON[1], **WAYNE H. ANDERSON, JACQUES R. CHIPAULT,** and **ELDON C. ELLISON,** University of Minnesota, The Hormel Institute, Austin, Minnesota 55912; **STUART W. FENTON,** Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455; and **JOANNE L. GELLERMAN, JOHN M. HAWKINS,** and **HERMANN SCHLENK,** University of Minnesota, The Hormel Institute, Austin, Minnesota 55912

ABSTRACT

Lipids of the moss, *Ceratodon purpureus*, yield up to 25% of an acetylenic acid which was identified as all *cis*-9,12,15-octadecatrien-6-ynoic acid. The methyl ester of this acid was isolated in 95% purity by gas liquid chromatography. Mass spectroscopy provided the mol wt and confirmed methyl stearate as product of hydrogenation. Ozonization indicated a triple bond in position 6 and a double bond in position 15. UV and IR spectra showed *cis*-double bonds, no conjugation, and no *trans*-double bonds. The Raman spectrum provided direct evidence for the triple bond and confirmed the presence of double bonds and the absence of conjugation. The ratio of intensities indicated 1 triple bond/3 double bonds. 9,12,15-Octadecatrien-6-ynoic acid has not previously been isolated from biological materials. It was found only in the triglycerides of *Ceratodon purpureus* and several other mosses. In contrast to arachidonic and eicosapentaenoic acids, the acetylenic acid seems to be restricted to few moss genera.

INTRODUCTION

Fatty acids with triple bonds occur in some higher plants, foremost in Santalaceae and Olacaceae and in some fungi (1-5) but, so far, have not been reported from bryophytes. In studies carried out in this laboratory on lipids of lower plants in relation to habitat and climate, an acetylenic fatty acid was isolated from the lipids of a few moss genera. The identification of this acid as all *cis*-9,12,15-octadecatrien-6-ynoic acid from *Ceratodon purpureus* is reported here. Polyen-ynoic acids with methylene interrupted pattern of unsaturation previously have not been found in biological sources. The acetylenic acid occurs exclusively in triglycerides. The latter can be fractionated by adsorption chromatography into 2 portions, one of which contains ca. 90% acetylenic acyl groups, whereas the other portion does not contain the acetylenic structure.

MATERIALS AND METHODS

Ceratodon purpureus moss was collected in Alaska and in Minnesota. The samples were picked clean from other plants and debris a few days later. Sporophytes and rhizoids were removed and the gametophores washed with water before extracting them twice with $CHCl_3$:CH_3OH, 2:1, v/v, and twice with the same solvents, mixed 1:1 together with 10% water. Filtrates from the latter extractions were adjusted to the solvent ratio 2:1 and the combined extracts shaken with 0.2 volume of 0.9% NaCl in water. Crude lipids were recovered from the chloroform phase, and their composition was evaluated by thin layer chromatography (TLC) on Silica Gel H (E. Merck, A.G., Darmstadt, Germany). Neutral lipid components were separated with hexane:diethyl ether:acetic acid, 80:20:1, as solvent and detected by charring with $K_2Cr_2O_7$-H_2SO_4; glycolipids with acetone:acetic acid:water, 100:2:1, detected by α-naphthol-H_2SO_4 (6); and phospholipids with $CHCl_3$:CH_3OH:14 N aqueous NH_4OH, 65:30:4, detected by MoO_3-H_2SO_4 (7). Lipid classes were isolated by column chromatography on silicic acid with a sequence of solvents and solvent mixtures, including petroleum ether, diethyl ether, chloroform, acetone, and methanol. The procedure is exemplified in Table I for the fractionation of 35 mg lipids from *C. purpureus* on 5.5 g Unisil (Clarkson Chemical Co., Williamsport, Pa.).

Fatty acids or esters were obtained from total lipids or individual lipid classes either by reflux with 2 N KOH in 15% aqueous methanol for 2 hr or by interesterification of the sample in 5% hydrochloric acid/methanol in a sealed tube for 40 min at 65 C with stirring. It was found that alkaline saponification of the mixed lipids, as well as the pure methyl ester of the polyen-ynoic acid, causes some conjugation. Therefore, acidic interesterification is the preferred procedure. Acids or esters were recovered, and the former was esterified with

[1]Hormel Fellow, 1972-73.

TABLE I

Column Chromatography of Moss Lipids

Fraction	Solvent, v/v	Ml	Lipids recovered	
			Mg	Class
1	Ether:hexane 1:99	62	3.1	steryl + wax esters, carotenes
2	Ether:hexane 4:96	40	0.1	
3	Ether:hexane 4:96	110	5.1	triglycerides
4	Chloroform	76	15.7	polyen-ynoyl triglycerides + sterols (trace)
5	Chloroform	12	2.3	chlorophyll pigments
6	Chloroform:acetone 1:1	35	3.2	monogalactosyl diglycerides + pigments
7	Acetone	20	1.0	digalactosyl diglycerides
8	Acetone	128	0.1	sulfolipids + trigalactosyl diglycerides
9	Acetone:methanol 7:3	26	1.9	phosphatidyl glycerols + inositols
10	Methanol	6	0.3	phosphatidyl ethanolamines + serines
11	Methanol	65	2.0	phosphatidyl cholines

CH_2N_2. When necessary, the methyl esters were purified from pigments and other contaminants by preparative TLC in hexane:diethyl ether:acetic acid, 80:20:1, on Silica Gel H.

Analytical gas liquid chromatography (GLC) of the methyl esters was carried out on ethylene glycol succinate (Applied Science Laboratories, State College, Pa.) and on cycloheptaamylose propionate (8). Identifications of the common acids were based upon equivalent chain length (ECL) (9) on the two phases in reference to authentic samples, and quantifications were checked by GLC of hydrogenated aliquots.

Preparative GLC was carried out on cycloheptaamylose valerate, a low-polarity phase (8), 15% on silicone treated Chromosorb W, 60-80 mesh (Johns Manville, Celite Division, New York, N.Y.) in a column 184 cm long and 0.8 cm wide at 232 C. The polyen-ynoate emerged pure at ECL 19.0. Artefacts, due to conjugation by alkaline treatment, have longer retention times on this and other GLC phases and are well separated from the genuine acetylenic acid methyl ester.

Ozonization of the acetylenic acid methyl ester was carried out with samples of 0.5 mg in 2.5 ml purified pentane at -60 C with a stream of 2-3% ozone in oxygen (10). Ca. 6 sec of exposure was sufficient for complete ozonization of the double bonds and excess O_3 was removed immediately by a stream of N_2. Ozonization of triple bonds proceeded more slowly than of double bonds (11). However, both reactions progressed sufficiently under the conditions specified here to provide for reliable identification of acidic compounds arising from triple bond and of aldehydic compounds from double bond unsaturation. After treatment of the ozonides with triphenylphosphine (12), the pentane solution was concentrated, and aliquots were analyzed by GLC before and after esterification with CH_2N_2. GLC on ethylene glycol succinate at 140 C was used to check for mono- and dimethyl esters of dibasic acids or aldehyde esters and on polyethylene glycol (Carbowax 4000, Carbide and Carbon Chemicals Co., New York, N.Y.) at 160 C for aldehydes. The authentic compounds were on hand for identification or proof of absence.

Mass spectra were recorded with an LKB 9000 mass spectrometer (LKB Produkter AB, Solna, Sweden), introducing the samples via a direct inlet probe. Ionization potentials were 70 eV and 20 eV with an ion source temperature at 290 C and an acceleration voltage of 3.5 kV.

IR spectra were obtained with a Perkin Elmer IR spectrophotometer model 21 (Perkin Elmer Corp., Norwalk, Conn.) equipped with a NaCl prism. Samples were analyzed as liquid films between NaCl plates or dissolved in CS_2 (4000-2400 cm^{-1}, 2000-1650 cm^{-1}, and 1400-650 cm^{-1}) or in tetrachloroethylene (2400-2000 cm^{-1} and 1650-1400 cm^{-1}).

UV spectra were measured in a Beckman DU or a DK-2 spectrophotometer (Beckman Instruments Co., Fullerton, Calif.), using purified hexane as solvent.

Raman spectra were obtained with a Laser Raman Spectrophotometer (Japan Electron Optics Lab., Tokyo, JRS-SL), using the 4880 Å exciting line from an Argon ion laser. Samples were placed neat in a sealed capillary tube.

RESULTS

The presence of a very highly unsaturated

TABLE II

Fatty Acids[a] in Lipids of *Ceratodon purpureus*

Acid	June 1972 Minnesota[b]	August 1972 Alaska[c]	Equivalent chain length High polarity phase[d]	Equivalent chain length Medium polarity phase[e]
16:0	9.7	10.9		
16:1	1.2	1.2	16.6	16.2
18:0	1.3	1.7		
18:1	5.2	3.2	18.6	18.2
9,12-18:2	16.1	10.4	19.5	18.6
6,9,12-18:3	1.5	1.2		
9,12,15-18:3	20.7	17.1	20.6	19.0
6,9,12,15-18:4	1.2	1.5		
5,8,11,14-20:4	9.3	8.1	22.6	20.8
5,8,11,14,17-20:5	6.8	6.3	23.7	21.3
22:0	1.7	1.3		
24:0	2.5	1.4		
9,12,15-18:3-6-ynoic	19.2	25.2	23.5[f]	20.5[f]

[a]Gas liquid chromatography area percent of methyl esters.

[b]Isanti County, bright sun on dry soil.

[c]Kantishna, deeply shaded rock ledge.

[d]Ethylene glycol succinate.

[e]Cycloheptaamylose propionate.

[f]Equivalent chain length 18.0 after hydrogenation.

C_{18} fatty acid was indicated by GLC of the methyl esters obtained from 5 out of more than 50 moss species. In samples of *C. purpureus*, this acid represented ca. 25% of the total fatty acids in lipids. ECL of the unidentified ester could not be brought in correlation with any of the common methyl esters (Table II), but quantification of chain lengths by GLC after hydrogenation suggested a highly unsaturated n-C_{18} structure for the acid. Increments of ECL for triple bonds (13) suggested a combination of such with several double bonds. From 2.6 g fresh moss tissue, 60 mg lipid, 20 mg methyl esters, and eventually 3.3 mg of the unknown ester was obtained by GLC in >95% purity. The fatty acid composition of the moss lipids and pertinent data on ECL are listed in Table II.

9,12,15-Octadecatrien-6-ynoate

Chemical degradation of the methyl ester by ozonization and subsequent hydrogenation yielded, according to GLC, propanal and monomethyl adipate. Methylation converted the latter to the expected dimethyl adipate. Carboxyl groups result from ozonization of triple bonds, whereas aldehyde groups are formed from double bonds in the procedure applied here. Since monomethyl adipate, but no aldehyde methyl ester, was found, it can be concluded that the triple bond is in position 6. Propanal indicates a double bond in position 15.

Mass spectrometry gave a peak m/e 288 which was identified as the molecular ion. Mass spectrometry also verified methyl stearate as the product after hydrogenation.

The IR spectrum indicated three isolated *cis*-double bonds by absorptions ν_{CH} 3020 cm^{-1} and $\nu_{C=C}$ 1650 cm^{-1}, as well as absence of CH deformation at 965 cm^{-1}. A triple bond was suggested by a small peak at 1335 cm^{-1} (14,15), but the absence of absorption at 2150 cm^{-1} indicated that acetylenic unsaturation would be neither terminal nor near the carboxyl group.

Three absorption bands at 1170, 1200, and 1245 cm^{-1} are characteristic of many common fatty acid methyl esters, but the ester in question showed a spectrum with a peak at 1150 cm^{-1}, broad absorption at 1200 cm^{-1}, and a band at 1260 cm^{-1} with a small inflection near 1235 cm^{-1}. The last two absorptions are shown also by methyl 6-octadecynoate, *cis*- and *trans*-6-octadecenoates, 6,9,12-heptadecatrienoate, and 6,9,12-octadecatrienoate which all have, in position 6, the unsaturation nearest to the ester group. Moreover, the intensities of the unknown between 1100-1350 cm^{-1} are nearly identical to those of methyl 6-octadecynoate. In contrast, several compounds with unsaturation at the 4 or 5 position show a different absorption profile in this region.

The UV spectrum did not show conjugation of unsaturated bonds in excess of 1-2% and thereby confirmed the results from IR spectroscopy in this respect.

The Raman spectrum of the ester showed absorption at 2250 cm^{-1} and at 1660 cm^{-1} which are characteristic for acetylenic and ethylenic bonds, respectively (16). The ratio of intensities was 0.55, while a ratio of 0.57 for triple bond/double bond was measured from a mixture of methyl 6-octadecynoate + 9,12,15-octadecatrienoate, 1:1. No appreciable conjugation was indicated.

According to the combined analytical evidence, the polyen-ynoic compound is the methyl ester of all *cis*-9,12,15-octadecatrien-6-ynoic acid.

Occurrence in Triglycerides

The major lipid (Table I, fraction 4) from *C. purpureus* migrates on SiO_2 with an R_f similar to that of acids, sterols, or diglycerides. The acetylenic acid was found only in this fraction. It was purified from minor contaminants by preparative TLC. Presence of a glycerol moiety was demonstrated by interesterification of the lipid fraction with 5% HCl/CH_3OH and acetylation of the water-soluble alcohol (17). The GLC retention time of the product was identical to that of triacetin. IR spectroscopy did not reveal a free hydroxyl group which would be detected from a diglyceride. Interpretation of the lipid as triglyceride is confirmed fully by the data from mass spectrometry.

The intensity of the major molecular ion peak m/e 860 was 6% of the base peak which is high compared with that from other triglycerides, e.g. 0.2% from glyceryl trioctadecanoate (18). The major fragment m/e 587 indicates loss of RCO_2 from the molecular ion where R is $C_{17}H_{25}$ (18-21). No major fragment derived by loss of other fatty acids could be detected. This confirms the prominence of a mono-acid triglyceride with $C_{17}H_{25}$ as alkyl of the acyl chains. Other fragments typical for triglycerides were m/e 257, $[RCO]^+$; m/e 331, $[RCO + 74]^+$; and m/e 385, $[RCO + 128]^+$.

A series of peaks m/e 831, 817, 805, 791, 777, 765, 751, 737, 725, and 711 was apparent which indicates the loss of a sequence of hydrocarbon fragments from the molecular ion. The glycerol moiety seems to have a charge retaining and stabilizing effect upon the ions containing unsaturated chains similar to that reported for pyrrolidides of unsaturated fatty acids (22,23). The series of m/e listed above fits that which is from a double bond in position 15 and makes likely positions 12 and 9 for additional double bonds. Such reasoning was supported by the similarity in the high mass region of the spectrum of authentic trilinolenin where the double bonds are in these positions of the chain.

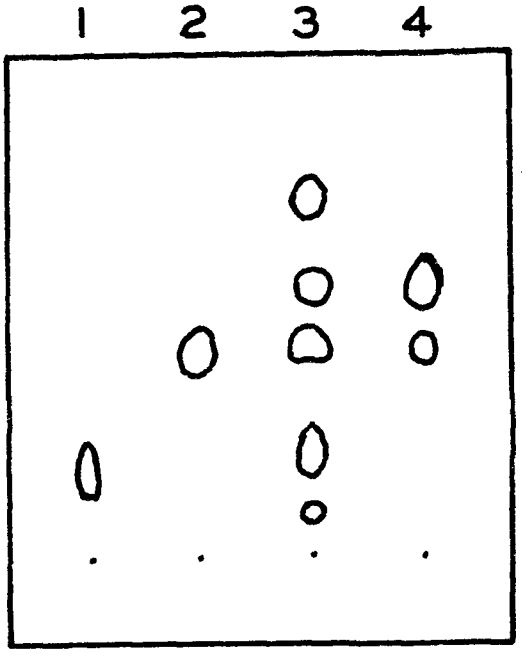

FIG. 1. Thin layer chromatography of moss lipids in hexane:diethyl ether:acetic acid, 80:20:1. (1) Acetylenic triglycerides from *C. purpureus*. (2) Methyl 9,12,15-octadecatrien-6-ynoate. (3) Standard:cholesteryl oleate, followed by methyl oleate, triolein, oleic acid, cholesterol. (4) Common methyl esters, followed by acetylenic methyl ester, from *Dicranaceae*.

The low R_f value of the acetylenic triglyceride (Fig. 1) could be caused by a substituent which is eliminated in the course of isolating the acetylenic methyl ester for structure identification. The IR, as well as the Raman, spectra of the methyl ester isolated by TLC and isolated by GLC are identical. The IR spectrum of the triglyceride is similar and does not show a hydroxyl group as substituent. Besides, it seems unlikely that such reaction would lead to a nonconjugated *cis*-double bond. The most likely explanation for the slow migration of the triglyceride is a cumulative effect of the triene-yne unsaturation in the majority of acyls. Comparison of R_f values listed in Table III supports such conclusion. Picramnia oil contains octadec-6-ynoic (tariric) acid as major acyl in the triglycerides (24), and this causes a difference of R_f values much greater between the triglycerides, picramnia oil and triolein, than between the methyl esters, tarirate, and oleate. Similarly, a difference is found in R_f of normal acyl triglycerides and of phytanoyl triglycerides, increasing with the number of phytanoyl substituents (25).

From the data presently available, we can not see any reason to assume for fraction 4 a structure other than that of triglycerides con-

TABLE III

R_f Values of Triglycerides and Methyl Esters

	Triglycerides	Methyl ester
Oleyl	0.42	0.55
Tariroyl	0.29	0.48
Ceratodon purpureus, fraction 3	0.34	0.53
Ceratodon purpureus, fraction 4	0.15	0.42
Tri-n-acyl[a]	0.24	
Di-n-acyl phytanoyl[a]	0.30	
n-Acyl di-phytanoyl[a]	0.36	
Tri-phytanoyl[a]	0.42	

[a]Measured from Figure 8-3, ref. 25.

taining 9,12,15-octadecatrien-6-ynoic acid as major component.

Occurrence of the Acetylenic Acid in Other Mosses

According to the GLC criteria discussed above, 9,12,15-octadecatrien-6-ynoic acid was detected in several other mosses besides *C. purpureus*. It represented in *Fontinalis pyretica* (England) about 13%; in *Aulacomnium turgidum* (Alaska), 3%; in *Bryum tortifolium* (Alaska), 8%; and in *Dicranum montanum* (Minnesota), 10% of all fatty acids. Segregation of triglycerides by TLC into acetylenic and non-acetylenic portions was observed also with lipids from these mosses. Besides distinction by migration, it is characteristic for the acetylenic triglycerides that they char with $K_2Cr_2O_7$-H_2SO_4 more rapidly and darker than any other lipid fraction.

DISCUSSION

The finding of arachidonic and related acids in mosses and some other lower plant families (26,27) had instigated our more detailed study on the lipid composition of mosses (28,29). In recent work, lipids of more than 50 species were analyzed, and the olefinic-acetylenic fatty acid identified here was detected in 5 of them. Acetylenic fatty acids had been reported so far only from a few families of higher plants. The occurrence in bryophytes seems to be similarly selective and may have taxonomic significance (3).

The particular structure, all *cis*-9,12,15-octadecatrien-6-ynoic acid, has not been encountered among acetylenic acids of higher plants, where *cis*-9-octadecen-12-ynoic acid is the only example of an olefinic-acetylenic acid with methylene interrupted unsaturation (2). None of the GLC analyses indicated acetylenic C_{20} acids although, in contrast to lipids from higher plants, up to 30% polyenoic C_{20} and C_{22} acids were present in the lipids of some mosses. In *C. purpureus*, the acetylenic acid occurs with ca. 15% of such polyenoic acids (Table II). It is bound exclusively in triglycerides of the mosses and such may be common for acetylenic acids in plants (3). In reference to other highly unsaturated fatty acids, this selectivity is unusual. For example, arachidonic acid occurs in all polar and nonpolar lipid classes of mosses, although the percentages may vary greatly.

The total lipids of *C. purpureus* contain ca. 50% triglycerides which is more than normally found in leafy tissue of other plants. According to preparative separations, ca. one-fourth of these triglycerides do not contain the acetylenic acid and their amount and composition conform much more closely to those of leaf lipids. The acetylenic acid is accumulated in the greater portion of triglycerides, and it represents there by far the majority of all fatty acids. Different cellular sites and, therewith, functions may account for this division into two types of triglycerides.

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In Vitro Incorporation of 1-^{14}C Nonanal-9-oic Acid into Plasma and Human Red Blood Cells Lipids

N. DOUSSET and **L. DOUSTE-BLAZY**, Institut National de la Sante et de la Recherche Medicale, C.H.R. Purpan, Toulouse Cedex, France

ABSTRACT

Nonanal-9-oic acid is incorporated principally into plasma phospholipids, whereas oleic acid is incorporated into red cells. This incorporation does not require the presence of adenosine 5′-triphosphate and Coenzyme A and is carried out in the absence of red cells. The incorporation of nonanal-9-oic acid in blood lipids takes place in the first 10 min of incubation.

INTRODUCTION

The turnover of fatty acids in blood phospholipids involves principally monocarboxylic acids. Studies done by Oliveira and Vaughan (1,2), Mulder and Van Deenen (3), and Mulder, et al. (4) have shown that the incorporation of 1-^{14}C labeled fatty acids in red blood cells occurs largely in phosphatidylcholines and in phosphatidylethanolamines.

The metabolism of aldehydes is less well known. According to Bell and White (5), these substances might be the direct precursors of plasmalogens. Because the peroxidation of oleic and linoleic acids, occurring as a consequence of the irradiation of plasma lipoproteins, produces an aldehydo-acid compound with 9-carbon atoms (6), we have studied the incorporation in vitro of this derivative in human blood in comparison to the incorporation of oleic acid.

MATERIALS AND METHODS

The 1-^{14}C oleic acid (specific activity 44 mCi/mmole) was obtained in hexane solution from the Atomic Energy Center, Saclay, France. The 1-^{14}C nonanal-9-oic acid was prepared through performic oxidation of 1-^{14}C labeled oleic acid (diluted 1/100 with nonlabeled oleic acid) (7), followed by periodic oxidation of the dihydroxy-acid produced (8). The 1-^{14}C labeled nonanal-9-oic acid thus derived then was purified by repeated thin layer chromatography (TLC) in a basic solvent of chloroform-methanol-7N aqueous ammonia (76:30:5 v/v/v), scraped off, and eluted in 100 ml ether. Its radioactivity was determined through the use of a Packard-Tricarb spectrometer using 10 ml of a scintillating solution (1 volume water for 5 volumes of dioxane containing 10% naphthalene, 0.03% dimethyl 1.4 bis [2(4 methyl-5-phenyl-oxazolyl)] benzene and 0.7% 2,5-diphenyloxazole). The identity of the nonanal-9-oic acid was confirmed by its transformation into azelaic acid by permanganic oxidation.

INCUBATION CONDITIONS

The majority of the experiments were conducted on human blood samples (whole blood, red blood cells, or plasma taken on Wintrobe's mixture from healthy subjects). Whole blood (1 ml) (or 0.5 ml red blood cells freed of leucocytes and washed 3 times with 0.9% NaCl or 0.5 ml plasma combined with 0.5 ml 0.9% NaCl) was incubated in the presence of ^{14}C labeled fatty acids (80,000 dpm) (specific activities: 1-^{14}C oleic acid 44 μCi/μmole and 1-^{14}C nonanal-9-oic acid 44 μCi/100 μmoles) and with 10 μmoles adenosine 5′-triphosphate (ATP); 0.5 μmole Coenzyme A (CoA); 250 μmoles phosphate buffer, pH 7.0; and 6 μmoles $MgCl_2$.

Parallel experiments were done with rat blood labeled with ^{32}P. A rat, having received an intraperitoneal injection of 4 mCi $Na_2H^{32}PO_4$, was sacrificed 15 hr later. Its blood was collected over heparin by cardiac puncture and incubated in the presence of 1-^{14}C nonanal-9-oic acid (specific activity 51 μCi/mmole). In some experiments, ATP, CoA, or both cofactors were omitted. The final volume was 3 ml and the concentration was 771 μosmoles/ml. After 1 hr of agitation at 37 C, the blood was centrifuged, the plasma removed, and the red blood cells were washed 3 times in 0.9% NaCl.

EXTRACTION AND LIPID CHROMATOGRAPHY

Plasma lipids were extracted according to Folch, et al., (9); red cell lipids were extracted by the method of Rose and Oklander (10). The lipid extracts were dissolved in 2 ml chloroform-methanol (1:1 v/v), and a known quantity was counted by liquid scintillation to determine the proportions of nonanal-9-oic acid and oleic acid in aqueous and chloroform layers.

The blood phospholipids were separated by TLC on Kieselgel G (500 μ) using the solvent system, chloroform-methanol-ammoniac 7N, (76:30:5 v/v/v). The labeled components were

localized either by TLC chromatogram scanner (Philips, model PW 4007-0) or by autogradiography done by contact with kodirex film (exposure time of 1-2 weeks). The radioactivity of each labeled lipid fraction was measured by liquid scintillation count.

An initial migration of plasma and red blood cell lipids from ^{32}P labeled rat blood in acid solvent ($CHCl_3$-CH_3OH-H_2O-CH_3COOH, 60:30:6:1 v/v/v/v) permitted the isolation of the phospholipid containing the nonanal-9-oic acid. After autoradiography, the phospholipids containing the nonanal-9-oic acid were extracted and eluted by chloroform-methanol (1:1 v/v) and by ether. A second chromatography in $CHCl_3$-CH_3OH-NH_4OH 7N, 76:30:5, v/v/v, allowed us to separate the phospholipids containing the nonanal-9-oic acid from each other. The spots marked by the autoradiography were scraped off and counted by liquid scintillation. The specific activities of the molecules containing nonanal-9-oic acid molecules and the phosphate were 11.3 x 10^7 dpm/mmole and 0.60 x 10^7 dpm/mmole, respectively. Some of the samples were submitted to chemical hydrolysis in acid solution (11).

RESULTS AND DISCUSSION

The average results of 14 experiments done with whole blood are for phospholipids 36,320 ± 5203 dpm in plasma and 68 ± 39 in red cells, while for neutral lipids 1680 ± 602 dpm in plasma and 42 ± 31 in red cells. The aldehydo acid localized itself almost exclusively in the plasma. The main component is cholinic and represents 84.5% of the plasma lipid radioactivity. The method of double labeling with ^{32}P and ^{14}C allows us to confirm the phospholipid nature of this compound. The ratio between radioactivities $^{14}C/^{32}P$ suggests a phospholipid containing 1 mole nonanal-9-oic acid for 1 mole phosphoric acid. The acid hydrolysis of this phospholipid with $HgCl_2$ causes the release of nonanal-9-oic acid (80-85% total dpm) and azelaic acid (15-20% total dpm), showing that, at the end of the reaction, most of the aldehydo acid remains in its original form.

The rate of the reaction is constant during the first 10 min and then decreases. After 10 min, 50 x 10^3 dpm represents 61% ^{14}C lipid incorporated into blood phospholipids.

The incorporation of the nonanal-9-oic acid has been compared with that of oleic acid in whole blood, plasma, and red blood cells. Most of oleic acid remains in the free form in the plasma.

Experiments done on plasma alone have shown incorporation of nonanal-9-oic acid into plasma phospholipids, but it is less than that observed with whole blood. Oleic acid under the same conditions is not incorporated; from ^{14}C-nonanal-9-oic acid or ^{14}C-oleic acid in same radioactivity (80,000 dpm), in the presence of CoA, ATP, $MgCl_2$, pH 7.0, plasma phospholipids contain essentially ^{14}C nonanal-9-oic acid (14,286 dpm) and not much oleic acid (450 dpm). This result confirms the work of Mulder and Van Deenen (3) and Winterbourn and Batt (12). Incubations with red cells alone confirm the results obtained with whole blood: a clear incorporation of oleic acid (20,255 dpm) but very low fixation of nonanal-9-oic acid (607 dpm) in red cell lipids. The incorporation of nonanal-9-oic acid in lipids does not require the presence of CoA and ATP.

The comparative study of the incorporation of oleic acid and nonanal-9-oic acid shows a clear difference in their activity in vitro. Nonanal-9-oic acid is incorporated preferentially into plasma phospholipids, whereas oleic acid is bound in the phospholipids and in neutral lipids of red blood cells.

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Dibutyryl Cyclic Adenosine 3′,5-Monophosphate and Brain Lipid Metabolism

BENJAMIN WEISS and **RICHARD L. STILLER**, Division of Neuroscience, New York State Psychiatric Institute and Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, N.Y. 10032

ABSTRACT

It was found that dibutyryl cyclic adenosine 3′,5′-monophosphate exerted its greatest suppressive effect upon total lipid synthesis in 7-10 day old rat bran slices incubated in the presence of 2-^{14}C-acetate, U-^{14}C-D-glucose, and 3-^{14}C-DL-serine. Fractionation of the neutral and polar lipids disclosed that the formation of each of their respective components was reduced to varying extents, thus suggesting that cyclic adenosine 3′,5′-monophosphate, arising from the hydrolysis of dibutyryl cyclic adenosine 3′,5′-monophosphate, may control the synthesis of brain lipids by regulating the utilization of acetyl-coenzyme A.

TABLE I

Effect of Age upon Incorporation of 2-^{14}C-Acetate into Total Lipids of Brain Slices in the Presence of Dibutyryl Cyclic Adenosine 3′,5′-monophosphate[a]

	Total lipid		
Age	Control	Experimental	Reduction
Days	10^3 cpm	10^3 cpm	Percent
1[b]	25	23	8
3	50	44	12
5	148	118	20
6	230	120	48
7	229	108	53
8	205	91	56
9	240	97	60
10	210	93	56
14	170	103	40
15	180	120	34
21	68	63	7
25	63	63	0
Adult[b]	23	20	13

[a]In each experiment, slices were prepared from a single rat brain after separation into hemispheres. Slices (3-4) each weighing ca. 100 mg were obtained from each hemisphere which served as a control and experimental. The slices were distributed among 3-4 serum bottles (15 ml) equipped with rubber septums and containing 2 ml Krebs-Ringer bicarbonate solution; each bottle received ca. 100 mg tissue. Each group of bottles contained 1.25 μCi of 2-^{14}C-sodium acetate. Both dbcAMP and theophylline were present at final concentrations of 1 mM in each experimental bottle. The bottles were flushed for several min with 95% O_2-5% CO_2 incubated with gyrotory shaking at 100 rpm in a water bath at 37 C for 2 hr. The reaction was terminated by addition of 10 volumes of methanol after which the total lipids were removed. (See text for details.)

[b]One hemisphere from each of 2 brains and 100 mg from each hemisphere of a single brain were used from 1 day old and adult rats, respectively.

INTRODUCTION

Cyclic adenosine 3′,5′-monophosphate (cAMP) in brain has been the subject of considerable study. The function of biogenic amines, depolarizing agents, and other factors, such as histamine, norepinephrine, and theophylline on the control of cAMP levels in incubated brain slices has been examined extensively (1-5). The phosphorylation of brain microsomal or ribosomal proteins has been shown to be stimulated by a cAMP-mediated protein kinase in rat brain microsomes (6-8). Although the role of cAMP on the regulation of lipolytic activity in various tissues has been well documented (9,10), little is known regarding its effect upon brain lipid metabolism. The study herein reported was undertaken to examine the action of exogenous cAMP, N,^{6}O2′-dibutyryl cyclic adenosine 3′,5′-monophosphate (dbcAMP), and agents known to affect the levels of cAMP in tissues on the synthesis of lipids in rat brain slices.

MATERIALS AND METHODS

All chemicals were reagent grade; solvents were distilled before use. Sprague-Dawley rats of either sex were purchased from Marland Farms, Hewitt, N.J. cAMP and dbcAMP were obtained from Sigma Chemical Co., 2-^{14}C-Sodium acetate, 24.4μCi/mg, U-^{14}C-D-glucose, 26.5μCi/mg and 3-^{14}C-DL-serine, 119μCi/mg were purchased from New England Nuclear, Boston, Mass. Lipid standards were from Applied Science Laboratories, State College, Pa., and Supelco, Bellefonte, Pa.

Silicic acid (Mallinckrodt, St. Louis, Mo.), washed several times with chloroform:methanol (C:M) (2:1) by suspension and centrigugation was dried overnight at 110 C; 6.0 g portions in chloroform were packed into columns, 1.5 x 50 cm. Acid-, alkali-, and water-washed diethylaminoethyl-cellulose (DEAE), 10 g portions, was packed into columns after conversion to the acetate by treatment with glacial acetic acid overnight. The columns were washed succes-

sively with methanol until free from acid and 10 column volumes (CV) of chloroform (11).

After incubation of the brain slices (12), the reaction was terminated by addition of 10 volumes of methanol. The brain slices from the same hemisphere in aqueous methanol were combined and homogenized in a teflon glass homogenizer. After centrifugation of the homogenate, the precipitate was rehomogenized 3 times in the same manner with 20 ml portions of C:M (2:1). The combined aqueous methanol and C:M supernates were adjusted to a final ratio of C:M:water of 8:4:3 to form a partition (13). After removal of the upper layer, the lower phase was repartitioned against an equal volume of fresh upper layer. The residue obtained after concentration of the lower phase under reduced pressure was dried over phosphorus pentoxide, weighed, and dissolved in chloroform. An aliquot, dried under a stream of nitrogen, was dissolved in 10 ml Omnifluor cotaining 0.1 ml solubilizer (Nuclear Chicago, Chicago, Ill.) and counted in a Packard Tri-Carb liquid scintillation spectrometer. The remainder of the chloroform solution containing ca. 60 mg lipid, derived from 4-5 pooled hemispheres, was applied to a silicic acid column. The neutral and polar lipids were eluted from the column with 10 CV of chloroform and methanol, respectively. Upon removal of solvent, the residues were dried, weighed, and stored in chloroform at -25 C until ready for use after assay of an aliquot for radioactivity.

Neutral lipids, 5-10 mg, were applied to four 0.25 mm Silica Gel G coated thin layer plates and developed with petroleum ether:diethyl ether:glacial acetic acid (90:10:1). After drying the plate in a hood at room temperature for ca. 30 min until no odor of acetic acid was apparent, chromatography was repeated in the same dimension (14,15) with petroleum ether: diethyl ether:glacial acetic acid (70:20:4). Cholesterol, mono-, and dimyristin, and tripalmitin were used as neutral lipid standards. Bands were detected by spraying either with water or 0.01% Rhodamine 6G in acetone and viewed under UV light; they were removed by successive treatments with hot methanol and twice with hot C:M (2:1) with centrifugation. After concentration of the combined supernates, the residues were counted. Ca. 50 mg polar lipids were applied from C:M (9:1) to a DEAE-cellulose column. After successive passage of 20 CV, each of C:M (9:1) and C:M (7:3), the combined eluates were concentrated, and the dried residues were weighed and dissolved in C:M (2:1). An aliquot was assayed for radioactivity, and another was analyzed by thin layer chromatography (TLC) in the same manner as used for the neutral lipids, except that the solvent system was chloroform:methanol:conc. NH_4OH (65:25:4). The polar lipid standards were choline lysophosphatide, sphingomyelin, serine-, choline-, and ethanolamine phosphatides and cerebroside. Bands, detected by spraying with water, were isolated and counted in the same manner as described for the neutral lipids.

RESULTS

Optimum age, concentration of components, and choice of buffers were determined prior to investigating the pattern of lipid metabolism. The greatest difference in incorporation of ^{14}C-acetate into the total lipids of brain slices incubated in the presence and absence of both dbcAMP and theophylline occurred in rats 7-10 days old (Table I). The effective concentration of dbcAMP was determined by varying its levels in the incubation medium through a range of .001 mM-10 mM. Although 0.1 mM was adequate in many cases, for purposes of certainty, the working concentration was maintained at 1 mM. The results obtained with cAMP were not consistent and reproducible. In similar manner, the optimum concentration of theophylline was found to be 1 mM. In a comparison of buffers in the absence of dbcAMP and theophylline, the utilization of isotope with 0.25 M Na_2HPO_4, pH 7.4, was as effective as Krebs-Ringer bicarbonate, whereas only 30% of the activity was obtained with 0.154 M sodium citrate, pH 7.4. Variation of the pH by ca. 1 unit below or above pH 7.4, eg. 0.154 M hepes, pH 6.4, 0.154 M glycylglycine, pH 6.6 or 0.154 M Tris, pH 8.4 sharply reduced the utilization to 10-15%.

The effects of various ions and compounds upon the incorporation of 2-^{14}C-acetate into the total lipids of brain slices also were studied. The experimental conditions were the same as those described in the legend of Table I, except for the presence of dbcAMP and theophylline in both control and experimental flasks and for the addition or omission of the compounds under examination. CsCl, LiCl, RbCl, and NaF, each at 100 mM concentration, reduced from 23 to 68% the incorporation of isotope into the total lipids. In a typical experiment, in the presence of Li^+, the lipids obtained from control and experimental determinations contained 91 x 10^3 cpm and 30 x 10^3 cpm, respectively, which represented a reduction in isotope utilization of 67%. The effect shown by Li^+ was ca. 3 times greater than that of Cs^+ and Rb^+ which gave reductions of 23 and 24%, respectively. Fluoride (68% reduction) was comparable to Li^+, whereas CN^- at 10 mM concentration

TABLE II

Separation into Classes of ^{14}C-Labeled Total Lipids from Rat Brain Slices[a]

	Lipids											
	Total			Neutral				Polar				
Substrate	Specific activity 10^3 cpm	Wt	Reduction in wt (a) specific activity (b)	Specific activity 10^3 cpm	Reduction in specific activity (b)	Wt	Reduction in wt (a)	Specific activity 10^3 cpm	Reduction in specific activity (b)	Wt	Reduction in wt (a)
	mg	mg	percent	mg	percent	mg	percent	mg	percent	mg	percent
2-^{14}C-Acetate											
					56[b]		35[b]		44[b]		65[b]
Control	10.6	66.1	6.2[c]	18.0	58	24.1	25	7.9	42	43.3	75
Experimental	7.4	62.0	30.0[d]	17.6	2.3[d]	15.2	37.0	4.3	45.6[d]	45.7	+5.5
U-^{14}C-D-Glucose											
					37		34		63		65
Control	6.6	58.6	4.3[c]	6.4		19.7		5.4		38.6	
					28		28		72		72
Experimental	4.1	56.1	38.0[d]	4.2	34.4[d]	15.4	22.2	4.0	36.0[d]	41.1	+6.3
3-^{14}C-DL-Serine											
					9		32		91		68
Control	6.6	59.9	8.8[c]	1.6		19.5		8.8		39.2	
					9		29		91		71
Experimental	3.5	54.6	47.0[d]	1.1	31.3[d]	16.4	15.8	4.5	48.8[d]	41.0	+4.6

[a]All values are the average of 3 experiments with 8 day old rats. The concentration of all isotopic compounds, as well as the experimental conditions were the same as those given in Table I. The radioactively labeled total lipids were fractionated on silicic acid columns into neutral and polar lipids, as described in the text.

[b]Values in these columns represent the percentages of either neutral or polar lipid and the percentages of total radioactivity associated with these lipids.

[c]Values represent the percent difference in total lipid wt between control and experimental. Ca. 1200 mg wet wt, 3 hemispheres, were processed to yield the wt indicated.

[d]Values are the percent difference in total radioactivity (specific activity x wt) between control and experimental determinations.

TABLE III

Fractionation of ^{14}C-Labeled Neutral Lipids from Rat Brain Slices[a]

Substrate	Neutral applied cpm 10 mg	Lipids recovered Percent[b]	Mono- and diglycerides cpm	Mono- and diglycerides Percent[c]	Cholesterol cpm	Cholesterol Percent[c]	Triglycerides cpm	Triglycerides Percent[c]
2-^{14}-Acetate								
Control	192,600	83	60,750	38	70,340	44	28,770	18
Experimental	181,000	87	88,180	56	55,115	35	14,170	9
U-^{14}C-D-Glucose								
Control	62,100	95	11,200	19	27,700	47	20,050	34
Experimental	43,500	88	15,310	40	15,690	41	7,270	19
^{14}C-DL-Serine								
Control	15,400	90	5,260	38	6,090	44	2,490	18
Experimental	10,900	90	5,980	61	2,840	29	980	10

[a]The neutral lipids obtained by silicic acid chromatography of the total lipids were fractionated into their components by thin layer chromatography. (See text for details.)

[b]Represents the sum of the radioactivities of all fractions divided by the total radioactivity applied to the thin layer plate.

[c]Represents the percent of total radioactivities of all components.

closed down the cell almost completely (97% reduction). The inhibition of ^{14}C uptake into the total lipids by Cs^+, Li^+, and Rb^+ was slightly less when dbcAMP and theophylline were omitted from both cotrol and experimental flasks. Little or no effect was observed with these ions at 10 mM concentration. The aggregate omission of K^+, Ca^{+2}, and Mg^{+2} from the Krebs-Ringer bicarbonate solution reduced the isotope uptake by 31%, whereas their individual effects were variable; a response of 70% was given by ethylenediamine tetraacetic acid (EDTA). DL-Epinephrine (0.1 mM) iodoacetate (5 mM) and puromycin (2.5 mM) inhibited the isotope utilization by 50, 45 and 30%, respectively.

The wt recovered in the neutral and polar lipids was ca. 100 ± 3% of the total lipids, whereas the total radioactivity recovered in these fractions was ca. 100 ± 10% (Table II). The wt of the total and neutral lipids in the controls from all three ^{14}C-precursors were slightly greater than those in the experimental determinations, whereas the corresponding wt differences in the polar lipids were reversed but to a smaller extent. In all instances, the specific activities of the controls were greater than their corresponding experimentals. The percent decrease in incorporation of isotope between control and experimental determinations with all of the substrates was greater than the corresponding changes in lipid wt, except for the neutral lipids from ^{14}C-acetate. The percentage of neutral and polar lipid wt from ^{14}C-acetate and from ^{14}C-DL-serine were disproportionate with the radioactivities in these fractions. The percentage of radioactivities in the neutral and polar lipids of both controls and experimentals from U-^{14}C-D-glucose corresponded with their respective wt percentages.

The radioactivity recovered in the TLC separations of the neutral lipid components was 83-95% of that applied to the plates (Table III). The radioactivities of the cholesterol and triglyceride controls derived from all 3 precursors were 20-65 percent greater than their corresponding experimentals. The mono- and diglycerides, however, showed a reversal with the experimentals showing 10-30% greater activity than the controls. Generally, the largest and smallest fractions were cholesterol and triglycerides, respectively.

The radioactivity recovered in the TLC separations of the polar lipid components was in the same range, 80-89%, as that obtained for the neutral lipids (Table IV). The radioactivities of the controls of all lipid components obtained from either of the 3 ^{14}C-precursors was 20-60% greater than their corresponding experimentals; the exception was the cerebroside derived from ^{14}C-DL-serine. The largest component was choline phosphatide; from 65-86% of the total radioactivity recovered was accounted for by both choline- and ethanolamine phosphatides.

DISCUSSION

The increase or decrease in the rate of cellular metabolism elicited by many hormones is mediated by cAMP. The gray matter of the central nervous system from mammalian tissues has one of the highest levels of adenyl cyclase and

TABLE IV

Fractionation of ^{14}C-Labeled Polar Lipids from Rat Brain Slices[a]

Substrate	Polar lipids Applied cpm	Polar lipids Recovered Percent[b]	Choline lysophosphatide cpm	Choline lysophosphatide Percent[c]	Sphingomyelin[d] cpm	Sphingomyelin[d] Percent	Choline phosphatide cpm	Choline phosphatide Percent	Ethanolamine phosphatide cpm	Ethanolamine phosphatide Percent	Cerebroside cpm	Cerebroside Percent
2-^{14}C-Acetate	10 mg											
Control	86,540	80	3,460	5	6,230	9	40,150	58	13,850	20	2,770	4[e]
Experimental	43,330	88	1,910	5	2,670	7	18,680	49	6,100	16	1,140	3[f]
U-^{14}C-D-Glucose												
Control	69,500	89	3,090	5	3,090	5	36,490	59	14,220	23	4,950	8
Experimental	39,820	81	1,290	4	1,610	5	20,320	63	6,770	21	2,260	7
3-^{14}C-DL-Serine												
Control	95,000	85	2,420	3	6,460	8	51,680	64	17,760	22	2,420	3
Experimental	48,180	84	2,020	5	2,830	7	22,260	55	10,120	25	3,240	8

[a]The polar lipids obtained by silicic acid chromatography of the total lipids were fractionated into their components by thin layer chromatography. (See text for details.

[b,c]Same as Table III.

[d]This fraction may have containd some serine phosphatide. (See text for details.)

[e]Unidentified material comprising 4% of the total radioactivity was recovered at the front.

[f]Unidentified material comprising 20% of the total radioactivity was recovered at the origin and front.

phosphodiesterase (16). In rat brain cerebellum slices, the adenyl cyclase receptors are not detectable for at least the first 3 days postpartum (17) and are localized primarily in the mitachondrial and microsomal fractions with the highest specific activity in those subfractions containing nerve endings (18). The greatest effect shown by dbcAMP on the suppression of lipid synthesis in the present experiments (Table I) did not coincide with the peak period, 14-21 days, of myelination. In the absence of dbcAMP (controls), the best preparations showed only 9.0% isotope utilization. Since cAMP is relatively impermeable to the cell, as are many phosphorylated compounds, and subject to rapid hydrolysis by phosphodiesterase, attempts to obtain slices or homogenates active in the presence of cAMP were unsuccessful.

Although the ionic environment influences the formation and action of cAMP, as well as affecting reactions not mediated by cAMP, generalizations as to the effects of ions on complex systems, such as brain slices, are difficult. It appears that Cs^+, Li^+, and Rb^+ exert their effects upon lipid metabolism independent of the presence of exogenous dbcAMP, although, possibly, these ions may act via the cell's endogenous cAMP. The effect of F^-, which stimulates adenyl cyclase, as well as that of CN^- and iodoacetate, most likely results from their roles as enzyme inhibitiors. Epinephrine and puromycin, both of which increase cAMP, elicited responses consistent with their known behavior in lipolysis (10). Other agents examined for their effects on 2-^{14}C-acetate utilization were cholate, desoxycholate, glucagon, and insulin, all of which yielded variable results. Theophylline alone showed an effect but much less than in the presence of dbcAMP.

In spite of the expected differential loss of lipid due to leakage during preparation of the slices, the recoveries of total lipid (Table II) in the controls were consistently greater than the experimentals by 4-9%. The reduction in isotope uptake produced by dbcAMP occurred throughout all components, except the mono- and diglycerides, of the neutral and polar fractions, although to different extents (Tables III, IV), thus, suggesting that cAMP may be involved in the synthesis of lipid by regulating the utilization of acetyl-coenzyme A (CoA). That the decrease in isotope content and lipid wt affected by dbcAMP is not due to increased utilization but rather to decreased incorporation was shown by the unchanged CO_2 production during incorporation of 2-^{14}C-acetate into cholesterol and fatty acids by rat liver slices (9). Since no discrete band for serine phosphatide was observed in the TLC, if present, some may have been processed along with the sphingomyelin fraction. The higher values found for the mono- and diglyceride fractions in the experimentals derived from all ^{14}C-precursors (Table III) are at variance with the surrounding data, and any explanation would be speculative. Although the effects exerted by cAMP may vary within the brain's heterogeneous cell population, it appears that it may mediate a major role in cell membrane synthesis, as well as regulate the rate and intensity of neuronal activity.

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Effects of Chronic Ethanol Ingestion upon Acyl-CoA: Carnitine Acyltransferase in Liver and Heart

SHARON L. PARKER, JOHN A. THOMPSON, and **RONALD C. REITZ,** Department of Biochemistry, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514

ABSTRACT

Chronic alcohol ingestion has been shown to cause a profound decrease in the activity of acyl-coenzyme A: carnitine acyltransferase in the liver. Twelve different acyl-coenzymes were used as substrates, and the decrease in acyltranser rates ranged from 22-60% of the control. This enzyme was not affected as drastically in the heart. Of 11 acyl-coenzymes tested, only two resulted in significantly lower rates of acyltransfer, even though the rates were decreased with all substrates tested. The specificities of this enzyme showed that increasing the chain length of the acyl group resulted in a decreased acyltransfer when the acyl groups were either saturated, monoenoic, dienoic, or trienoic. Also, increasing the number of ethylenic bonds present in the acyl group of fatty acyl-coenzymes of the same chain length resulted in increased rates of acyltransfer. One exception to this ethylenic bond effect was noted in the heart. Linolenate resulted in an acyltransfer rate lower than linoleate. Ethanol had little or no effect upon these specificities.

INTRODUCTION

Carnitine has been shown to stimulate mitochondrial β-oxidation (1-4), and the mechanism of this stimulation is known to involve the translocation of fatty acids across the mitochondrial membrane (5,6). The enzyme mediating this translocation, acyl-coenzyme A (CoA):carnitine acyltransferase (EC 2.3 1.23), has been studied from the standpoint of chain length specificity; and the existence of three distinct enzymes has been postulated—one which acts on short chain acids, on medium chain acids, and on long chain acids (7). Further, the reactivity of the long chain acyltransferase has been investigated using several different acylcarnitines as substrates (8). The substrate specificity varied according to chain length and to the number of ethylenic bonds present in the acyl moiety. The specificity of the enzyme toward different acyl-CoAs, however, has not been examined. It has been postulated that this enzyme(s) may have a role in the regulation of glucose metabolism, as well as fatty acid metabolism (9).

Alcohol ingestion has been reported to decrease the oxidation of fatty acids in the liver (10), and other studies have indicated that alcohol also may cause a decrease in β-oxidation in the heart (11,12). The concentration of free and acylcarnitine and the levels of free and acyl-CoA have been reported to be elevated in rat liver after acute ethanol administration (13), and chronic ethanol administration also has been shown to result in increased long chain acyl-CoA (14). Thus, it is quite possible that alcohol may affect the oxidation of fatty acids by exerting an influence upon the acyl-CoA: carnitine acyltransferase. Since both heart (15) and, to a lesser extent, liver (4) derive a significant portion of their oxidative energy from fatty acids, it is important to determine what effect alcohol has upon this enzyme. Therefore, the purpose of this study was to determine the effect of chronic alcohol ingestion on the acyl-CoA:carnitine acyltransferase and also to determine the specificity of this enzyme toward different acyl-CoAs.

METHODS

Animals: Wt-paired Sprague-Dawley rats initially weighing 140-160 g were used. The alcohol test animals were given 20% ethanol as the sole source of drinking water, and they were allowed to eat Purina Lab Chow ad libitum. The volumes of ethanol and the amounts of chow consumed were measured and recorded every 2 days. The wt-paired control animals were given isocaloric amounts of glucose in their drinking water, and each animal was given an amount of chow equal to that consumed by its alcohol pair. Wt gain in both groups of animals followed the normal wt curve for animals of this age (16). The animals were ca. 36 days old at the start of the experiment and were kept on the experimental regime for 42 days. The wt of the animals on the alcohol diets increased from a mean ± standard error of the mean of 151 ± 2.8 g at the start of the experiment to 340 ± 10.2 g at the end of the experiment, while the control animals gained

from 153 ± 2.4 g to 310 ± 10.1 g during the experimental feeding. The ethanol treated animals consumed an average of 30% of their total calories in the form of ethanol.

Preparation of mitochondria: The animals were sacrificed by decapitation. The chest and abdomen then were opened along the midline to expose the liver and the heart completely. After a thorough perfusion of both the liver and the heart with ice cold isotonic saline, these organs were removed and homogenized in ice cold 0.25 M sucrose. The procedures described by Chappell and Hansford (17) were followed for the isolation of mitochondria from both tissues. Basically this involved collecting the fraction of the homogenate sedimenting between 5000 g/min and 45,000 g/min. The isolated mitochondria were resuspended in 0.25 M sucrose. Protein was determined by the procedure of Lowry, et al. (18).

Spectrophotometric assay of acyl-CoA: carnitine acyltransferase: The reaction mixture contained 25 nmoles acyl-CoA and 1.78 μmoles ℓ(−) carnitine in a mixture of 0.9 ml 0.154 M phosphate buffer (pH 7.25) and 0.1 ml 0.01 M 5,5′-dithiobis (2-nitro-benzoic acid) (DTNB). The reactions were done at 27 C and were initiated by the addition of about 0.05 mg mitochondrial protein. The activity of the enzyme was monitored continuously at 412 nm in a Beckman 25 double beam spectrophotometer. The blank cuvette contained all reactants in the test cuvette except carnitine. The concentrations of several of the acyl-CoAs were varied, and no change in activity was observed between 20-30 μM. At 25 μM acyl CoA, the optimal concentration of carnitine was determined to be 1.78 mM.

Radioactivity assay of acyl-CoA:carnitine acyltransferase: Seventy-five nmoles of [1-^{14}C] palmityl-CoA (194 cpm/nmole) and 5.34 μmoles ℓ(−) carnitine were incubated at 27 C with 0.15 mg of rat heart mitochondrial protein in a total volume of 3 ml phosphate buffer (0.154 M, pH-7.25). At 1,2, and 3 min, 1 ml aliquots were removed, immediately added to 3 ml of MeOH:$CHCl_3$ (2:1 v/v), and mixed thoroughly. The total lipids then were extracted by the procedure of Bligh and Dyer (19). The lower $CHCl_3$ phase was removed and evaporated to dryness under N_2. The lipids were dissolved in benzene and chromatographed using Gelman SG ITLC glass fiber papers and a solvent containing 100 ml $CHCl_3$ and 6 ml 10% glacial acetic acid in methanol. The palmityl-carnitine spots were visualized with I_2, cut out, and counted in toluene scintillation fluid after the I_2 was allowed to vaporize from the chromatogram. Appropriate phospholipid standards were utilized to ensure that there was no contamination of the acyl-carnitine by these lipids.

To check the extraction of acyl-carnitine, palmitoyl-carnitine (Sigma Chemical Co., St. Louis, Mo.) was dissolved in water and extracted, according to Bligh and Dyer (19). The organic phase was evaporated to dryness under nitrogen, and then both the dried organic and aqueous phases were saponified with 10% KOH, acidified, an internal standard of 17:0 added and extracted with petroleum ether. The petroleum ether extracts then were methylated with BF_3-methanol and quantitated by gas chromatography (GLC) as previously reported (20). All the palmitic acid was found in the organic phase indicating that the extraction procedure was quantitative for palmitoyl-carnitine.

Preparation of acyl-CoAs: All acyl-CoAs were prepared by the micromodification of Seubert's procedure (21), described by Reitz, et al. (22). The saturated and monoenoic acyl-CoAs were quantitated by their absorption at 232 nm and at 260 nm and by phosphate determinations (23). The polyunsaturated acyl-CoAs contained Santoquin (Monsanto Corp., St. Louis, Mo.) as an antioxident; therefore, only phosphate determinations could be done on these compounds. The usual A_{232}:A_{260} assay for CoA thiol esters (21) (used where applicable) and the phosphorous content were compared with the amount of CoA released by the acyltransferase system. The agreement we found between these values indicated that the synthesized material was 100% reactive.

RESULTS

Assay Method

The translocation of fatty acids from the cytoplasm into the mitochondria involves two reactions, (6) both of which are catalyzed by carnitine palmitoyltransferase. The first reaction is the transfer of a fatty acid from CoA to carnitine, and the second reaction is simply the reverse of the first reaction. We have been concerned with the first reaction, the formation of the acyl-carnitine. This reaction has been assayed several different ways (7), but the use of DTNB (5,5′-dithiobis-(2-nitrobenzoic acid) is preferred with impure preparations of the transferase, such as mitochondria (24). Bieber, et al., (25) described this procedure in detail, but he did not isolate the product, palmitoyl-carnitine, and conclusively demonstrate that the release of free CoA was directly proportional to the product formed. We have, therefore, carried these experiments this last step

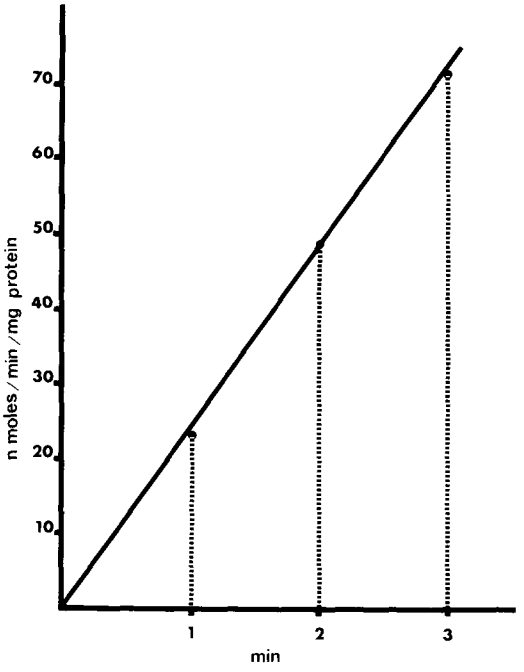

FIG. 1. Comparison of a continuous spectral assay with a radioactive assay for acyl-coenzyme A (CoA): carnitine acyltransferase. The reaction mixture for the spectrophotometric assay contained 25 nmoles of acyl-CoA and 1.78 μmoles of ℓ(−) carnitine in a mixture of 0.9 ml 0.154 M phosphate buffer (pH 7.25) and 0.1 ml 0.01 M 5,5'-dithiobis (2-nitro-benzoic acid). The reaction was initiated by adding ca. 0.05 mg rat heart mitochondrial protein, and it was monitored continuously at 412 nm in a double beam spectrophotometer. The blank cuvette contained all reactants, except carnitine. The reaction rate for this spectral assay is indicated by the solid black line. The reaction mixture for the radioactive assay contained 75 nmoles of [1-^{14}C] palmitoyl-CoA (194 cpm/nmole) and 5.34 μmoles of ℓ(−) carnitine in a total volume of 3 ml 0.154 M phosphate buffer (pH 7.25). Ca. 0.15 mg rat heart mitochondrial protein was used to catalyze the reaction. At 1, 2, and 3 min, 1 ml aliquots were removed and the lipids extracted. The lipids were chromatographed on Gelman ITLC glass fiber papers in a solvent system of 100 ml $CHCl_3$ and 6 ml 10% glacial acetic acid in methanol. The palmitoyl-carnitine spots were visualized with I_2, and, after the I_2 had vaporized, they were cut out and counted. The specific activity determined for the enzyme by this method is represented by the vertical dashed lines. The cmp/min/mg protein were converted to nmoles/min/mg protein to allow comparisons to be made easily.

and the data in Figure 1 show that the rate of release of CoA was exactly the same as the rate of formation of palmitoyl-carnitine. Therefore, the use of DTNB represents an easy, accurate, and sensitive assay for the formation of acyl-carnitine from acyl-CoA.

Substrate Specificity in Rat Heart Mitochondria

We were interested in examining the substrate specificity of the acyl-CoA:carnitine acyltransferase in rat heart mitochondria; therefore, the activity of the enzyme was examined with 11 different acyl-CoAs using the DTNB assay. The results are presented in Table I under the control column. Considerable differences were noted in the activity of the enzyme toward the various acyl groups and some definite specificity patterns were apparent. The enzyme clearly exhibited chain length specificity. Of the saturated compounds investigated, 15:0-CoA resulted in the greatest activity. Lengthening the carbon chain by only one carbon atom resulted in a loss of ca. 40% of the activity, and a further decrease in activity was obvious when stearoyl-CoA was the substrate. This same specificity for chain length was observed when the monoene, diene, and triene substrates were compared. Thus, the activity of the enzyme appeared to be inversely proportional to length of the hydrocarbon chain of the acyl group within a homologous series of fatty acyl-CoAs.

In addition to the selectivity for chain length, the enzyme also exhibited a specificity toward the number of double bonds present in the hydrocarbon chain of the substrate. The presence of one double bond in the C_{16} series resulted in almost twice the activity of that seen when the saturated acyl-CoA, palmitoyl-CoA, was used. In comparing the members of the C_{18} group of acyl-CoAs this direct relationship between enzyme activity and the number of ethylenic bonds present in the hydrocarbon chain existed until there were three ethylenic bonds present, at this point, the activity toward linolenate was slightly lower than that toward linoleate. A similar observation was made in the C_{20} series, but in this case the diene and triene had almost identical rates of transfer. It may be important to point out that the diene and triene in the C_{18} series were from the same family ω6, while in the C_{20} series, the diene was from the ω6 family and the triene was from the ω3 family.

Substrate Specificity in Rat Liver Mitochondria

The acyl-CoA:carnitine acyltransferase found in rat liver mitochondria (Table II) exhibited much the same specificity patterns as those seen in rat heart mitochondria (Table I). Increasing the chain length of the substrate resulted in a loss of activity, and, in general, increasing the number of ethylenic bonds resulted in an increase in activity for acids of the same chain length. Here again little difference was noted in the activity when 20:2ω6 or 20:3ω3 served as substrates. An obvious difference between the heart and liver enzymes was their relative activity toward 18:2ω6 and

TABLE I

Effect of Alcohol upon Heart Acyl-Coenzyme A: Carnitine Acyltransferase[a]

	nmoles/min/mg mitochondrial protein		
Fatty acid	Control	Ethanol	Percent change
15:0	38.7 ± 7.3	36.4 ± 11.2	− 5.9
16:0	23.0 ± 1.7	20.8 ± 0.4	− 9.6
16:1ω7[b]	41.2 ± 6.3	14.3 ± 1.2	− 65.3
18:0	6.2 ± 0.7	7.0 ± 1.1	+ 12.9
18:1ω9	10.1 ± 0.6	9.9 ± 1.3	− 2.0
18:2ω6	45.6 ± 4.6	43.3 ± 5.3	− 5.1
18:3ω6	33.1 ± 2.5	28.8 ± 0.9	− 12.9
20:1ω9	4.4 ± 0.6	3.7 ± 0.3	− 15.9
20:2ω6	12.6 ± 0.9	10.3 ± 0.9	− 18.3
20:3ω3	12.2 ± 0.6	11.1 ± 0.9	− 9.0
20:4ω6[c]	21.3 ± 2.5	11.8 ± 4.7	− 44.6

[a]The components of the reaction mixture are the same as for the spectrophotometric assay in Figure 1. All values represent the mean ± standard error of the mean from 4-7 analyses. No comparisons were statistically significant except those noted.

[b]$P < 0.001$.

[c]$0.05 > P > 0.02$.

TABLE II

Effect of Alcohol upon Liver Acyl-Coenzyme A: Carnitine Acyltransferase[a]

	nmoles/min/mg mitochondrial protein		
Fatty acid	Control	Ethanol	Percent change
15:0	48.0 ± 3.7	28.7 ± 1.4	− 40.2
16:0[b]	28.6 ± 1.4	20.4 ± 1.3	− 28.7
16:1ω7[c]	43.3 ± 4.7	30.1 ± 2.1	− 30.5
18:0	7.7 ± 0.3	5.3 ± 0.4	− 31.2
18:1ω9[d]	10.6 ± 0.4	8.2 ± 0.5	− 22.6
18:2ω6[b]	47.6 ± 4.6	34.0 ± 1.6	− 28.6
18:3ω6	84.9 ± 2.4	34.0 ± 1.9	− 60.0
20:1ω9[b]	5.0 ± 0.3	2.8 ± 0.5	− 44.0
20:2ω6	15.5 ± 1.1	10.8 ± 1.0	− 30.3
20:3ω3	17.3 ± 0.4	7.9 ± 0.4	− 54.3
20:4ω6	35.3 ± 3.4	23.5 ± 2.4	− 33.4
22:6ω3	45.9 ± 3.5	29.8 ± 2.9	− 35.1

[a]The components of the reaction mixture are the same as for the spectrophotometric assay in Figure 1. All values present the mean ± standard error of mean from 4-7 analyses. All comparisons were statistically significant at $P < 0.001$, except those noted.

[b]$0.01 >, P > 0.001$.

[c]$0.02 > P > 0.01$.

[d]$0.05 > P > 0.02$.

18:3ω6. In the heart, the activity with linoleate was greater than with linolenate; however, in the liver, just the opposite was seen. It was interesting to note that the activity of the enzyme in the heart and liver was almost identical with several substrates. These included all but 18:3ω6 of the C_{18} series and 20:1ω9 and 20:2ω6 of the C_{20} series. Also the difference between the heart and liver with the C_{15} and C_{16} substrates was not large.

Effects of Ethanol

Next, we were interested in determining the effects of alcohol upon the acyl-CoA:carnitine acyltransferase from both the heart and the liver. These date are shown in Tables I and II, respectively. In general, there were decreases in the activity of the enzyme toward all the substrates after alcohol ingestion. In the heart (Table II), a significant decrease was recorded for only two of the acyl-CoAs tested. These were palmitoleate (65%) and arachidonate (45%). The percentage decrease with the other acyl-CoAs ranged from 2% with 18:1ω0 to 18% with 20:2ω6. In the liver, however, a significant decrease was observed with all the acyl-CoA substrates. The lowest percentage decrease was with oleate (22%) and the highest decrease was with linolenate (60%). Alcohol ingestion resulted in roughly a 30% decrease in activity toward the other substrates, except for 15:0, 20:1ω9, and 20:3ω3. The decrease in activity toward these 3 substrates was 40, 44, and 54%, respectively. Obviously then, the effects of alcohol were more profoundly evident in the liver compared to the heart. This may be significant from the point of view that the heart relies more heavily upon the oxidation of fatty acids for energy than does the liver. It also should be pointed out that ethanol had little effect upon the specificities of the enzyme which were discussed earlier.

DISCUSSION

The long chain acyl-CoA:carnitine acyltransferase catalyzes the reversible reaction of acyl-CoA with carnitine shown below:

$$\text{Acyl-CoA} + \text{carnitine} \rightleftharpoons \text{acylcarnitine} + \text{CoA-SH}$$

Since the purpose of this reaction is to mediate the translocation of long chain fatty acids into the mitochondria, the forward reaction would be expected to take place on the outer surface of the mitochondria, while the reverse reaction would occur inside the mitochondria. The recent work of Yates and Garland (26), West, et al. (27), and Hoppel and Tomec (28) provide data to support the thesis of Fritz and Yue (6) of an inner and outer enzyme; however, it should be pointed out that inner and outer refer to the inner membrane only. The substrate specificity has only been determined on the reverse reaction, and, with the exception of one report (8), only saturated acyl-carnitines have been used (7,24,27). In general, maximal rates seem to be obtained with palmitoyl-carnitine. Using a solubilized acyltransferase prepara-

tion from liver, Christophersen and Bremer (8) noted a decrease in activity as the C_{16} substrate was increased in chain length to C_{18} and the activity of the enzyme decreased with the insertion of one ethylenic bond, but, with the successive insertion of ethylenic bonds, the activity increased significantly above that of the saturated acylcarnitine. Our data provide the first report of experiments to determine the substrate specificity of the forward reaction. Twelve Acyl-CoAs were used, and the specificity was quite similar to that noted by Christopherson and Bremer (8) for the reverse reaction. Some differences were apparent, but, in general, increasing the chain length of the acyl group resulted in decreased acyltransfer, whether the acyl groups were saturates, monoenes, dienes, or trienes. Also, an increase in the number of ethylenic bonds present in the acyl group resulted in an increased acyltransfer. This specificity pattern is remarkably similar to that reported by Bjorntorp (29) who examined the V_{max} for the β-oxidation of several long chain fatty acids.

Ethanol has been shown to decrease the rate of β-oxidation in the heart, and, although we have shown a definite tendency for alcohol to decrease the activity of acyl-CoA:carnitine acyltransferase in the heart, we were unable to explain this decrease on the basis of a significant effect on this enzyme. This may be due to the relatively large biological variances in activity. A possible explanation might involve inhibition of the reaction by increased amounts of carnitine. Bode, et al., (13) quoted unpublished data which is said to show a twofold increase in serum carnitine after alcohol ingestion. However, Rodis, et al., (30) has shown that negligible amounts of carnitine are taken up by the heart making this an unlikely explanation. Our data, then, suggest an effect upon the enzyme independent of the carnitine concentration, especially since our assay includes carnitine in excess. Finally, of the two acyl-CoAs which resulted in a significant decreased rate of acyltransfer, only arachidonate is a substantial component of the total myocardial fatty acid composition (20). Therefore, the effect of ethanol upon this enzyme in heart may be minimal with respect to the decrease in β-oxidation.

On the other hand, the liver, which accounts for greater than 90% of the total oxidation of ethanol in the body (31), displayed striking reductions in the transfer of all the acyl-CoAs to carnitine after chronic ethanol ingestion. This suggests very strongly that in the liver this enzyme may play a significant role with respect to the decreased β-oxidation which has been demonstrated repeatedly. If alcohol does decrease the activity of this enzyme, then acyl-CoA would be expected to increase, and this has been observed (13,14). Acyl-carnitine, the product, also has been reported to be increased (13,32) which may suggest an impairment in the translocation of acyl-carnitine across the mitochondrial membrane. Gordon (14) has demonstrated that ethanol ingestion significantly interferes with the ability of the mitochondria to translocate adenosine diphosphate (ADP); therefore, the general phenomenon of translocation may be impaired. This is supported by the observation of striking changes in mitochondrial shape after alcohol ingestion (33) which suggests that the integrity of the membrane may well be affected. Since an acyl-CoA generating system (34) has been shown to produce mitochondrial swelling similar to that caused by alcohol (33), it is possible that the alterations in mitochondrial shape are a direct result of the inhibition of acyl-CoA: carnitine acyltransferase which would cause an increase in acyl-CoA. The increased acyl-CoA then would produce an altered membrane, presumably causing a defect in translocation and resulting in increased acyl-carnitine in spite of its severely reduced synthesis. Further work must be done to validate this mechanism.

ACKNOWLEDGMENTS

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Cholesteryl Ester Metabolism in Tissue Culture Cells. I. Accumulation in Fu5AH Rat Hepatoma Cells

GEORGE H. ROTHBLAT, The Wistar Institute, Philadelphia, Pennsylvania 19104

ABSTRACT

Exposure of Fu5AH rat hepatoma tissue culture cells to hyperlipemic rabbit serum results in the accumulation of cellular cholesteryl esters. Accumulation is not a characteristic of all cells in culture, as evidenced by the lack of response of mouse and human fibroblasts. Fu5AH cells grown for 24 hr on 5% hyperlipemic rabbit serum have an 8- to 12-fold increase of cellular cholesteryl esters, small increases in free cholesterol and triglycerides, and no change in phospholipids, when compared to cells grown in normal rabbit serum. Rapid accumulation of cholesteryl esters occurs during the first 8-12 hr of incubation, and maximum cellular concentration is achieved within 24 hr. The maximum level of cellular cholesteryl esters obtained with individual samples of hyperlipemic rabbit serum is correlated with the cholesterol content of the original sera, even when the incubation medium is adjusted to a constant concentration of cholesterol. Heating hyperlipemic rabbit serum (60 C/30 min) does not destroy activity; however, no cholesteryl ester accumulation occurs in heated cells. The stimulatory activity of hyperlipemic rabbit serum primarily is associated with lipoproteins having densities <1.006. High levels of cellular cholesteryl ester are associated with the appearance of cytoplasmic vacuoles containing cholesteryl esters. The increase in cellular cholesteryl esters is accompanied by a decrease of the cholesteryl esters in the growth medium. Cellular cholesteryl esters are not rapidly hydrolyzed or released upon removal of hyperlipemic rabbit serum.

INTRODUCTION

The metabolism of cholesteryl esters (CE) in tissue culture cells has received only a limited investigation. A variety of cells has been shown to contain only small quantities of CE when grown on normal sera (1,2); however, Bailey and Keller (3) have reported that human skin fibroblasts grown in the presence of hyperlipemic rabbit serum (HRS) rapidly accumulate CE. Similar observations have been reported by Chen, et al., (4) using rabbit aortic cells in culture grown on HRS. The subject of this article presents an investigation of factors involved in the accumulation and metabolism of cholesteryl esters in rat hepatoma tissue culture cells.

EXPERIMENTAL PROCEDURES

Cells and Growth Conditions

L-cell mouse fibroblasts (Clone 1-D) have been maintained in this laboratory for 3 years. HF fibroblasts, originating from human foreskin, and the W-18VA$_2$ cells (SV$_{40}$ transformed buccal mucosa) were obtained from colleagues at The Wistar Institute. The above cells were grown routinely in Eagle's minimal essential medium (MEM, Auto-Pow, Flow Labs) supplemented with 5% calf serum, pencillin (200 μ/ml), and streptomycin (0.1 mg/ml). Incubations were at 37 C in an atmosphere of 5% CO_2 in air. All cultures were tested routinely and found free of mycoplasma contamination. The Fu5AH rat hepatoma cell line was initiated from the Reuber H-35 rat hepatoma and exhibits a large number of stable, differentiated functions (5,6). Stock monolayer cultures of Fu5AH cells were maintained routinely in MEM medium supplemented with either 5% calf serum or 5 mg/ml delipidized calf serum protein (DLP). Experimental cultures were obtained by refeeding stock cultures with MEM supplemented with varying concentrations of normal rabbit serum or HRS, when the cultures were ca. one-third to one-half confluent. No evidence of cellular toxicity was apparent when established monolayers of Fu5AH cells were grown in HRS. However, HRS reduced cellular plating efficiency, and, therefore, these cells were not routinely maintained in the presence of HRS. After washing with phosphate-buffered salt solution (7), cellular monolayers were harvested with 0.25% trypsin in 0.1% versene. Cell suspensions were washed by recentrifugation in the buffered salt solution. Cells obtained from a single 60 mm petri dish (Falcon Plastic, Oxnard, Calif.) provided sufficient material for routine sterol quantitation. Cells were grown in 1 liter Blake bottles for experiments requiring larger amounts of cellular material.

Serum and Serum Lipoproteins

DLP was prepared as previously described (8,9). Sterile normal rabbit serum was purchased from Flow Laboratories, Rockville, Md. Unless otherwise noted, the HRS was pooled from rabbits fed with rabbit chow augmented with 2% cholesterol and 6% corn oil for at least 2 months (10). The total sterol concentration of these sera ranged between 1536-2530 mg% (mean = 1969 mg%) with ester to free cholesterol ratios between 3.2-3.6 (mean = 3.3). Pooled normal and hyperlipemic monkey (*Macaca mulatta*) and pigeon (White Carneau) sera were obtained from Bowman Gray School of Medicine. In experiments requiring incubation periods longer than 12 hr, sera were sterilized by filtration through a 0.45 mμ Millipore filter after prefilteration through a glass fiber filter. Lipoproteins were separated from normal and hyperlipemic sera and washed by recentrifugation following the methods published by Hatch and Lees (11).

Analytical Procedure

Washed cell pellets were resuspended in saline and sonicated (Branson sonifier, setting no. 1, 20 sec) to obtain aliquots for lipid extraction and cellular protein quantitation by the Lowry method (12). The Bligh-Dyer procedure (13) was used to extract cellular homogenates, aliquots of incubation media, serum, and serum lipoproteins. Free cholesterol, i.e. unesterified, (FC) and total cholesterol were analyzed quantitatively by gas liquid chromatography (GLC); coprostanol (5β-cholestan-3β-ol, Applied Science Laboratories, State College, Pa.) was added at the time of extraction as an internal standard. GLC analysis of cholesterol was performed on a Hewlett-Packard no. 402 chromatograph equipped with a hydrogen flame detector and a Hewlett-Packard electronic integrator. A 6 ft column was packed with 100-120 mesh Gas-Chrom Q coated with 3% OV-17 (Applied Science Laboratories) and operated at 245 C. CE levels were calculated as the difference between the free and total cholesterol and are expressed as μg of CE cholesterol. All values represent the average of at least two replicate cultures. Saponification of total lipids was carried out with 10% ethanolic KOH, as previously described (14). Triglycerides were quantitated following the procedure described by Levy and Keyloun (15) using corn oil as a standard and phospholipids by the method of Bartlett (16).

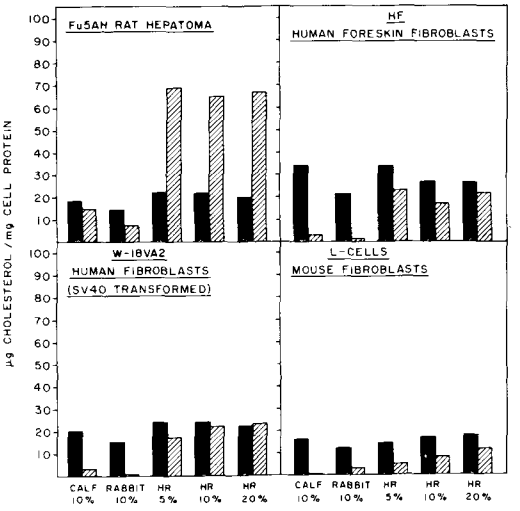

FIG. 1. Sterol content of tissue culture cells grown in normal serum and hyperlipemic rabbit serum. Incubation time, 3 days; ■ = free cholesterol; ///// = esterified cholesterol.

RESULTS

Cholesteryl Ester Accumulation in Various Cell Lines

Figure 1 illustrates the FC and CE content of 4 different tissue culture cell lines

TABLE I

Free and Ester Cholesterol Content of Fu5AH Cells

Serum	Number of determinations	μg/mg Cell protein: Free cholesterol (FC)	Cholesteryl ester (CE)	CE/FC
5% HRS[a]	55	21.95 ± 0.59[b]	72.52 ± 2.41	3.30
5% Normal rabbit[a]	24	12.89 ± 0.44	6.30 ± 0.79	0.49
10% Calf[c]	11	15.68 ± 0.49	10.19 ± 1.54	0.65
Delipidized calf[c]	10	12.64 ± 0.64	0.98 ± 0.21	0.08

[a]Stock cultures of calf serum grown cells refed with MEM supplemented with normal or hyperlipemic rabbit serum (HRS). Incubation time, 24 hr.

[b]Mean ± standard error.

[c]Stock cultures grown continuously in MEM supplemented with calf serum or delipidized calf serum.

TABLE II

Lipid Content of Fu5AH Rat Hepatoma Cells Grown on Normal and Hyperlipemic Rabbit Serum

Lipid	μg/mg cell protein		Hyperlipemic / normal
	Normal rabbit[a]	Hyperlipemic rabbit[a]	
Cholesterol[b]			
Free	12.9	22.0	1.7
Ester[c]	6.3	72.5	11.5
Total	19.2	94.5	4.9
Phospholipid[d]	4.8	4.9	1.0
Triglyceride	66.9	109.8	1.6

[a]Cells incubated 24 hr in 5% serum.
[b]Cholesterol values taken from Table I.
[c]As cholesterol of cholesteryl ester.
[d]As phospholipid P.

grown for 3 days in MEM, supplemented with either HRS, normal rabbit serum, or calf serum. There is a large increase in cholesteryl esters in the Fu5AH hepatoma cells grown on HRS, resulting in a CE/FC ratio of 3. By comparison, the other cell lines contained considerably less sterol, and in no instance are CE/FC ratios significantly greater than 1. Thus, the ability to accumulate CE may be a rather specific phenomena and not a characteristic of all cells maintained in tissue culture.

Lipids in Fu5AH Cells

Table I presents the sterol content of Fu5AH cells expressed as the mean of a large number of determinations. Some variation in cellular sterol content has been observed between individual experiments. Such variation probably reflects differences in the metabolic state of the cells and the pools of HRS being used. The CE content of the hepatoma cells grown in calf serum is somewhat higher than that of hepatoma cells maintained in normal rabbit serum, and when grown in medium supplemented with DLP, the cells contain very low levels of CE. Table II compares the lipid content of Fu5AH cells grown for 24 hr on 5% normal rabbit serum or 5% HRS. Of the lipid classes examined, CE showed the greatest increase.

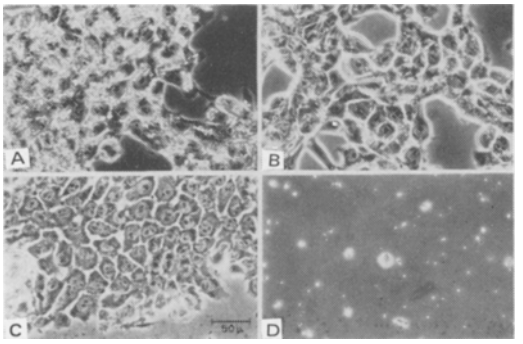

FIG. 2. Fu5AH rat hepatoma cells grown for 24 hr in presence of: A 5% hyperlipemic rabbit serum; B 5% normal rabbit serum; C delipidized calf serum protein (5 mg/ml); D vacuoles isolated from cells grown on 5% hyperlipemic rabbit serum.

Figure 2A shows that the accumulation of CE in HRS-grown cells is accompanied by the appearance of cellular vacuoles which often almost completely fill the cytoplasm. Cells grown on normal rabbit serum (Fig. 2B) contain fewer vacuoles, while cells maintained on DLP (Fig. 2C) have only occasional vacuoles. Figure 2D shows vacuoles recovered by centrifugation at 100,000 x g for 1 hr after disruption, by Dounce homogenization, of the cells suspended in buffered salt solution. These vacuoles, which collect at the surface of the centrifuge tube, were composed of CE:FC:phospholipid in the ratio of 1:0.06:0.20. The ratio of CE:triglyceride varied, ranging from 1:0.68 to 1:1.73. The distribution of FC and CE between pelleted and supernatant fractions after centrifugation (100,000 x g/1 hr) was determined. A mean of 72% (range 56-91%) of CE was recovered in the supernatant (including vacuole layer) while the value for FC was 15% (range 5%-25%).

Factors that Influence CE Accumulation

Figure 3 shows the free and esterified cholesterol content of Fu5AH cells throughout a 24 hr incubation period. Two different stock sublines of cells were used, one previously maintained on calf serum, the second on DLP. In both sublines, a rapid, nearly linear accumulation of CE was observed for the first 8 hr of incubation. In the experiment shown in Figure

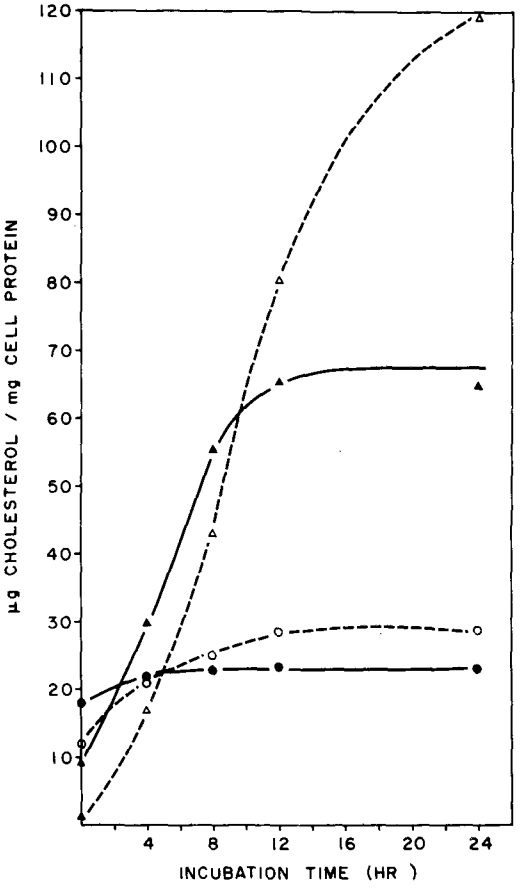

FIG. 3. Sterol content of Fu5AH cells incubated in medium supplemented with 5% hyperlipemic rabbit serum.—— subline of cells previously grown in calf serum. ––– subline previously grown in medium supplemented with delipidized calf serum protein. △,▲, cholesteryl ester; ○,●, free cholesterol.

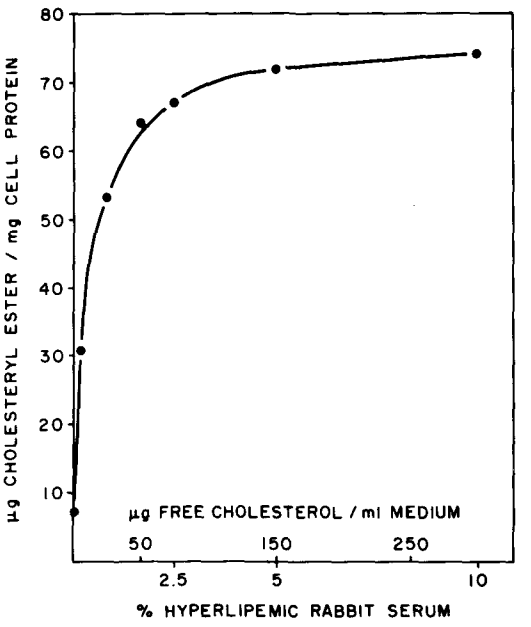

FIG. 4. Effect of serum concentration upon cholesteryl ester content of Fu5AH cells. Incubation time, 24 hr.

3, accumulation of CE by the DLP subline continued beyond 8 hr and resulted by 24 hr in a higher CE content. No appreciable change in cellular CE was observed when incubations were extended for more than 24 hr. The difference in pattern and maximum cellular accumulation of CE as illustrated by the two extremes shown in Figure 3, could not be linked consistently to specific sublines of Fu5AH cells, cell concentration, or incubation conditions and probably represents more subtle differences in metabolic state. The free cholesterol content of the cells grown in DLP medium and transferred to HRS showed ca. a two-fold increase (Table I and Fig. 3). A similar increase in free cholesterol has been observed in DLP grown L cells transferred to medium supplemented with cholesterol (14) or serum (17). Figure 4 shows that, at low levels of HRS (<2.5% serum; <75 µg/ml free cholesterol), there was a direct correlation between concentration of serum in the medium and cellular level of CE. Increasing the concentration of HRS in the medium above this level had no appreciable effect of cellular CE. The concentration of CE in these hepatoma cells when grown on normal rabbit serum was independent of serum concentration over 5-40% serum.

The observation that the cells achieved a maximum CE concentration which was unaffected by the presence of high concentrations of HRS suggests that this maximum concentration does not result from a limitation or exhaustion of some component in the HRS. The results presented in Table III demonstrate that refeeding cells previously grown in HRS with fresh HRS produces no further increase in cellular CE. In addition, used HRS medium taken from 24 hr cultures still retains its ability to induce CE accumulation when incubated with fresh cells for 24 hr.

The factors in HRS responsible for CE accumulation were stable to heating (60 C/30 min) and freezing and thawing (6 x), with cellular CE concentrations being 99 and 73%, respectively, of untreated HRS controls, (Table IV). If, however, the HRS was delipidized (9,18) and the protein and lipids added together to the culture medium after sonication at 40 C (18), no significant CE accumulation was observed (Table IV). Varying the pH of the

TABLE III

Effect of Refeeding and Used Medium on Cholesteryl Ester Accumulation in Fu5AH Cells

Culture number	Treatment	Incubation time in HRS[a] (in hours)	μg cholesterol/mg cell protein[b] Free	Ester
1	0 time cells	0	14.5	18.4
2	HRS	24	26.0	103.4
3	Refed at 24 hr with HRS	48	20.2	103.0
4	used HRS[c]	24	20.2	94.3

[a]All hyperlipemic rabbit serum (HRS) at 5%.

[b]Average of two replicate cultures.

[c]Media taken from culture 3 after first 24 hr incubation.

culture medium over pH 6.7-pH 8.3 did not influence cellular CE content. In contrast to the observed lack of effect of heating HRS, heating the Fu5AH cells eliminated the accumulation of cellular CE, although the FC content of heated cells increased after 24 hr incubation in either HRS or normal rabbit serum. Inhibiting protein synthesis with cycloheximide (2 μg/ml) resulted, after 24 hr incubation in HRS, in an increase in cellular CE/mg cell protein as compared to untreated control cells, (Table IV).

Effect of Degree of Hypercholesteremia upon Cellular CE Content

The HRS used in all of the previous experiments was obtained from rabbits maintained on hyperlipemic diets for at least 2 months, and these sera had total cholesterol levels in the range of 2000 mg%. Figure 5 shows the CE content of cells exposed to individual samples of serum obtained from rabbits maintained on hyperlipemic diets for various time periods up to 21 days. In the experiment shown in Figure 5A, the serum was added to the growth medium at a constant percentage (2.5%). Thus, the total cholesterol concentration in the growth medium ranged from 18-260 μg/ml. Figure 5B illustrates the cellular CE content when the same sera were added to the culture medium to obtain a constant concentration of 300 μg/ml of total cholesterol. Incubation times were reduced, because the sera were not filtered to ensure sterility. The results shown in both Figures 5A and 5B demonstrate that the cellular level of CE can be correlated with the cholesterol level of the individual serum. This result, in the case of a constant serum dilution (Fig. 5A) could be explained simply as a correlation between cellular CE and the concentration of cholesterol in the medium. However, this cannot be the explanation for the results

TABLE IV

Cholesteryl Ester Accumulation in Fu5AH Cells Exposed to 5% Hyperlipemic Rabbit Serum

Treatment[a]	Cholesterol ester accumulation % of untreated controls[b,c]
Serum	
heated (60 C/30 min)	99
frozen/thawed (6 x)	73
delipidized/relipidized	0
Cells	
heated (60 C/30 min)	0
cycloheximide (2 μg/ml)	167

[a]All cultures incubated 24 hr.

[b]$\frac{\mu g \text{ cholesteryl ester treated} - \mu g \text{ cholesteryl ester normal rabbit serum}}{\mu g \text{ cholesterol ester untreated} - \mu g \text{ cholesterol ester normal rabbit serum}} \times 100$

[c]All values are average of at least two independent experiments, each with at least two replicate cultures.

TABLE V

Cholesteryl Ester Accumulation in Fu5AH Cells: Effect of sera from rabbits upon regression diets

Diet	Time on diet (month)	Serum[a] total cholesterol (mg/%)	Cell cholesterol (μg/mg cell protein)[b] Free	Ester
Corn oil + cholesterol	2	1530	21.6	66.3
Corn oil + cholesterol corn oil	2	1097	22.0	66.0
Corn oil + cholesterol chow	2	695	19.8	57.7

[a]Each serum added to culture medium to give 75 μg/ml free cholesterol. Incubation = 24 hr.

[b]Average of two replicate cultures.

seen in Figure 5B, where cholesterol levels in the medium are similar in all samples (300 μg/ml). These data suggest that cellular CE accumulation is induced by specific serum lipoproteins, the level of which is a function of the degree of hypercholesteremia. The results presented in Table V demonstrate that the ability of HRS to induce high CE levels in Fu5AH cells persists after the rabbits have been removed from the cholesterol diet for a period of 2 months.

As shown in Table VI, the ability to promote CE accumulation is not unique to HRS. An increased CE content was observed in cells exposed for 24 hr to hyperlipemic monkey and pigeon sera as compared to cells grown in normal sera.

Table VII shows the free and esterified cholesterol content of Fu5AH cells grown for 24 hr on HRS and the individual lipoproteins isolated from these sera. The serum used in experiment I (Table VII) was obtained from an animal on the cholesterol diet for 4 months, while that used in experiment 2 was from an animal on the diet for only 3 weeks. The concentration of lipoproteins was adjusted to similar total cholesterol levels in the growth medium. In both instances, the greatest accumulation occurred in the lipoprotein fractions designated as very low density lipoproteins and chylomicrons. Considerably less accumulation was evident in cells grown in the presence of low density lipoprotein and high density lipoprotein, particularly in the case of lipoproteins obtained from the animal on a cholesterol diet for 3 weeks (experiment 2).

Origin and Stability of Cellular CE

In an effort to gain information on the origin of the cellular CE, balance studies were conducted in which changes in free and esterified cholesterol content were quantitated in both cells and incubation medium. Cells were incubated 24 hr in HRS previously heated to 60 C/30 min. Increase in the cellular cholesterol was determined as the difference between zero time cellular cholesterol and that recovered in the HRS-grown cells. Loss of exogenous sterol was quantitated by the differences in the level of cholesterol in medium taken from the incubated cultures and similar medium incubated in the absence of cells. Results from a

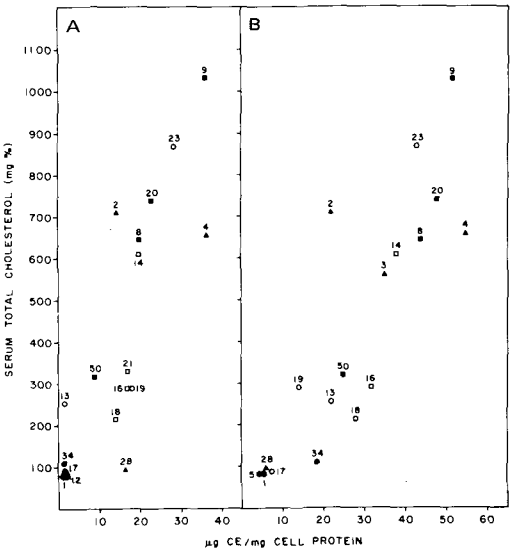

FIG. 5. Cholesteryl ester content of Fu5AH cells incubated in presence of rabbit sera having varying cholesterol levels (mg %). Experiment A: sera added to medium at constant level of 2.5%. Experiment B: sera added to equivalent total cholesterol concentration of 300 μg/ml. Incubation time, 10 hr. Numbers on figure denote individual sera. Symbols denote number of day rabbits maintained on cholesterol diet. ● = 0 days; ○ = 3; ■ = 7; ▲ = 11; □ = 21.

TABLE VI

Cholesterol Content of Fu5AH Cells Exposed to Normal and Hyperlipemic Rabbit, Monkey, and Pigeon Sera

Serum	Percent serum in medium	Serum total cholesterol (mg %)	Cellular cholesterol content μg/mg cell protein[a]	
			Free	Ester
Rabbit				
Normal	5	54	10.5	7.2
	10	54	10.1	9.2
Hyperlipemic	5	2295	20.0	79.1
	10	2295	20.9	82.2
Monkey				
Normal	5	137	14.8	2.9
	10	137	12.1	3.1
Hyperlipemic	5	894	17.9	63.5
	10	894	20.4	74.3
Pigeon				
Normal	5	368	17.2	22.6
	10	368	16.4	23.3
Hyperlipemic	5	837	19.9	59.3
	10	837	17.4	57.5

[a]Average of two replicate cultures.

typical experiment are presented in Table VIII. During the 24 hr incubation period the cellular pools of free and ester cholesterol increased by 55 μg and 332 μg, respectively. The decrease in exogenous unesterified sterol was considerably larger than the cellular increase, while the decrease in exogenous ester was 82% of the cellular increase. Thus, the data indicate that 80% of the CE that accumulated in the cells could be derived from the serum CE and 20% a product of cellular esterification of incorporated free cholesterol.

The stability of cellular CE also was assayed in a balance study, and the results from a representative experiment are presented in Table IX. In this experiment, Fu5AH cells, previously grown for 24 hr in 5% HRS and thus containing CE (82.3 μg/mg protein, Table IX), were refed with medium containing DLP (5 mg/ml) and lecithin (100 μg/ml). Sterol levels in cells and medium were quantitated 24 hr and 48 hr after the removal of the HRS. The results, expressed as μg cholesterol/culture, show that during the first 24 hr of incubation, the cellular pool of CE decreased by only 11% (152-135 μg). This small decrease was accompanied by the appearance of 25 μg CE in the incubation medium. A similar decrease in cellular CE

TABLE VII

Cholesteryl Ester Accumulation in Fu5AH Cells: Effect of Lipoproteins from Hyperlipemic Rabbit Serum

Serum or lipoprotein[a]	Experiment 1[b]		Experiment 2[c]	
	μg/mg cell protein[d]			
	FC	CE	FC	CE
Hyperlipemic rabbit serum	17.7	66.6	16.0	45.1
Chylomicron	17.0	61.4	15.6	41.0
VLDL	19.1	82.2	18.7	50.1
LDL	17.4	54.8	13.9	15.3
HDL	15.2	35.5	10.2	10.8
Normal rabbit serum	11.6	4.6	16.7	7.8

[a]Serum and lipoproteins in medium to yield 300 μg/ml total cholesterol. VLDL = very low density lipoproteins, LDL = low density lipoproteins, HDL = high density lipoproteins.

[b]Experiment 1 serum from rabbit on cholesterol diet 4 months, 1705 mg % total cholesterol.

[c]Experiment 2 serum from rabbit on diet 3 weeks, 1051 mg % total cholesterol.

[d]Average of two replicate cultures. FC = free cholesterol and CE = cholesteryl ester.

(13%) was observed during the following 24 hr incubation period. However, no additional CE were recovered in the medium. Although the amount of CE/culture (cells plus medium) remained constant, the free cholesterol increased substantially (254%) with most of the increase attributed to the appearance of FC in the incubation medium. This increase in FC presumably results, in part, from the initiation of de novo cholesterol synthesis together with the efflux of FC which occurs in the presence of DLP plus lecithin. This explanation is consistent with the results of previous investigations with L-cell mouse fibroblasts grown in medium containing DLP (8,14,19).

TABLE VIII

Balance Study: Recovery of Serum Cholesterol in Fu5AH Hepatoma Cells

	μg Cholesterol/culture[a]		
	Free	Ester	Total
Medium[b]			
Control medium[c]	203	537	740
Medium from cells	91	266	357
Loss from medium	112	271	383
Cells			
0 time[d]	37	23	60
24 hr incubation[e]	92	355	447
Increase in cells	55	332	387
$\frac{\text{Medium loss}}{\text{Cell gain}} \times 100$	204%	82%	99%

[a]All values average of four replicate cultures.

[b]3 ml Medium/T25 flask containing heated (60 C/30 min) hyperlipemic rabbit serum.

[c]Medium incubated 24 hr in absence of cells.

[d]Average protein/culture 1.38 mg.

[e]Average protein/culture 4.25 mg.

DISCUSSION

The accumulation of lipids within tissue culture cells has been reported by a number of investigators. A variety of cells has been shown to form lipid vacuoles in response to manipulations of the growth media (20-26). Although no consistent pattern of cell types is evident from these studies, the results can best be divided into two general phenomena. The first is an accumulation of triglycerides induced by the presence of free fatty acids in the culture medium (23,24), while the second is primarily an increase in cholesteryl esters observed when cells are grown in media supplemented with hyperlipemic sera (3,4) or β-lipoprotein (27). Such cholesteryl ester accumulation has now been observed in primary aortic cells (4); MAF cells derived from human embryonic skin and muscle (3); a continuous line of mouse fibroblasts (27); and, as demonstrated in this article, rat hepatoma cells (Fu5AH). In addition, human skin fibroblasts obtained from an individual with cholesteryl ester storage disease have been reported to contain more CE than normal cells even in the absence of hyperlipemic serum (28).

When exposed to HRS, all of the cells shown in Figure 1 demonstrated an increase in both free and esterified sterol. FC increased by ca. 40% as compared to normal rabbit serum controls, while CE increased 4- to 20-fold. However, even with this rise in CE, the CE/FC ratio did not exceed 1.0 in L-cells, HF-cells, and W-18VA$_2$-cells, while the CE/FC ratio in Fu5AH cells was greater than 3. Similar high CE/FC ratios have been reported for MAF fibroblasts grown in HRS (3), and in NCTC 2445 mouse fibroblasts exposed to human or bovine LDL (27). Thus, all cells in culture may be capable of accumulating CE above the level observed when the cell is grown in normal serum; however, the accumulation of CE in concentrations substantially greater than the FC level may be a more specific phenomenon.

The response of Fu5AH cells is not directly related to the concentration of FC or CE in the culture medium but is a function of the degree of hypercholesteremia (Fig. 5). The results obtained using isolated lipoproteins (Table VII) indicate that the activity of HRS is primarily associated with HRS lipoproteins having a density <1.006 g/ml. Marked increases in the concentration of these lipoproteins have been observed in cholesterol-fed rabbits together with a change in composition resulting in an increase in the percentage of CE and a decrease in triglycerides (29). It is probable, therefore, that it is these modified lipoproteins which are responsible for the cellular response.

Vacuoles from cells which have accumulated lipid in response to exogenous fatty acids have been shown to be composed primarily of triglycerides (30). In the present investigation, the vacuoles isolated from the hepatoma cells contained high percentages of both CE and triglycerides and low levels of FC and phospholipids. More detailed investigations are required to determine if the vacuoles isolated from these cells are a homogenous population containing both triglycerides and CE or are of two types, one enriched in CE and a second containing triglycerides. The fractionation studies indicate, however, that not all of the cellular CE is stored in lipid vacuoles, since substantial percentages of the CE were recovered in the cellular

TABLE IX

Sterol Content of Fu5AH Cells upon Removal of Hyperlipemic Rabbit Serum

	μg Cholesterol/culture[a]									μg/mg Cell protein		
	Cells			Medium			Total (cells + medium)			Cells		
Time[b]	FC	CE	TC	FC	CE	TC	FC	CE	TC	FC	CE	TC
0 time	44.6	152.2	196.8	0	0	0	44.6	152.2	196.8	24.1	82.3	106.4
24 hr	45.1	135.4	180.5	44.7	25.2	69.9	89.8	160.6	250.4	18.6	55.8	74.4
48 hr	48.2	118.3	166.5	65.0	23.8	88.8	113.2	142.1	255.3	19.9	48.8	68.7

[a]Average of two replicate cultures. FC = free cholesterol, CE = cholesterol ester, and TC=total cholesterol.

[b]Time incubated in delipidized calf serum protein medium (5 mg/ml delipidized calf serum protein + 100 μg/ml lecithin). Cells incubated in 5% hyperlipemic rabbit serum 24 hr prior to 0 time.

fraction which pelleted at 100,000 x g. In addition, the results presented in Table IX demonstrate that once present, the cellular CE undergoes relatively little hydrolysis or excretion upon replacement of HRS with medium containing DLP.

The results from the balance study shown in Table VIII indicated that much of the cholesterol in cellular CE could be derived from the CE in the growth medium. These studies show that the amount of CE lost from the growth medium was ca. 80% of the amount of CE recovered in the cells. Intact CE can be incorporated by the liver (31); however, data obtained in the present study do not indicate whether the exogenous CE accumulates in the cell without modification or has undergone hydrolysis with subsequent reesterification of the cholesterol. Additional cellular CE probably is derived through the esterification of exogenous free cholesterol incorporated by the cells. It has been demonstrated that exposure of MAF skin fibroblasts to HRS resulted in the stimulation of both fatty acid synthesis and the incorporation of these fatty acids into CE and triglycerides (3). The relationship between CE accumulation and fatty acid synthesis in hepatoma cells will be investigated in future studies.

If the CE is incorporated from the growth medium, the experiment utilizing heated cells (Table IV) suggests that the mechanism for CE influx is different from that of FC uptake. Previous studies with heat-treated tissue culture cells (32) and boiled aorta (33) have demonstrated continued exchange of free sterol, whereas such drastic treatment eliminated CE accumulation in Fu5AH cells. The uptake of exogenous CE could occur through the cellular incorporation of an entire lipoprotein or by the incorporation of CE from serum lipoproteins enriched with CE in the diet-induced hyperlipemic animal.

Many of the questions left unanswered by this investigation can be studied in more detailed experiments. The present study does demonstrate that Fu5AH hepatoma cells provide a readily available and easily manipulated experimental system for the study of cellular cholesteryl ester metabolism.

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Effects of Age, Sex, and Diet upon Carcass and Liver Fatty Acid Composition of Pitman-Moore Miniature Pigs

L.J. FILER, JR., SAMUEL J. FOMON, THOMAS A. ANDERSON, THOMAS L. NIXT, and DEAN W. ANDERSEN, Department of Pediatrics, University of Iowa, Iowa City, Iowa 52242

ABSTRACT

Fatty acid composition of carcass and liver and proximate analysis of liver were studied in 14-28 day old Pitman-Moore miniature pigs, 26 sow-reared and 30 fed a semisynthetic diet in which the fat was lard. With increasing age, fat of carcass, but not of liver, became significantly more unsaturated. The percentage of palmitic acid (16:0) and total saturated fatty acids was significantly greater and the percentage of linoleic acid (18:2) and total unsaturated fatty acids significantly less in carcasses of male than of female pigs. No sex-related differences in proximate or fatty acid composition of the liver were noted. Carcasses of sow-reared pigs contained significantly greater percentages of myristic (14:0), palmitoleic (16:1), and linoleic acids and significantly lesser percentages of stearic (18:0) and oleic (18:1) acids than did those of pigs fed the semisynthetic diet. Diet-related differences in fatty acid composition of liver closely paralleled those of carcass, although liver contained markedly greater percentages of stearic and arachidonic (20:4) acids and lesser percentages of palmitoleic and oleic acids than did carcass. Diet-related differences in fatty acid composition of carcass and liver are discussed in relation to the fatty acid composition of dietary fat (sow milk and lard).

INTRODUCTION

Wt gain by the human infant in the first 4 months of life is accounted for primarily by increase in mass of adipose tissue (1). Fatty acid composition of lipid stored in this compartment is diet- and, probably, sex-related. The limited fat stores of the pig at birth and its rapid rate of growth and accumulation of fat postnatally make it an ideal animal in which to study the influence of age, sex, and diet upon fatty acid composition of carcass and liver. Studies of the relation of age, sex, diet, and fatty acid composition of depot fat of the infant pig may provide insight into similar relationships in the human infant, a consideration of probable importance in view of current infant feeding practices. Fat content of commercially prepared formulas frequently differs remarkably from that of human milk. In addition, unconventional fats (e.g., medium chain triglycerides) may be fed.

We have summarized data on the influence of age, sex, and diet upon growth, serum biochemical values, and carcass composition of Pitman-Moore miniature pigs (2,3). In an earlier study, Baker, et al., (4) examined the effect of dietary fat source upon composition of intrascapular adipose tissue of Pitman-Moore pigs 2-8 weeks of age. This article compares fatty acid composition of carcass and liver of sow-reared Pitman-Moore pigs with corresponding data concerning Pitman-Moore pigs fed a semisynthetic diet in which the fat was lard.

MATERIALS AND METHODS

Management of the pigs, composition of the semisynthetic diet, and preparation of the carcass for analysis have been described previously (2,3). Chemical analysis of liver was performed in a manner identical to that used for carcass (2). Extracts of lipid obtained in the course of chemical analysis of carcass and liver were methylated with boron trifluoride and methanol, according to the method of Metcalfe and Schmitz (5). Gas chromatographic (GLC) separation of the methyl esters was carried out with an apparatus (Beckman model GC-5, Beckman Instrument Co., Fullerton, Calif.) equipped with a hydrogen flame detector and a 183 x 0.32 cm stainless steel column packed with 15% EGSS-X on Chromosorb-W, 100-200 mesh (Supelco, Inc., Supelco Park, Bellefonte, Pa.) Helium flow rate was 20 ml/min, column temperature 180 C, and the percentage distribution of fatty acids was calculated using the products of retention times and peak heights, as described by Hill (6). Methyl ester peaks were identified by comparison with standards of fatty acid methyl esters and with lipid from beef testis, as described by Holman and Hof-

TABLE I

Effect of Age, Sex, and Diet upon Carcass Fatty Acid Composition of *Pitman-Moore Miniature* Pigs

	Age (days)				Sex			Diet			
	14	21	28	Level of probability[a]	M	F	Level of probability	Sow milk	Semisynthetic	Level of probability	Error mean square[b]
Number of animals	18	17[c]	20		28	27		25	30		
Fatty acid (%)											
14:0	1.7	1.4	1.6	N.S.[d]	1.7	1.5	N.S.	2.0	1.3	<0.001	0.36
16:0	20.7	20.3	17.4	<0.01	20.6	18.1	<0.01	20.1	18.8	N.S.	8.70
16:1	7.1	5.9	6.8	N.S.	6.6	6.6	N.S.	8.7	4.9	<0.001	2.16
18:0	5.5	5.3	5.3	N.S.	5.3	4.9	N.S.	4.0	6.5	<0.001	1.47
18:1	43.0	46.1	46.2	N.S.	45.1	45.2	N.S.	41.4	48.3	<0.001	22.37
18:2	16.0	16.5	16.9	N.S.	15.4	17.6	<0.01	19.0	14.4	<0.001	7.40
18:3-20:0	1.6	1.3	1.6	<0.05	1.5	1.5	N.S.	1.4	1.6	N.S.	0.18
20:3	0.2	0.1	0.2	<0.001	0.2	0.2	N.S.	0.1	0.2	<0.001	0.01
20:4	1.5	0.9	1.3	N.S.	1.2	1.3	N.S.	0.9	1.6	<0.01	0.62
Other	2.7	2.2	2.7	N.S.	2.4	3.1	N.S.	2.4	2.4	N.S.	1.46
Percent saturated	30.1	28.9	26.6	<0.05	29.6	27.2	<0.05	28.2	28.6	N.S.	12.38
Percent unsaturated	69.9	71.1	73.4	<0.05	70.4	72.8	<0.05	71.8	71.4	N.S.	12.38

[a]Based on F-test.

[b]$SEM = \sqrt{\frac{EMS}{N}}$, where EMS is error mean square and N is number of observations used in computing mean.

[c]Sample lost from 1 female sow-reared pig.

[d]N.S. = not significant at $P<0.05$ level.

TABLE II

Effect of Age and Diet upon Liver Composition of Pitman-Moore Miniature Pigs

	Age (days)				Diet			Error
	14	21	28	Level of probability	Sow milk	Semisynthetic	Level of probability	mean square
Number of animals	17[a]	18	20		26	29		
Liver wt								
g	97.9	136.6	194.6	<0.001	151.8	140.2	N.S.	1582.54
Percent body wt	4.55	4.49	4.26	N.S.	4.16	4.66	N.S.	1.130
Liver composition (%)								
Wet								
Water	80.5	80.2	80.0	N.S.	81.0	79.6	<0.001	1.69
Protein	14.4	14.8	15.1	N.S.	14.7	14.9	N.S.	1.34
Fat	3.7	3.7	3.7	N.S.	3.1	4.2	<0.001	0.55
Ash	1.4	1.3	1.2	<0.01	1.2	1.3	N.S.	0.01
Fat-free wet								
Water	83.7	83.3	83.0	N.S.	83.5	83.1	N.S.	1.44
Protein	14.9	15.4	15.7	N.S.	15.2	15.6	N.S.	1.42
Ash	1.4	1.3	1.3	<0.01	1.3	1.3	N.S.	0.013

[a]Sample lost from 1 male pig fed the semisynthetic diet.

stetter (7).

The data were subjected to a three way analysis of variance for age, sex, diet, and interaction effects, as outlined by Snedecor (8). Stated significant differences refer to results of the F-test.

RESULTS AND DISCUSSION

Data concerning 56 pigs between 14-28 days of age are presented in Tables I-III. Thirty of the pigs (15 males and 15 females) were fed a semisynthetic diet and 26 (13 males and 13 females) were sow-reared. Data on fatty acid composition of carcass are presented in Table I, chemical analysis of liver in Table II, and fatty acid composition of liver in Table III.

Carcass fatty acid composition: The percentage of palmitic acid (16:0), the predominant saturated fatty acid in carcass (Table I), decreased significantly between 14-28 days of age both in sow-reared pigs and in those fed the semisynthetic diet. Carcass fat of male pigs included a greater percentage of palmitic acid than did that of female pigs. The percentage of palmitic acid in carcass fat was slightly greater for sow-reared pigs than for those fed the semisynthetic diet. On the other hand, the percentage of stearic acid (18:0), the only other saturated fatty acid present in carcass in relatively large amounts, was significantly greater in carcass fat of pigs fed the semisynthetic diet.

Of the unsaturated fatty acids in carcass fat at all ages, oleic acid (18:1) was present in largest amount followed by linoleic acid (18:2) and palmitoleic acid (16:1); together, these unsaturated fatty acids comprised 66-70% of total carcass fatty acids. Carcass fat of sow-reared pigs contained significantly more palmitoleic and linoleic acids and significantly less oleic acid than did carcass fat of pigs fed the semisynthetic diet. The concentration of linoleic acid was significantly less in carcass fat of male than of female pigs.

The percentage of unsaturated fatty acids in carcass fat increased significantly with increasing age, both in sow-reared pigs and in those fed the semisynthetic diet. The concentration of saturated fatty acids was significantly greater in carcass fat of male than of female pigs.

The sex-related differences in fatty acid composition of carcass fat may, therefore, be summarized as follows: males demonstrated significantly greater percentages of palmitic acid and of total saturated fatty acids and significantly lesser percentages of linoleic and total unsaturated fatty acids (Table I). Similar sex-related differences have been reported by Koch, et al., (9) with respect to backfat of 4-5 month old Hampshire x Yorkshire barrows and gilts. These investigators also found a significantly greater percentage of stearic acid in fat of the castrate males than in that of the females.

Pudelkewicz, et al., (10) reported that tissue lipids of female rats contain 1.3-1.6 times more double bonds than do those of male rats. A sex-related difference in susceptibility of the rat to essential fatty acid deficiency has been described by Aaes-Jorgensen (11) and Aftergood and Alfin-Slater (12). The latter investigators reported that fatty acids are more saturated in plasma and liver of male than of female

TABLE III

Effect of Age and Diet upon Liver Fatty Acid Composition of Pitman-Moore Miniature Pigs

	Age (days)				Diet			
	14	21	28	Level of probability	Sow milk	Semisynthetic	Level of probability	Error mean square
Number of animals	15[a]	16[b]	20		24	27		
Fatty acid (%)								
14:0	0.4	0.5	0.4	N.S.	0.5	0.4	N.S.	0.05
16:0	16.0	17.0	15.5	<0.05	17.5	14.8	<0.001	2.71
16:1	2.2	1.8	2.1	N.S.	2.6	1.5	<0.001	0.33
18:0	20.0	20.8	21.1	N.S.	19.6	21.6	<0.01	5.49
18:1	18.5	18.4	15.6	<0.01	13.9	20.4	<0.001	7.94
18:2	13.8	14.3	14.8	N.S.	17.0	12.0	<0.001	1.90
18:3-20:0	0.4	0.3	0.4	N.S.	0.4	0.4	N.S.	0.04
20:3	0.8	0.7	0.8	N.S.	0.8	0.8	N.S.	0.08
20:4	18.3	18.3	19.9	N.S.	18.1	19.7	N.S.	8.77
Other	9.6	7.9	9.4	N.S.	9.6	8.4	N.S.	6.05
Percent saturated	37.4	39.0	38.1	N.S.	38.3	38.1	N.S.	8.67
Percent unsaturated	62.6	61.0	61.9	N.S.	61.7	61.9	N.S.	8.67

[a]Samples lost from 2 male pigs fed the semisynthetic diet and 1 sow-reared male pig.

[b]Samples lost from 1 sow-reared male pig and 1 female pig fed the semisynthetic diet.

rats. Liver phospholipid concentrations are more reduced and essential fatty acid deficiency symptoms mroe severe among male animals.

Comparison of data of Baker, et al., (4) with the present data suggests that the fatty acid composition of intrascapular adipose tissue of Pitman-Moore miniature pigs is similar to that of whole carcass. Fat of intrascapular adipose tissue from sow-reared miniature pigs from 14-28 days of age contained a slightly greater percentage of palmitic acid (25.2%) and oleic acid (43.8%) and a lesser percentage of linoleic acid (14.0%) than did fat of whole carcass in the present study (Table I). Fat of intrascapular adipose tissue of sow-reared pigs was somewhat less unsaturated (65.8%) than was fat of whole carcass.

Sink, et al., (13) analyzed 7 adipose tissue depots (perirenal fat, and 2 samples each, inside and outside, from shoulder, loin, and rump) from carcasses of 3 groups of Yorkshire x Poland China and Hampshire x Poland China pigs averaging 34, 59, and 86 kg. No data on age of these pigs were presented, but one could speculate from body wts that the groups of pigs were ca. 3, 4, and 5 months of age, respectively. Mean fatty acid content of the 7 depot sites in the first group of pigs was (in %, with range in parentheses): palmitic acid, 22.2 (20.9-26.0); stearic acid, 9.8 (8.4-11.4); oleic acid, 44.0 (42.1-45.6); and linoleic acid, 14.5 (12.1-15.2). Percentages of palmitic and stearic acids were greater and those of oleic and linoleic acids were less in perirenal fat than in fat from any of the other sampling sites. These investigators found that adipose tissue in the 7 sampling sites became more saturated with increasing age (group 1, 34.9%; group 2, 35.9%; group 3, 37.3%). Unfortunately, the diet of these animals was not specified.

Sweeney, et al., (14) reported that fatty acid distributions of subcutaneous fat of human infants by age 6-8 weeks bears considerable resemblance to the dietary fats being fed. The percentage of linoleic acid was markedly higher and that of palmitic acid markedly lower in subcutaneous fat of infants fed formulas containing vegetable oils than that found in infants fed a formula containing butterfat. Bagdade and Hirsch (15) have reported that the degree of unsaturation in fat of fetal abdominal and buccal adipose tissue decreases throughout gestation. After birth, abdominal and buccal adipose tissue became more unsaturated with increasing age; however, the diets of these infants were not described.

Fatty acid composition of carcass of pigs in the present study seemed to reflect to some extent the composition of the diet. Lard, the fat of the semisynthetic diet, provides lesser percentages of palmitoleic and linoleic acids and greater percentages of stearic and oleic acids (16) than does the fat of milk from sows fed standard diets (17,18). Similarly, carcasses of pigs fed the semisynthetic diet contained lesser percentages of palmitoleic and linoleic acids and greater percentages of stearic and oleic acids than did those of sow-reared pigs. Hill (6) determined the fatty acid composition of adipose tissue obtained from 8 week old Hormel miniature pigs fed a fortified milk diet

for 4 weeks and a semipurified diet containing 2% corn oil for 4 weeks. Carcass fat of Pitman-Moore miniature pigs fed the semisynthetic diet was more saturated than were the samples of Hormel pig adipose tissue (6) or of lard (16).

Proximate composition of liver: With the exception of a decrease in percentage of ash, the proximate composition of liver did not change significantly with increasing age (Table II). We have reported previously (3) that, between 14-28 days of age, the percentage of whole carcass accounted for by water and ash decreases, whereas the percentage accounted for by fat increases. It would appear that composition of liver, unlike that of carcass, does not change significantly with age. Percentage of water was significantly less and percentage of fat significantly greater in livers of pigs fed the semisynthetic diet than in livers of sow-reared pigs.

Liver fatty acid composition: Fatty acid composition of liver (Table III) differed markedly from that of carcass (Table I). Stearic, rather than palmitic acid was the predominant saturated fatty acid in liver, the combined wts of stearic and palmitic acid accounting for 36-38% of total fatty acids. Oleic acid comprised a much lesser percentage of fatty acids in liver than in whole carcass. Arachidonic acid (20:4) accounted for more than 18% of fatty acids of liver compared with less than 2% of fatty acids of carcass. Fatty acids of liver were somewhat less unsaturated than were those of carcass. In contrast to the findings in carcass, the degree of unsaturation in liver did not increase with increasing age.

Diet-related differences in fatty acid composition of liver were similar to those described for carcass. In pigs fed the semisynthetic diet, percentage of fatty acids accounted for by palmitic, palmitoleic, and linoleic acids was significantly less and that of stearic and oleic acids significantly greater than corresponding values for sow-reared pigs.

Data presented here on fatty acid composition of livers of Pitman-Moore miniature pigs in the present study are quite similar to those reported by Hill (6) for Hormel miniature pigs.

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Fatty Acids and Phospholipids of Adult and Newborn Rat Hearts and of Cultured, Beating Neonatal Rat Myocardial Cells

C.G. ROGERS, Research Laboratories, Health Protection Branch, Department of National Health and Welfare, Ottawa, KIA 0L2, Canada

ABSTRACT

Fatty acids and phospholipids of adult and newborn rat hearts and of cultured, neonatal rat heart cells were determined by gas liquid and thin layer chromatographies. In adult heart, the proportion of linoleic acid was higher and that of palmitic acid lower than in newborn hearts or in cultured cells. The relative amounts of linoleic and arachidonic acids in adult heart were affected by the source and amount of dietary fat. In heart cells, after 3 days in culture, the proportion of arachidonic acid resembled that in the newborn and adult rat hearts but showed a gradual and significant decline with age. The gradual shift in fatty acid composition as the cells aged in culture was attributed to outgrowth of mesenchymal cells (fibroblasts and endothelioid cells) characterized by a low relative proportion of arachidonic acid. The amounts of phospholipids in heart cells after 3 days in culture differed from those in the newborn or adult rat hearts. Phosphatidylethanolamine was highest in adult heart (34% of lipid phosphorus) and lowest in cells (26%); lecithin was higher in newborn heart (43%) than in adult heart (37%) or in cells (39%), while sphingomyelin was higher in cells (8%) than in newborn (5%) or adult heart (3%). Phospholipid levels in cultured heart cells were unrelated to those of serum in the growth medium. The absence of a significant change in phospholipid composition after continued incubation of the heart cell cultures for periods up to 3 weeks reflected the major structural role of these lipid components in cell membranes.

INTRODUCTION

Primary cultures of beating, neonatal rat heart cells were first used successfully by Harary, et al., (1-3) as a model system for the study of fatty acid metabolism and its relationship to cell function within the myocardium. In studies related to the effect of fatty acids upon beating and mitochondrial phosphorylation, Gerschenson, et al., (4) described the fatty acid composition of rat heart cells after 2 and 4 days in culture. Employing similar cultural techniques, Szuhaj (5) compared the fatty acid composition of the neutral and polar lipid fractions of cultured rat heart cells to that of the intact, newborn rat heart and described the fatty acid composition of hearts from rats of different ages (6) but obtained low values for arachidonic acid and did not detect C_{22}-polyunsaturates.

The purpose of the present study was to examine more closely the total fatty acid and phospholipid composition of neonatal rat heart cells in culture and to compare the results to values for intact cardiac tissue from adult and newborn rats.

MATERIALS AND METHODS

Animals and diets: Adult hearts for lipid analyses were obtained from 2 groups of male, Wistar rats, 10-12 weeks of age (Bio-Breeding Laboratories, Ottawa, Canada). One group of rats was fed for 2 weeks a purified diet containing, by wt, 20% casein, 20% sucrose, 30% cornstarch, 1% vitamin mixture, 4% salt mixture U.S. Pharmacopeia XIV, 5% alphacel, and 20% of a 3:1 mixture of lard and corn oil; the other group of rats was given laboratory chow (3.5% fat), Ralston-Purina Co., Toronto, Canada.

Medium: The neonatal rat heart cells were grown in Eagle's minimum essential medium (MEM) supplemented with 20% calf serum; L-glutamine, 2mM; penicillin, 100 units/ml; streptomycin, 100 μg/ml; and amphotericin B, 2.5 μg/ml. The MEM medium and calf serum (no. 14-401) were obtained from Microbiological Associates, Bethesda, Md. The L-glutamine and antibiotics were from Grand Island Biological Co.

Preparation of heart cells: From 24-36 newborn male and female rats of the Wistar strain, age 3-5 days (Bio-Breeding Laboratories, Ottawa, Canada) were used in each experiment. The pups were obtained from dams fed a standard diet of laboratory chow. The hearts were excised aseptically, placed immediately in

TABLE I

Comparison of Fatty Acid Composition of Adult and Newborn Rat Hearts with That of Heart Cells in Culture and of Calf Serum

	Relative proportions (wt percentage) of major fatty acids						
	Adult heart		Newborn heart	Heart cells in culture[a] days			
Fatty acid	Purified diet	Chow diet		3	7	21	Calf serum[f]
	(4)	(6)	(4)	(7)	(10)	(3)	(3)
16:0[b]	11.2 ± 0.4[c,d]	14.6 ± 0.3[d]	18.3 ± 1.6	19.8 ± 0.6	19.6 ± 0.6	20.4 ± 1.2	17.4 ± 1.5
18:0	21.1 ± 0.5[e]	19.8 ± 0.3	18.8 ± 0.7	22.2 ± 0.8[e]	21.8 ± 0.5[d]	23.4 ± 0.4[d]	13.8 ± 0.8[d]
18:1	12.4 ± 0.9	9.1 ± 0.2[d]	12.7 ± 0.3	13.0 ± 0.3	16.0 ± 0.5[d]	17.1 ± 0.6[d]	21.2 ± 1.1[d]
18:2	18.2 ± 0.7[d]	29.2 ± 0.6[d]	7.4 ± 0.1	6.4 ± 0.2[d]	10.4 ± 0.3[d]	12.8 ± 0.6[d]	27.6 ± 0.5[d]
20:4	23.9 ± 0.8	13.9 ± 0.3[d]	24.7 ± 0.5	23.5 ± 0.6	19.7 ± 0.8[d]	17.9 ± 0.1[d]	4.7 ± 0.3[d]
22:5	2.0 ± 0.4	1.4 ± 0.2[e]	2.1 ± 0.2	2.6 ± 0.2	2.0 ± 0.2	1.8 ± 0.4	1.8 ± 0.4
22:6	5.0 ± 0.7[d]	10.1 ± 0.3[e]	8.9 ± 0.5	6.2 ± 0.8	3.8 ± 0.3[d]	2.2 ± 0.3[d]	1.9 ± 0.2[d]

[a]Samples contained minor to trace amounts of 16:1, 17:0, 20:2, 20:3, and 22:4.

[b]Number of carbon-atoms; number of double bonds.

[c]Mean ± standard error of the mean. Figures in parentheses denote number of separate experiments analyzed.

[d]Signficantly different from the newborn heart value at $P<0.01$.

[e]Significantly different from the newborn heart value at $P<0.05$.

[f]Microbiological Associates, Bethesda, Md., Catalog no. 14-401.

TABLE II

Arachidonate to Linoleate Ratios in Intact Hearts, Heart Cells in Culture, and in Calf Serum

Source	Fatty acids, wt % values	
	Arachidonate/linoleate	Ratio
Adult heart (purified diet)	23.9/18.2	1.3
Adult heart (chow diet)	13.9/29.2	0.5
Newborn heart (3-5 days)	24.7/ 7.4	3.3
Heart cells (3 days)	23.5/ 6.4	3.7
Heart cells (7 days)	19.7/10.4	1.9
Heart cells (21 days)	17.9/12.8	1.4
Calf serum	4.7/27.6	0.2

cold Tyrode's salt solution (pH 7.4, free of Ca^{++} and Mg^{++} ions) and rinsed once in the saline, after which the hearts were trimmed of blood vessels and auricular tissue. The ventricles were minced with fine scissors, rinsed twice in the saline, and transferred to a 50 ml Erlenmeyer flask equipped with a magnetic stirrer for digestion with 15 ml of trypsin solution (Schwarz-Mann, Orangeburg, N.Y., 1:300, at 0.150% in Tyrode's saline + 0.020% ethylenediamine tetraacetic acid pH 7.4). The enzyme solution was presterilized by filtration under N_2 through a Gelman pressure-filtration unit fitted with a Nuclepore membrane filter (Nuclepore Co., Pleasanton, Calif., 0.45 μ, 47 mm diameter). A modification of the digestion procedure of Wenzel, et al., (7) was used in which the enzyme solution was discarded after each of two 15 min digestion cycles, and the cells were retained in MEM medium on ice following each of 6 additional 15 min cycles of enzyme digestion. The cells were collected by centrifugation (1200 x g, 5 min), resuspended in 3 ml of cold, fresh MEM medium, filtered to remove clumps (Nitex, 80 mesh cloth), pooled, and diluted to a cell concentration equivalent to 1.1-1.3 x 10^6 cells/ml. Initially, the cells were plated in 10 ml aliquots in 100 mm culture dishes (Falcon plastics, Beckton and Dickinson, Clarkson, Canada) and incubated at 37 C (95% air and 5% CO_2), according to the procedure of Blondel, et al., (8). After 3 hr of incubation, the cells remaining in suspension were replated in 60 mm culture dishes (Falcon plastics) at an inoculum density of ca. 4 x 10^6 cells/plate. After incubation for 24 hr (95% air and 5% CO_2) at 37 C, the plates were rinsed 3 times with sterile, Tyrode's saline to remove dead cells and debris, then given 4 ml fresh growth medium and returned to the incubator. Each plate was given a complete medium change every 48 hr.

For experiments in which pure cultures of mesenchymal cells (fibroblasts and endothelioid cells) were required, the large culture dishes (Falcon Plastics, 100 mm diamter), used in the selection procedure for myoblasts, were incubated 1 hr at 37 C. Then, the supernatant fluid (containing myoblasts) was poured off, and the cultures rinsed once in Tyrode's saline and returned to the incubator with 10 ml fresh MEM medium. The pure cultures of mesenchymal cells obtained by this procedure were given a complete change of medium every 48 hr.

Extraction of lipid: The hearts of adult and newborn rats were extracted of lipid by the Hanson and Olley (9) modification of the method of Bligh and Dyer (10). The adult hearts were weighed and extracted individually, while from 10-12 newborn hearts were weighed, pooled, and then extracted of lipid.

Heart cells were prepared for lipid analyses after 3, 7, and 21 days in culture. These time intervals were chosen to represent a newly formed monolayer of cells after 3 days, an established and functional culture after 7 days, and an aging culture undergoing dedifferentiation at 21 days. The monolayer of cells in each culture dish was rinsed three times with sterile Tyrode's saline and gently scraped from the floor of the dish with a rubber policeman. The cells were collected by centrifugation (3000 x g, RC2B, 15 min), quantitatively transferred in distilled water to a 50 ml glass tube, and extracted of lipid by the method of Bligh and Dyer (10). The lipid extract in $CHCl_3$ was concentrated to a volume of 5 ml and stored under N_2 in a deep freeze until used.

Fatty acid and phospholipid analysis: The fatty acid composition of the lipid extracts was analyzed by gas liquid chromatography (GLC) (11). The data obtained were expressed as relative percentages of fatty acids. The phospholipid composition of the lipid extracts was determined by thin layer chromatography (TLC) on Silica Gel H employing the conditions described previously (12). Phosphorus in lipid extracts and in phospholipids from TLC chro-

TABLE III

Phospholipid Composition of Adult and Newborn Rat Hearts, Heart Cells in Culture, and of Calf Serum

	Phospholipid phosphorus (% of phosphorus recovered)					
			Heart cells in culture days			
Spot[a]	Adult heart[b]	Newborn heart	3	7	21	Calf serum[c]
	(6)	(4)	(3)	(3)	(4)	(3)
Front	12.9 ± 0.1[d,e]	7.1 ± 0.6	6.5 ± 0.2	6.5 ± 0.1	5.4 ± 0.3[f]	1.0 ± 0.1[e]
DPG+PA	2.5 ± 0.2	2.9 ± 0.2	5.0 ± 0.3[e]	4.7 ± 0.3[e]	4.2 ± 0.3[e]	1.5 ± 0.1[e]
PE	33.7 ± 0.4[e]	31.1 ± 0.7	25.8 ± 0.9[e]	25.3 ± 0.7[e]	25.2 ± 0.7[e]	2.0 ± 0.1[e]
PS+PI	7.5 ± 0.2	8.4 ± 1.1	10.9 ± 0.4	11.9 ± 0.3[e]	13.8 ± 0.4[e]	3.3 ± 0.2[e]
PC	37.5 ± 0.3[e]	42.5 ± 0.9	40.1 ± 0.4	38.5 ± 0.3[e]	38.6 ± 0.4[e]	59.8 ± 0.5[e]
SPH	3.3 ± 0.1[e]	5.1 ± 0.2	7.6 ± 0.7[e]	8.3 ± 0.1[e]	9.6 ± 0.4[e]	18.4 ± 0.8[e]
LPC	1.5 ± 0.2	2.0 ± 0.2	3.3 ± 0.4[f]	3.2 ± 0.1[e]	2.9 ± 0.1[e]	12.7 ± 0.8[e]
Origin	1.1 ± 0.2	1.0 ± 0.2	0.9 ± 0.1	1.9 ± 0.6	1.1 ± 0.1	1.5 ± 0.1[e]
Ratio PC/PE[g]	1.11	1.37	1.55	1.52	1.53	29.90

[a]SPH = sphingomyelin, LPC = lysophosphatidylcholine, PC = phosphatidylcholine, PS = phosphatidylserine, PI = phosphatidylinositol, PE = phosphatidylethanolamine, DPG = diphosphatidylglycerol, and PA = phosphatidic acid.

[b]Chow diet.

[c]Microbiological Associates, Bethesda, Maryland, Catalog No. 14-401.

[d]Mean ± standard error of the mean. Figures in parentheses denote number of separate experiments analyzed.

[e]Significantly different from the newborn heart value at $P<0.01$.

[f]Significantly different from the newborn heart value at $P<0.05$.

[g]Ratio of phosphatidylcholine phosphorus to phosphatidylethanolamine phosphorus.

matograms was measured by the method of Bartlett (13), as modified by Parker and Peterson (14).

Protein analysis: Protein in cell cultures was determined by the method of Lowry, et al., (15) as modified by Oyama and Eagle (16).

RESULTS

Total Cell Protein

An assessment of the rate of growth of the rat myocardial cells in culture was made by measurement of total cell protein. When expressed in mg/culture plate, protein concentration increased throughout the 21 day incubation period (Fig. 1). The similarity between the two curves, each of which represents a separate preparation of primary hearts cells, is indicative of the reproducibility of the culture procedure.

Fatty Acid Composition

The relative percentages of major fatty acids of adult heart, newborn rat heart, heart cells in culture, and of calf serum were compared, as shown in Table I. The wt percentage values for major fatty acids in adult rat heart were influenced by diet, and this was most evident in the values for linoleic and arachidonic acids. Adult heart, regardless of diet, was characterized by higher relative values for linoleic acid than in the newborn heart or cells in culture.

In heart cells, after 3 days in culture, the relative proportions of fatty acids resembled closely that of the newborn rat heart from which the cells were derived. However, as the cells aged in culture, the pattern of fatty acids showed a gradual but significant shift. This was evident in higher relative values for oleic and linoleic acids and by lower values for arachidonic and docosahexaenoic acids. At no time, did the wt percentage values for heart cells in culture reflect those of calf serum in which the cells were grown.

A comparison of the ratios of arachidonic acid to linoleic acid (Table II) further emphasized the effect of diet upon fatty acid composition. The ratio was 1.3 with adult rats fed a purified diet (20% fat) but dropped to 0.5 when the diet was chow (3.5% fat). In the newborn heart, in contrast to the adult, a three- to fourfold decrease in the percentage of linoleate produced a ratio of 3.3. The ratio for heart cells resembled this value after 3 days but showed a decline after 7 and 21 days in culture. The very low ratio for calf serum was further evidence that the fatty acid composition of the cells in culture did not reflect that of serum in the growth medium.

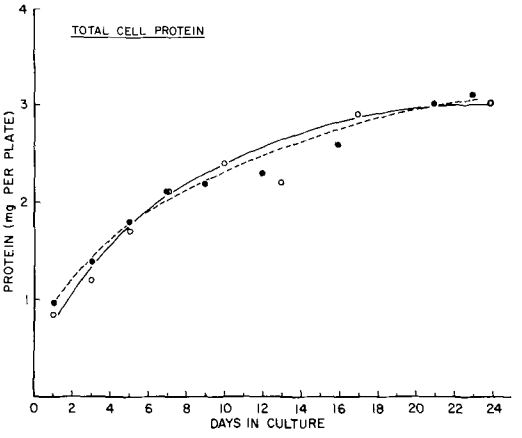

FIG. 1. Growth of primary heart cell cultures. Relationship between total cell protein and incubation time. Open and closed circles represent separate preparations of primary heart cells.

TABLE IV

Major Fatty Acids of Mesenchymal Cells after 3,7, and 21 Days in Culture[a]

	Relative proportions (wt %)		
	Heart cells in culture[b]		
Fatty acid	3 days	7 days	21 days
	(3)	(3)	(1)
16:0[c]	23.5 ± 1.4[d]	17.1 ± 0.7	19.9
18:0	25.2 ± 1.4	21.9 ± 0.9	26.1
18:1	21.3 ± 0.3	19.3 ± 0.2	20.0
18:2	8.4 ± 0.4	10.5 ± 0.2	10.0
20:4	9.5 ± 1.2	13.4 ± 0.7	10.0
22:5	2.4 ± 0.1	4.0 ± 0.5	1.9
22:6	0.4 ± 0.1	0.5 ± 0.1	0.3

[a]Figures in brackets denote number of individual determinations.

[b]Contained minor to trace amounts of 16:1, 17:0, 20:2, 20:3, 22:3, and 22:4.

[c]Number of carbon-atoms:number of double bonds.

[d]Standard error of the mean.

Phospholipid Composition

The phospholipid composition of adult heart, newborn heart, cells in culture, and of calf serum is compared in Table III. The phospholipids detected by TLC were lysophosphatidylcholine, sphingomyelin, lecithin, phosphatidylserine + phosphatidylinositol, phosphatidylethanolamine, and diphosphatidylglycerol + phosphatidicic acid. From 60-70% of the total lipid phosphorus in each sample was attributed to the major phospholipid components, phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Although there was broad, general agreement in myocardial phospholipid composition some important differences were evident.

Sphingomyelin in cultured cells showed a 1.5- to twofold increase over that in the young or adult heart; phosphatidylcholine was higher in young heart than in adult heart or in cells; while phosphatidylethanolamine was higher in adult heart than in newborn heart or in cultured cells. However, the amounts of phospholipids in adult and newborn myocardial tissue or in cultured cells appeared unrelated to those of serum in the growth medium. Little or no change in phospholipid levels of cultured heart cells occurred throughout the 21 day incubation period. This observation was given emphasis by the phosphatidylcholine to phosphatidylethanolamine ratios (Table III). The PC to PE ratio in calf serum was large (29.90) and appeared unrelated to that of adult or newborn rat heart or of cultured cells. However, the ratio in cells was similar to that in the young heart and remained unchanged throughout the 21 days in culture.

The relative proportions of major fatty acids of the total lipid fraction of mesenchymal cells (fibroblasts and endothelioid cells) are shown in Table IV. While minor fluctuations were seen in the wt percentage values, particularly for palmitic (16:0) and arachidonic (20:4) acids, the proportions of major fatty acids in mesenchymal cells, generally, were similar after 3,7, or 21 days in culture. The low values for arachidonic acid in mesenchymal cells (9.5-13.4%) were in contrast to the higher values for this fatty acid seen in normal, primary heart cell cultures (Table I).

DISCUSSION

Primary cultures of rat myocardial cells, after incubation for 3 days, were found to be similar in total fatty acid composition to newborn heart tissue from which the cells were derived, but showed a change in fatty acid composition with age. The principal alterations in the fatty acid pattern were related to linoleic and arachidonic acids. The former increased with age, while the latter showed a gradual but significant decline. The present findings were in good agreement with the values reported by Gerschenson, et al., (4) for 4 day old cultures of primary rat heart cells.

Dedifferentiation or loss of function (1,17,18) could explain the decline in the ratio of arachidonate to linoleate seen in primary heart cells (Table I) after 7 and 21 days in culture. One also may speculate that the cells, which started with a fatty acid composition similar to that of newborn hearts (Table I),

gradually may increase their proportion of linoleic acid (18:2) at the expense of arachidonic acid (20:4) when grown in the MEM medium which contained calf serum rich in 18:2 but poor in 20:4. Alternatively, however, this result may be attributed to the outgrowth of mesenchymal cells (fibroblasts and endothelioid cells) similar to that described by other workers (7,8,19) and also to the presence of a lower proportion of arachidonic acid in the mesenchymal cells after 3, 7, or 21 days in culture (Table IV).

Studies in tissue culture with a variety of mammalian systems showed that the fatty acid composition of the cells, in general, reflected that of the serum in which they were grown (4,20,21). In contrast to these reports, the total fatty acid composition of rat heart cells in culture at no time reflected that of the serum in the growth medium. Furthermore, Szuhaj (5) observed that the fatty acid composition of the polar lipids of cultured rat heart cells similarly did not reflect that of the growth medium. Apparently, heart cells in culture are capable of rapidly metabolizing the fatty acids taken up fom the surrounding medium.

Total cell protein, used to assess heart cell growth, increased during the first 10-12 days in culture but showed a decline with continued incubation. Wenzel, et al. (7) observed similar growth characteristics with rat heart cell cultures following myoblastic enrichment. In the absence of enrichment procedures, Orloff and McCarl (22) reported a linear increase in total protein in rat heart cell cultures over a 3 week period. Along with the present findings, these studies emphasize the sensitivity of heart cells to the experimental conditions under which they are grown.

Several workers (23-25) have suggested that the phospholipid composition of cultured mammalian cells is similar to that obtained with most animal tissues. In agreement with these earlier studies, the present findings showed a broad, general similarity in phospholipid composition within the adult heart, newborn heart, and cells in culture. However, the amounts of phospholipids in heart cells after 3 days of incubation, differed from those in the young or adult rat heart, particularly with respect to sphingomyelin, lecithin, and phosphatidylethanolamine (Table III). The absence of a further change in the amounts of each phospholipid after continued incubation of the cultures for periods up to 3 weeks reflected the major structural role of these lipid components in cell membranes (26).

Continued incubation of primary heart cell cultures resulted in a progressive increase in the number of mesenchymal cells (fibroblasts and endothelioid cells) but little or no increase in the number of myoblasts, thus confirming the findings of other workers (7,8,19). Furthermore, the amounts of phospholipids in heart cells were essentially unchanged after 3, 7, and 21 days in culture (Table III). Together, these findings suggest that the phospholipid composition of myoblasts and of mesenchymal cells is similar in cultures derived from rat myocardial tissue.

The phospholipid composition of cultured heart cells in the present study resembled that reported by Fletcher (27) for human ventricular tissue; while the values for newborn rat heart were similar to those found by Soula and Champanet (28) for adult rat myocardial tissue. In comparison to cultured heart cells, lower values for phosphatidylethanolamine and higher values for sphingomyelin were obtained by Marinetti, et al., (29) who were amoung the first to describe the phospholipid composition of rat myocardial tissue. The phospholipid values for calf serum displayed a similar pattern to that of the plasma phospholipids reported for other mammmalian species by Dawson, et al., (30).

Despite obvious differences which exist between the cell in culture and the tissue from which it is derived, it is apparent from these investigations that cultured heart cells can, potentially, contribute significantly to our understanding of lipid metabolism within the myocardium.

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Positional Specificity of Adipose Tissue Lipoprotein Lipase

P. NILSSON-EHLE, Department of Physiological Chemistry, University of Lund, Lund, Sweden; **A.S. GARFINKEL,** Research, Veterans Administrations, Wadsworth Hospital Center, Los Angeles, California 90073; and **M.C. SCHOTZ,** Department of Medicine, UCLA School of Medicine, Los Angeles, California 90024

ABSTRACT

The positional specificity of preparations of lipoprotein lipase derived from rat epididymal adipose tissue was investigated. The enzyme preparations were a crude extract of acetone powder of the whole tissue, partially purified lipoprotein lipase fractions a and b separated by gel chromatography from such an extract, and lipoprotein lipase activity eluted from adipose tissue into a medium by incubation with heparin in vitro. The enzyme preparations were incubated with triglyceride substrate labeled with ^{3}H in the glycerol moiety and with ^{14}C in the fatty acid esterified to the 2 position of the glycerol. The reaction products were separated by thin layer chromatography. All preparations preferentially hydrolyzed the 1(3) ester bonds of the tri- and diglycerides, indicating that, like lipoprotein lipase from other sources, the adipose tissue enzyme has a specificity for the 1(3) position.

INTRODUCTION

Lipoprotein lipase, present chiefly in adipose, heart, and mammary tissues, is known to play a role in the transport of triglyceride fatty acids into tissues (1). The specificity of this enzyme for hydrolysis of primary and secondary ester bonds of its triglyceride substrate has been studied by several groups. On the basis of experiments with extracts of acetone powder of adipose tissue, Korn concluded that lipoprotein lipase had no positional specificity (2). In contrast, Björntorp and Furman, using lipoprotein lipase activity eluted from adipose tissue with heparin, reported that lipoprotein lipase preferentially hydrolyzed 1(3) glyceride bonds (3). Since these studies were reported more than 10 years ago, no further work has been done to resolve these conflicting data with regard to adipose tissue. However, purified preparations of lipoprotein lipase from human postheparin plasma (4) and bovine skim milk (5) have been reported to have marked positional specificity for the 1(3) ester bonds of triglycerides. In this study, employing a doubly labeled radioactive triglyceride substrate, we have reexamined the specificity of lipoprotein lipase preparations similar to those used by Korn (2) and Bjorntorp and Furman (3). Further, we have determined the positional specificity of two additional preparations of adipose tissue lipoprotein lipase separated and partially purified by gel chromatography (6). Using a triglyceride substrate labeled with ^{3}H in the glycerol and with ^{14}C in the fatty acid esterified to the 2 position of the glycerol, we found that all adipose tissue lipoprotein lipase preparations preferentially hydrolyzed the 1(3) ester bonds of the tri- and diglyceride.

MATERIALS AND METHODS

Sources of materials were as follows: glycerol trioleate (purity>99%), the Hormel Institute, University of Minnesota, Austin, Minnesota; [2-^{3}H]glycerol trioleate (266 mCi/mmole), Amersham-Searle, Arlington Heights, Ill.; bovine albumin, fatty acid-poor, Miles Research Products, Kankakee, Ill.; Triton X-100, Rohm and Haas, Philadelphia, Pa.; and egg lecithin, Schwartz-Mann, Orangeburg, N.H. The egg lecithin was purified by a water wash of a chloroform-methanol, 2:1 (v/v), extract (7). All other chemicals used were reagent grade.

1,3-Dioleoyl-2-[1-^{14}C]oleoyl glycerol was prepared as described (8) and purified by Florisil column chromatography (>99.6% radiopurity). [1(3)-^{3}H]glycerol trioleate was prepared and purified as earlier described (4).

Enzyme Preparations

Four preparations of lipoprotein lipase were employed. All were obtained from epididymal adipose tissue of male Sprague-Dawley rats weighing 180-240 g. The animals were fed either Purina Chow ad libitum or fasted 18 hr and refed Chow and 20% glucose in the water for 3 hr. The lipoprotein lipase activity, designated heparin eluate, was eluted in vitro from fat pads into a Krebs-Ringer bicarbonate buffer medium (pH 7.4) containing 1% fatty acid-poor bovine albumin, 13 mM glucose, 0.12% cas-

amino acids, 12 mU insulin/ml and 3U heparin/ml. Also, lipoprotein lipase was extracted with 0.05 M NH_4OH-NH_4Cl buffer (pH 8.6) from acetone powders prepared from adipose tissue (6). Acetone powder extract was used either directly as a second type of enzyme preparation, designated unfractionated enzyme, or the extracts were concentrated under nitrogen pressure ca. 30-fold through a UM-10 Amicon membrane. Previous experiments showed that recovery from Amicon filtration was quantitative. The concentrates were applied to 8% agarose gel chromatographic colums to separate two lipoprotein lipase fractions, one of which appears in the void volume of a Biogel A-1.5 M column, lipoprotein lipase $(LPL)_a$, and one of which is retarded on such a gel, LPL_b. It has been shown that both of these fractions have the characteristics of lipoprotein lipase but respond differently to the addition of heparin to the assay system (9). Lipoprotein lipase fractions a and b were the third and fourth kinds of enzyme preparations tested for positional specificity. In four experiments, recovery of the LPL activity from the gel columns in the LPL_a and LPL_b fractions was 104% (range 82-138%). For the positional specificity studies, samples of lipoprotein lipase a and b activities were selected from column fractions in such a manner that each pool represented a separate lipoprotein lipase activity peak. These pools were concentrated about threefold; medium containing the lipoprotein lipase activity eluted from fat pads by heparin was concentrated 25-fold.

The lipoprotein lipase specific activity (munits/ml enzyme solution) varied from one preparation to another. In the experiments reported here, initial rates of lipolysis ranged from 5.0 (Fig. 3) to 50.0 (Fig. 4) nmoles of triglyceride hydrolyzed/min. Therefore, instead of combined data from several different experiments, data from individual representative experiments are reported.

Incubations, Separation, and Radioactive Assay

The incubations were carried out using as substrate a mixture of [1(3)-^{3}H]glyceryl trioleate and 1,3-dioleoyl-2-[1-^{14}C]oleoyl glycerol. The triglycerides (1.6 mg) were sonicated with 0.3 ml 1% bovine serum albumin, 0.3 ml 1% Triton X-100, and 2.4 ml 0.2 M Tris-HCl buffer, pH 7.5 or 8.0 (4). After sonication, the triglyceride emulsion was activated by incubating it at 37 C for 30 min with 1 ml serum obtained from dogs fasted overnight. In some experiments, egg lecithin (0.1 mg) was used as emulsifier instead of Triton X-100. This did not alter the results. The substrate mixture (1/2 ml) was employed, and the incubations started by addition of enzyme and buffer to a final volume of 1 ml. Incubation was carried out at 37 C and the reaction stopped by addition of 1 ml 0.15 M KH_2PO_4 and 6.0 ml ether-heptane-ethanol (1:1:1, v/v/v). The lipids were extracted and separated by thin layer chromatography (TLC) on Silica Gel H, impregnated with boric acid (4), with 10% acetone in chloroform as eluent. The glyceride fractions clearly were separated as visualized by iodine vapor. Radioactivity of the separated fractions (triglyceride, 1,3-diglyceride, 1,2[2,3]-diglyceride, fatty acid, 2-monoglyceride, and 1[3]-monoglyceride) and of glycerol in the aqueous phase of the lipid extract was measured (4). Recovery of ^{3}H- and ^{14}C-activity during the entire procedure was 76-84%.

To check the extent of isomerization of partial glycerides possibly taking place during the extraction and separation procedures, [^{3}H]-glycerol labeled 1,3-diglyceride, 2-monoglyceride, and 1(3)-monoglyceride were chromatographed as described above. The separated fractions of the mentioned partial glycerides were scraped and extracted with ether:heptane 1:1, concentrated and immediately rechromatographed in the same system. More than 97% of the ^{3}H-label then was recovered in the proper fractions, indicating that essentially no isomerization of 1,3-diglycerides or of monoglycerides occurred during extraction or separation on the silica gel plates impregnated with boric acid.

Calculations

Molar composition of the ^{3}H-labeled constituents (glycerides and glycerol) of the reaction mixtures has been calculated. The $^{14}C/^3H$ ratios of the individual glyceride fractions also have been calculated and related to that of the triglyceride substrate, which has been given a value of 1.0. Since the triglyceride substrate was labeled with ^{3}H in the glycerol moiety and with ^{14}C in the fatty acid at position 2, hydrolysis of the 2 ester bond yields a glyceride with a ratio <1. A 1(3)-monoglyceride with a ratio <1, thus, probably would have been formed by hydrolysis of the 2 ester bond of a 1,2(2,3)-diglyceride, whereas a 1(3)-monoglyceride with a ratio ca. 1 still retains the fatty acid originally at position 2, indicating that isomerization from position 2 to 1(3) has taken place.

RESULTS

The $^{14}C/^3H$ ratios of the di- and monoglycerides formed in incubations were consistently around 1 (0.90-1.17), indicating that all four enzyme preparations preferentially hydrolyzed the 1(3) ester bond of the triglyceride sub-

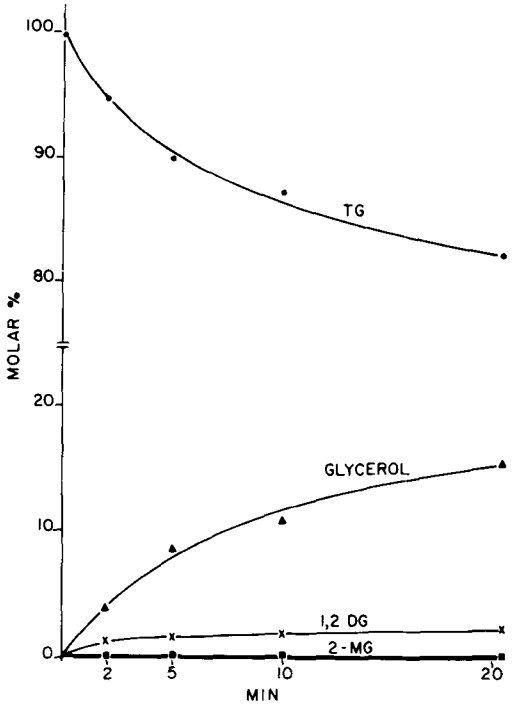


FIG. 1. Products of hydrolysis of triglyceride by an unfractionated acetone powder extract. The extract was prepared from epididymal fat pads (8.9 g tissue) of 10 rats fasted 18 hr, then fed chow and 20% glucose in the drinking water ad libitum for 3 hr. To determine positional specificity, the extract was incubated with doubly labeled triglyceride at pH 8.0 as described in the text. Results are given in molar percentage of glyceride constituents in the reaction mixture at times shown on the abscissa. TG = triglyceride; 1,2-DG = 1,1(2,3)-diglyceride; 2-MG = 2-monoglyceride. No measurable amount of 1(3)-monoglyceride was formed.

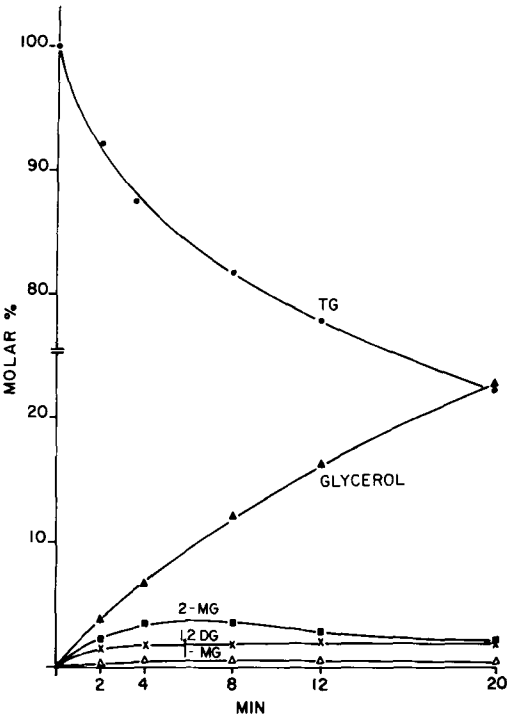


FIG. 2. Products of hydrolysis of triglyceride by lipoprotein lipase a. An aliquot (21 rats, 18.5 g tissue) of the unfractionated extract shown in Figure 1 was applied to an agarose gel column, as described in the text. Eluted fractions of the lipoprotein lipase a activity peak were pooled, concentrated, and incubated at pH 7.5 with labeled substrate to determine positional specificity. Results are given in molar percentage of glyceride constituents in the reaction mixture at times shown on the abscissa. 1-MG = 1(3)-monoglyceride; for explanation of other symbols, see Figure 1.

strate. Table I shows data obtained in a representative experiment with the heparin eluate, chosen since the degree of hydrolysis and the accumulation of intermediates were greatest with this enzyme preparation. Similar ratios were obtained in the glyceride fractions formed in incubations with the other enzyme sources. Only with the crude acetone powder extract was the amount of 2-monoglyceride formed too small to allow calculation of $^{14}C/^{3}H$ ratio. No measurable amount of 1,3-diglyceride was formed in any incubation. The ratio of the 1(3)-monoglyceride fraction, being essentially the same as that of the 2-monoglyceride, indicates that most of the 1(3)-monoglyceride must have been formed by isomerization from the 2 isomer and not by hydrolysis of the 2 ester bond of the 1,2(2,3)-diglycerides. The slightly higher ratios of the 1,2(2,3)-diglyceride fractions (1.09-1.17) can be explained by a resynthesis of diglyceride from monoglyceride with a ratio of ca. 1.0 and the partially ^{14}C-labeled free fatty acid pool. Such an acylation has been shown to occur in incubations with monoglyceride substrates (unpublished results).

Although all four lipoprotein lipase preparations used in our study initially hydrolyzed the 1(3) ester bonds of the triolein, the patterns of hydrolysis differed (Figs. 1-4). The unfractionated extracts of acetone powder hydrolyzed the triglyceride substrate to glycerol with essentially no accumulation of monoglyceride (Fig. 1). A small amount of 1,2(2,3)-diglyceride was found but no 1,3-diglyceride. The LPL_a fraction hydrolyzed the triglyceride substrate to glycerol with some accumulation of monoglyceride (Fig. 2). Glycerol production was ca. linear with time until ca. 10% of the substrate had been hydrolyzed. At that time point, the monoglyceride constituted ca. 4% of the total re-

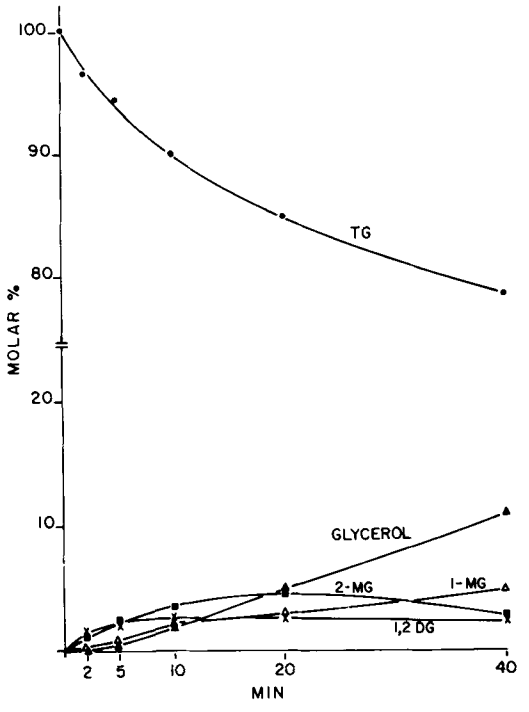


FIG. 3. Products of hydrolysis of triglyceride by lipoprotein lipase b. Epididymal adipose tissue from 20 rats (27 g tissue) fed ad libitum, was incubated for 45 min at 23 C in 100 ml Krebs-Ringer bicarbonate buffer (pH 7.4) containing 13 mM glucose, 0.12% casamino acids, 1.2 U insulin, and 300 U heparin An acetone powder of the tissue was prepared after rinsing the incubated fat pads 3 times with ice cold 0.85% NaCl. After gel chromatography, the lipoprotein lipase b column fractions were pooled, concentrated, and incubated at pH 8.0 with labeled substrate to determine positional specificity. Results are given in molar percentage of glyceride constituents in the reaction mixture at times shown on the abscissa. For explanation of symbols see Figures 1 and 2.

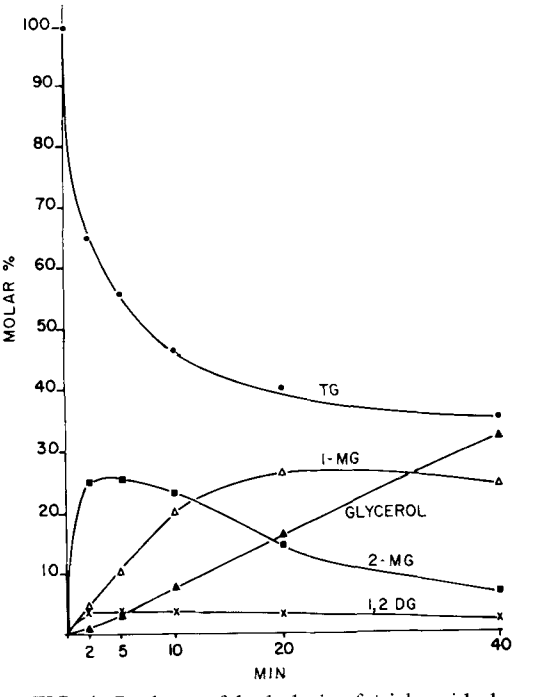


FIG. 4. Products of hydrolysis of triglyceride by lipoprotein lipase eluted with heparin from adipose tissue in vitro. Epididymal fat pads (19 g tissue) from 30 rats, fed ad libitum, were incubated for 45 min at 37 C in 100 ml Krebs-Ringer bicarbonate buffer (pH 7.4) containing 1% albumin, 13 mM glucose, 0.12 casamino acids, 1.2 U insulin, and 300 U heparin. The medium was separated from the tissue, concentrated 25-fold, and incubated of pH 8.0 with labeled substrate to determine positional specificity. Results are given in molar percentage of glyceride constituents in the reaction mixture at times shown on the abscissa. For explanation of symbols see Figures 1 and 2.

action mixture and was almost exclusively in the 2 configuration. Throughout the incubation, a small but constant amount of 1,2(2,3)-diglyceride was found, but no 1,3-diglyceride was detected.

Experiments with the LPL_b fraction showed a different pattern. In contrast to the rapid appearance of glycerol seen in Figures 1 and 2, an initial lag in glycerol production (Fig. 3) was observed with LPL_b preparations. Also the accumulated monoglyceride constituted a much larger proportion of the reaction products than did glycerol. At short incubation times, the 2 isomer constituted more than 70% of the total monoglyceride. The diglyceride, all of which had 1,2(2,3) configuration, was constantly 1-3% of the reaction mixture.

Heparin eluates showed the same pattern as the LPL_b fractions (Fig. 4), except for a larger accumulation of 1(3)-monoglyceride at longer incubation times. The large amount of albumin in this enzyme extract may have promoted isomerization from the 2 isomer (10). However, the high degree of hydrolysis at short incubation times in this experiment clearly shows the rapid production of 2-monoglyceride.

Figure 5 shows the appearance of ^{14}C-radioactivity in the fatty acid fractions of the experiments given in Fig. 1-4. In incubations with unfractionated extracts and LPL_a preparations, the ^{14}C-labeled fatty acid, originally in the 2 position, was released rapidly without a lag period. In contrast, appearance of labeled fatty acid in incubations with LPL_b preparations and heparin eluates showed a distinct lag phase. Also, with these latter preparations, disappearance of triglyceride was not accompanied by as large a release of labeled fatty acid as was observed with the unfractionated and LPL_a preparations.

TABLE I

$^{14}C/^{3}H$ Ratio of Glyceride Fractions Obtained on Incubation of Doubly Labeled Substrate with Lipoprotein Lipase Eluted from Adipose Tissue with Heparin in Vitro[a]

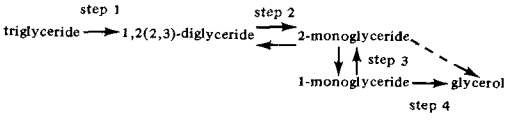
Incubation time (min)	Triolein	Diolein	Monoolein 1(3)-	Monoolein 2-
2	1.02	1.11	0.98	1.04
5	1.03	1.11	0.97	1.02
10	0.99	1.13	0.99	1.00
20	0.99	1.17	0.98	1.03
40	1.00	1.09	1.01	1.03

[a] [^{3}H]Glycerol 2-[1-^{14}C]oleoyl trioleate was incubated with lipoprotein lipase eluted from adipose tissue with heparin. Conditions of the experiment are given in the legend to Figure 4. The radioactivity ratio of the reaction products has been related to the $^{14}C/^{3}H$ ratio of the triolein substrate, which has been given a value of 1.00.

DISCUSSION

Our results support and extend those of Björntorp and Furman (3) that adipose tissue lipoprotein lipase catalyzes the rapid hydrolysis of the 1- and 3-ester bonds of the substrate. However, their experimental procedure, using triglyceride substrate with nonrandom fatty acid distribution, did not allow them to study the mechanism beyond the diglyceride step. Moreover, there are inherent difficulties in the interpretation of their data, since fatty acid specificity by lipoprotein lipase cannot be ruled out (2,11).

On the basis of the $^{14}C/^{3}H$ ratios and the pattern of glycerides found in the reaction mixtures, we suggest that the lipoprotein lipase of adipose tissue hydrolyzes its triglyceride substrate with a high specificity for the 1(3) ester bonds according to the following reaction sequence:



Although the evidence for the first two steps is not subject to other interpretations, there is the possiblity of an additional alternative pathway for the 2-monoglyceride hydrolysis, namely a direct hydrolysis of 2-monoglyceride to glycerol (dotted line in scheme above).

It has been reported that the 1 ester bond is attacked more rapidly than the 3 ester bond (12), but our experimental procedure did not distinguish between hydrolysis at these two positions. Triglyceride is hydrolyzed to 1,2(2,3)-diglyceride. The 1,2(2,3)-diglyceride is split rapidly to the 2-monoglyceride. This compound then isomerizes to its 1(3) isomer. Such a mechanism is supported by the fact that the 1(3)-monoglyceride isolated from the reaction mixture retains the ^{14}C-label originally present in the 2 position of the labeled triglyceride substrate. Presumably, the isomerization step is nonenzymatic and is rate limiting (13). The 1(3)-monoglyceride then is hydrolyzed yielding glycerol and ^{14}C-labeled fatty acid.

A possible reacylation of monoglyceride to higher glyceride (step 2 in the scheme above) was indicated by the $^{14}C/^{3}H$ ratios of the 1,2(2,3)-diglyceride formed in incubations of triglyceride with all enzyme preparations. The diglyceride $^{14}C/^{3}H$ ratio was constantly > 1.0 (1.09-1.17) (Table I). This higher ratio could be explained by a resynthesis of diglyceride from monoglyceride with a ratio of ca. 1.0 and the partially ^{14}C-labeled free fatty acid pool.

The lipoprotein lipase fraction b isolated

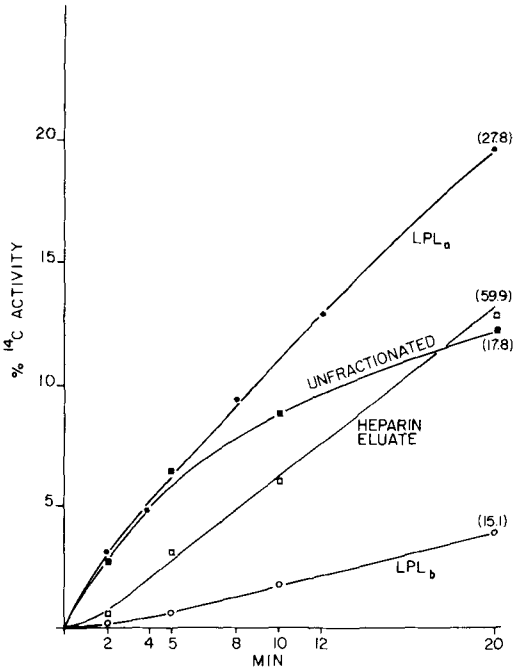


FIG. 5. Appearance of ^{14}C-labeled free fatty acid during hydrolysis of triglyceride by lipoprotein lipase preparations. The ^{14}C-labeled fatty acid originally was esterified to the 2-position of the triglyceride substrate. Hydrolysis of triglyceride substrate by enzyme preparations was stopped and lipids extracted and separated by thin layer chromatography, as described in the text. Results are given as percentage of total ^{14}C activity. The numbers in parenthesis indicate the percentage of triglyceride hydrolyzed after a 20 min incubation. LPL = lipoprotein lipase.

from adipose tissue and heparin eluates from incubated fat pads hydrolyze triglycerides, according to the scheme shown above. However, the LPL_a column fractions and crude acetone powder extracts differ from the LPL_b and heparin eluted enzyme in the proportion of monoglyceride and glycerol found in the reaction mixture, i.e. with regard to steps 3 and 4. The rapid hydrolysis of the triglyceride substrate to glycerol and ^{14}C-labeled fatty acid essentially without any accumulation of monoglyceride by crude extracts from acetone powder and by the LPL_a gel fractions suggests the presence in these preparations of a separate monoglyceride esterase activity capable of splitting 2-monoglyceride. Such a monoglyceride ester hydrolase activity in adipose tissue has been described by several groups (14,15), and such an enzyme recently has been characterized and partially purified from rat adipose tissue (H. Tornqvist, personal communication). The presence of a monoglyceride esterase in unfractionated acetone powder extracts and the possible elution of this activity with LPL_a in the gel filtration fractions would explain the absence of a lag and the rapid appearance of labeled glycerol and fatty acid during triglyceride hydrolysis by unfractionated and LPL_a preparations from acetone powders (dotted arrow in scheme above). Korn's conclusion that there was no positional specificity for lipoprotein lipase from chicken adipose tissue also could be explained by the presence of monoglyceride ester hydrolase in his preparation (2).

Thus, the reaction mechanism for the hydrolysis of triglyceride and diglyceride catalyzed by adipose tissue lipoprotein lipase appears to be similar to that of lipoprotein lipase from post heparin plasma (4) and bovine milk (5).

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Isovaleric Acid as a Precursor of Odd Numbered Iso Fatty Acids in Tetrahymena

ROBERT L. CONNER, KIM-ENG KOO, and **J.R. LANDREY,**
Department of Biology, Bryn Mawr College, Bryn Mawr, Pennsylvania 19010

ABSTRACT

Tris isovalerate-supplemented *Tetrahymena pyriformis* W showed no qualitative change in fatty acid composition; however, an increase in polar lipids that contain odd numbered iso acids (C_{13}, C_{15}, C_{17}, C_{19}) occurred. This change was accompanied by a decrease in the proportional amount of even numbered normal acids (C_{14}, C_{16}, C_{18}). The neutral and polar lipids from cells incubated with [1-^{14}C]isovaleric acid were found to contain radioactivity. The methyl esters of the saturated fatty acids obtained from the polar lipids by alkaline methanolysis were separated by reversed phase chromatography, the identities confirmed by gas chromatography-mass spectrometry, and the specific activities determined. Iso acids were found to be the most heavily labeled materials. In addition to ceramide, two sphingolipid components were detected. One yielded saturated fatty acids after acidic methanolysis, while the other contained >93% α-hydroxy fatty acids. Radioactivity was noted in the long chain base fraction derived from the sphingolipids. Progressive growth inhibition occurred as the isovalerate concentration was increased in the culture medium; however, the ciliates were morphologically indistinguishable from unsupplemented cells.

INTRODUCTION

Nutritional supplementation experiments with *Tetrahymena pyriformis* have shown that several short chain fatty acid precursors are incorporated by the cells into a variety of long chain fatty acids (1-3). Chain elongation occurs when sodium acetate, propionate, isobutyrate, or α-methyl butyrate are provided. The whole cell fatty acid composition is altered when any of the latter three precursors are supplied with an increase in odd numbered normal acids, even numbered iso acids, and odd numbered anteiso acids, respectively (2). The influence of isovaleric acid supplementation on lipid composition has not been reported.

Erwin and Bloch (1) found that exogenous leucine served as a precursor for odd numbered iso acids which constitute 23% of the total fatty acids of log phase cultures of *Tetrahymena paravorax*. When [methyl-^{14}C] methionine was added, little radioactivity was recovered in the iso fatty acids which led to the conclusion that odd numbered iso fatty acids were formed by chain elongation of isovalerate derived from leucine rather than by methylation of straight chain analogues. Isovaleric acid, however, was not employed as a nutritional supplement.

Ceramides in *T. pyriformis* W contain branched long chain bases (15-methyl-C_{16}-sphinganine and 17-methyl-C_{18}-sphinganine) (5-7) which could arise by chain elongation of isovalerate followed by formation of sphinganine. Ferguson, et al., (4) noted that odd numbered iso acids and the α-OH derivatives constitute ca. 50% of the fatty acids found in the sphingolipids of this ciliate. No anteiso acids were detected; however, small amounts of iso acids were found in triglycerides and in phosphoglycerides (1,4).

This investigation was undertaken to determine if isovaleric acid could serve as a direct precursor for odd numbered iso fatty acids and long chain bases in *T. pyriformis*, to ascertain if the fatty acid composition of the cellular phospholipids could be altered by nutritional supplementation, and to note if the viability of the cells was influenced by the presence of the short chain acid.

MATERIALS AND METHODS

Isovaleric acid (Eastman Chemical Co., Rochester, N.Y.) was distilled at 172-173 C (uncorrected). Purity was ascertained on a model 402 F & M biomedical gas chromatograph (Hewlett-Packard, Avondale, Pa.) using a 6 ft stainless steel column (3 mm inside diameter) packed with 60-80 mesh Diatoport S coated with 10% diethylene glycol succinate and 2% phosphoric acid at an oven temperature of 110 C and with a carrier gas flow of 20 ml/min. Only a single component was detected.

The Tris (2-amino-2-hydroxymethyl-propane-1,3-diol methane) salt of isovaleric acid was prepared by mixing equivalent amounts of a solution of Trizma base (Sigma Chemical Co., St. Louis, Mo.) in 95% ethanol and redistilled

isovaleric acid. Diethyl ether addition gave a white crystalline solid which was recrystallized twice from absolute ethanol. The yield was 88.5%.

Growth studies were carried out using a series of concentrations of Tris isovalerate. The salt was dissolved in water, autoclaved at 121 C for 15 min and added aseptically to a peptone based culture fluid (8). The inoculum consisted of 48 hr old (stationary phase) cells grown in the culture fluid without isovalerate supplementation.

Mass cultures of supplemented (2.5 mM Tris isovalerate) and unsupplemented *T. pyriformis* W were grown at 28.5 ± 0.5 C for 21 hr. A separate incubation was carried out in which 8.4 μC_i sodium [1-^{14}C] isovalerate (specific activity 2 mCi/mmole, International Chemical and Nuclear Corp., Irvine, Calif.) was provided in 500 ml culture fluid. The cells from all incubations were harvested, lyophilized, and extracted with chloroform-methanol, 2:1 (v/v), as previously described (9).

The total lipid fraction was separated from nonlipid contaminants by Sephadex column filtration (10). The purified lipids were fractionated into neutral and polar lipids by absorption chromatography on a column packed with methanol-washed Unisil (Clarkson Chemical Co., Williamsport, Pa.) (11). The neutral lipids were eluted with chloroform, while the polar lipids were removed with chloroform-methanol, 2:1 (v/v). Both fractions were subjected to thin layer chromatography (TLC) on 20 x 20 cm plates coated with 0.25 mm Silica Gel H (Brinkmann Instruments, Des Plaines, Ill.) which had been activated for 20 min at 100 C. The neutral lipid plates were developed in benzene-ethyl acetate, 10:1 (v/v), and stained with phosphomolybdic acid. The solvent system used for polar lipid chromatograms was chloroform-methanol-water, 95:35:4 (by volume). The developed plate was stained first with ninhydrin and then with phosphomolybdic acid. The major separated components were identified by staining reactions and by comparison of the R_f values with standards. Radiolabeled materials were located by autoradiography (9).

The polar lipids collected from the Unisil column were subjected to differential methanolysis (5). Glycerophospholipids were cleaved by 1 N NaOH in methanol to yield a mixture of fatty acid methyl esters, phosphatides, and mild alkali-stable sphingolipids. The fatty acid methyl esters were obtained from the mixture by Unisil column chromatography as described by Ferguson, et al. (4). The saturated fatty acid methyl esters were separated from the unsaturated fatty acid esters by argentation chromatography (12). The saturated fatty acid esters from the [1-^{14}C] isovalerate and 2.5 mM supplemented incubations were combined and resolved further into individual components by reversed-phase chromatography on a hydrophobic Sephadex column as described by Beijer and Nystrom (13). A glass column (5 mm inside diameter) was filled with a slurry of Lipidex TM-5000 (Packard Instrument Co., Downers Grove, Ill.) in a solvent mixture of water-methanol-ethylene chloride, 20:80:10 (by volume), to a height of 350 mm. The fatty acid esters were eluted with the same solvent mixture at a flow rate of 12 ml/hr and were collected in 2 ml fractions. Every third fraction was analyzed for radioactivity and fatty acid content. Fractions showing only a single component were combined and the solvents removed. The samples were dissolved in a known volume of benzene, and aliquots were taken for liquid scintillation spectrometry and quantitative fatty acid assays. The identification of each of the isolated fatty acid methyl esters was confirmed by gas liquid chromatography (GLC)-mass spectrometry (MS).

The unsaturated fatty acid methyl esters were subjected to catalytic hydrogenation using platinum oxide (Pfaltz-Bauer, Inc., Flushing, N.Y.).

Fatty acid methyl esters were prepared from the sphingolipid fraction by acid methanolysis for 16 hr at 75 C (4). The fatty acid esters were separated from the long chain bases by Unisil column chromatography, as previously described (4). The trimethylsilyl ethers of the sphinosines were analyzed by GLC on a 4 ft 0.75% SE-52 column at 190 C with a nitrogen flow of 70 ml/min. The trimethylsilyl ethers of dihydrosphingosine (Sigma Chemical Co.) and sphingosine (Applied Science Laboratories, State College, Pa.) were used as reference standards.

All fatty acid methyl ester mixtures were analyzed isothermally on the model 402 F & M biomedical gas chromatograph (Hewlett-Packard) using a 6 ft glass column packed with 80-100 mesh Gas Chrom P coated with 12% diethylene glycol succinate at 160 C for the fatty acid esters from glycerophospholipids and 180 C for those from sphingolipids. Nitrogen flow was 35 ml/min. Each component was identified by comparison with relative retention times (methyl stearate) and by mass spectrometry (Perkin-Elmer Hitachi RMS 4 mass spectrometer interfaced with a Perkin-Elmer model 990 gas chromatograph equipped with a 6 ft stainless steel column packed with 12% diethylene glycol succinate on 80-100 mesh Gas Chrom P and operated isothermally at 140 C).

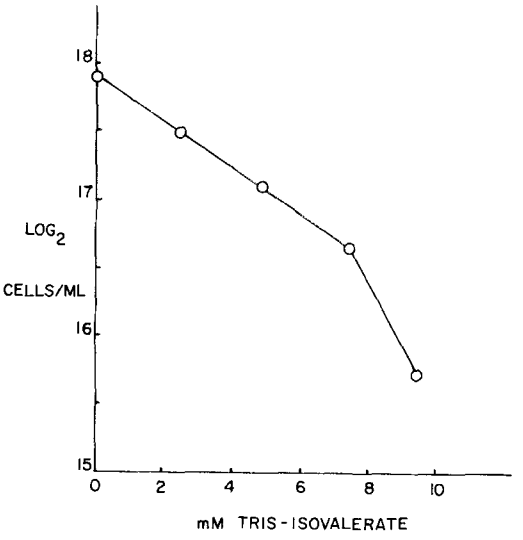

FIG. 1. Cellular density of control and isovalerate-supplemented *Tetrahymena pyriformis* W cultures after 21 hr at 25 ± 1 C. Cells were grown in 100 ml of culture fluid (2% proteose-peptone, 0.1% yeast extract, and 90 μM iron-ethylenediaminetetraacetic acid complex (8). Final concentrations of Tris isovalerate were 2.5, 5.0, 7.5, and 10 mM. The control contained 10 mM of Tris chloride buffer. The pH value of the medium ranged from 7.0 with Tris chloride to 6.6 with 10 mM Tris isovalerate. These pH values are well within the optimal growth range for these cells.

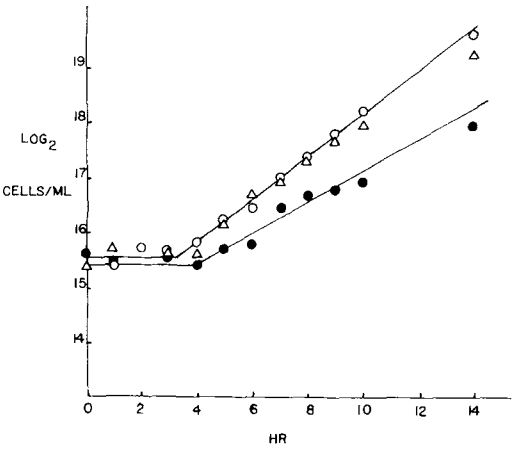

FIG. 2. The growth profiles of control and isovalerate-supplemented *Tetrahymena pyriformis* W. Final concentrations of isovalerate were 2.5 mM (Δ—Δ) and 7.5 mM (•—•). The control cells (○—○) and supplemented cells were grown at 28.5 ± 0.5 C.

RESULTS

The growth studies showed that the cell population decreased proportionally with increasing concentrations of Tris isovalerate, except at the highest concentration (10 mM) which lowered the cell yield markedly (Fig. 1). Ten mM Tris chloride (HCl) had no influence upon the growth rate or population density compared to an unsupplemented control. Cells supplemented with 2.5 mM Tris isovalerate had a growth rate comparable to the control (Fig. 2); however, at a concentration of 7.5 mM a prolonged lag phase was seen. Logarithmic growth was achieved only after 6 hr of incubation and the generation time was lengthened. The ciliates were morphologically normal in all cases, although reduced motility was observed with the higher concentrations of Tris isovalerate.

Little difference was noted between Tris isovalerate (2.5 mM) and Tris chloride (10 mM) supplemented cells with regard to dry wt/1 x 10^8 cells (628 vs 604 mg), lipid content (after Sephadex filtration)/g dry wt (117 vs 112 mg), or neutral (17.6 vs 13.4 mg), or polar (91.4 vs 88.7 mg) lipid content/g dry wt cells.

Cells incubated with 8.4 μC_i sodium isovalerate accumulated 9.6% (1.77 x 10^6 dpm) of the added label into the cellular lipids. Radioactivity recovery in the neutral lipids equalled 13.3% of the purified lipid fraction while 79.7% was found in the polar lipid fraction.

Thin layer chromatograms of the neutral lipids revealed one heavily radiolabeled region similar in R_f value to the wax esters reported by Holmlund and coworkers (14,15) to contain a number of fatty acids and alcohols. Tetrahymanol and all other neutral lipid components were labeled lightly. This fraction was not examined further.

The polar lipid autoradiogram (Fig. 3) showed radioactivity in the cardiolipin (I), the ceramide (II), the phosphatidylethanolamine (III), and the phosphatidylcholine (VI) regions. In addition, two labeled ninhydrin-positive areas (IV,V) were detected. The appropriate regions from preparative plates were scraped and the lipids eluted from the Silica Gel H with methanol. An analysis for mild-alkali stable fatty acids derived from sphingolipids in region IV revealed the methyl esters of palmitic, isoheptadecanoic, stearic, and small amounts of two C_{19} fatty acids with, at most, traces of α-hydroxy acids (Table I).

The material recovered from region V also contained mild-alkali stable fatty acids, however, a preponderance (>93%) were found to be methyl esters of α-hydroxypalmitic, α-hydroxyisoheptadecanoic, α-hydroxystearic, and smaller amounts of two C_{19} α-hydroxy fatty acids (4). Only trace amounts of sphingolipid fatty acids were detected in region VI. The fatty acid composition of the mild-alkali stable sphingolipid fraction of cells incubated with [1-^{14}C] isovalerate did not show a significant change

TABLE I

Mild Alkali Stable Fatty Acid Composition from Chromatographic Regions IV and V as Determined by Gas Liquid Chromatographic Analyses

Fatty acid	Region IV (% of total)	Region V (% of total)
16:0(n)	5.5	0
17:0(i)	39.1	0
18:0(n)	55.5	6.5
16:0(n,OH)	0	1.9
17:0(i,OH)	0	31.4
18:0(n,OH)	<1	52.0
19:0(?,OH) (2 components)	0	8.1

TABLE II

Saturated Fatty Acid Composition of Mild Alkali Labile Lipids (Glycerophospholipids) from Control and Isovalerate-Supplemented *Tetrahymena pyriformis* W

Fatty acid	Control (%)	Isoval[a] (%)	Δ(Isoval-control)
12:0(n)[b]	5.6	5.6	0
13:0(i)	trace[c]	2.5	2.5
13:0(n)	trace	trace	-
14:0(n)	34.7	28.7	-6.0
15:0(i)	7.6	15.0	7.4
15:0(n)	3.5	1.9	-1.6
16:0(i)	1.4	1.2	-0.2
16:0(n)	29.2	19.4	-9.8
17:0(i)	11.8	20.6	8.8
17:0(n)	trace	trace	-
18:0(n)	6.3	3.1	-3.2
19:0(i)	trace	1.9	1.9

[a]Cells were supplemented with 2.5 mM Tris isovalerate, average values from three experiments.

[b]Carbon chain length: number of double bonds (normal or iso).

[c]Fatty acid detected in trace amounts only.

from the controls. GLC analysis revealed two long chain bases (5,6) (relative retention time [RRT] compared to dihydrosphingosine, 0.53 and 1.09) in both regions IV and V.

The mild alkali labile fatty acid methyl esters obtained from the glycerophospholipids contained 52.5% of the radioactivity associated with the polar lipid fraction, the mild alkali stable fatty acids derived from sphingolipids, 20.6%, and the long chain base fraction, 12.9%.

The mild alkali labile fatty acid methyl esters were resolved into saturate and unsaturate fractions by argentation chromatography. Radioactivity (64%) was present in the saturated acids with the remainder in the unsaturates.

Odd numbered iso fatty acids comprise 40.0% of the total saturates from isovalerate-supplemented (2.5 mM) and 19.4% of the control glycerophospholipid fraction as determined by quantitative GLC (Table II). The iso acids increased at the expense of myristic, palmitic, and stearic acids.

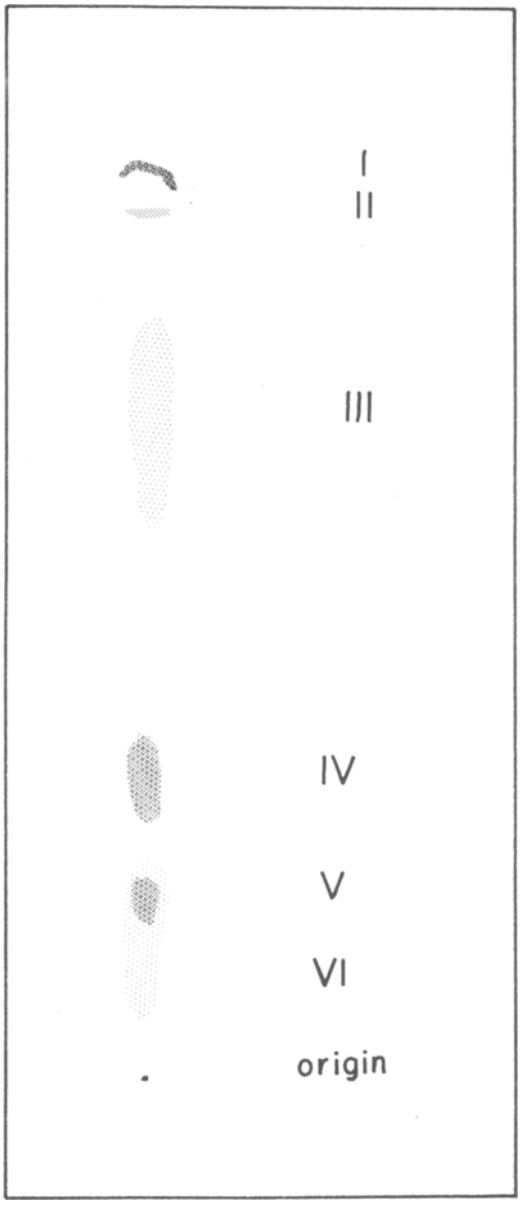

FIG. 3. A facsimile of an autoradiogram of the polar lipids obtained from *Tetrahymena pyriformis* incubated with Tris [1-^{14}C] isovalerate. The plates were developed with chloroform:methanol:water, 95:35:4 (by volume). Eastman Kodak Royal Blue X-ray No-Screen was employed. After 21 days of exposure the film was developed in the usual manner. Major components are identified as: cardiolipin (I), ceramide (II), phosphatidylethanolamine plus glyceroaminoethyl phosphonate (III), ceramide-aminoethylphosphonate (AEP) (saturated fatty acids) (IV), ceramide-AEP (α-hydroxy acids) (V), and phosphatidylcholine (VI).

TABLE III

Specific Activity of Saturated Fatty Acids Obtained from the Glycerophospholipids of Isovalerate-Supplemented *Tetrahymena pyriformis* W.

Fatty acid	Fraction number[b]	RRT (Methyl stearate)	Specific activity[c]
12:0(n)[a]	30-31	0.12	0
14:0(n)	50-56	0.28	7
15:0(i)	61-66	0.32	171
16:0(n)	91-99	0.53	4
17:0(i)	106-114	0.63	175

[a]Fatty acid methyl esters (2 x 10^5 dpm) were separated by hydrophobic Sephadex chromatography and identified by gas liquid chromatography and mass spectrometry. Amounts were determined by peak wt relative to methyl stearate. Fatty acids with chain lengths greater than C_{17} were not satisfactorily resolved by the procedure employed.

[b]Fractions shown by gas liquid chromatography to contain only one component were pooled.

[c]dpm/μg; radioactivity recovered from the pooled samples was 89.6% of that applied to the column.

TABLE IV

Unsaturated Fatty Acid (Hydrogenated) Composition of Glycerophospholipids from Control and Isovalerate-Supplemented *Tetrahymena pyriformis* W

Fatty acid	Control (%)	Isoval[a] (%)	Δ(Isoval-control)
12:0(n)	trace	trace	
14:0(n)	trace	trace	
16:0(n)	11.2	8.6	-2.6
17:0(i)	0.4	2.7	2.3
17:0(n)	2.4	3.5	1.1
18:0(n)	81.2	80.4	-0.8
19:0(i)	0.4	1.2	0.8
19:0(n)	1.5	1.2	-0.3
20:0(n)	1.9	1.4	-0.5

[a]Cells were supplemented with 2.5 mM Tris isovalerate, average values from three experiments.

The individual saturated fatty acids were isolated by reversed-phase chromatography employing hydrophobic Sephadex (Table III). The specific activities clearly indicate that the radioactivity is associated primarily with the iso acids, while little incorporation of the carboxyl carbon of isovaleric acid into acids of the normal series occurred.

A GLC analysis of the unsaturated fatty acids of glycerophospholipids gives a number of composite peaks which does not allow proportional changes observed with isovalerate supplementation to be assigned to a particular acid (unpublished data). To circumvent this difficulty the unsaturated fatty acids were hydrogenated, subjected to GLC, and the distribution by carbon skeleton ascertained (Table IV). In contrast to the saturated acids, C_{13}(i) and C_{15}(i) were present in only trace amounts in the unsaturates. The odd numbered iso fatty acids, C_{17} and C_{19}, showed an increase from less than 1% in controls to ca. 4% of the total unsaturates in isovalerate-supplemented cells.

DISCUSSION

The present experiments demonstrate that isovaleric acid is a direct precursor for long chain iso fatty acid biosynthesis in *T. pyriformis*. The data also provide presumptive evidence that branched long chain bases found in the sphingolipids arise from the same short chain acid. Since leucine can serve in a similar fashion in another species of *Tetrahymena* (1), it is reasonable to assume that transamination of the amino acid gives rise to α-ketoisocaproic acid (16) which in turn undergoes oxidative decarboxylation to isovaleric acid or the Coenzyme A (CoA) derivative (17-20). It is not known if isovalerate can spare the essential amino acid, leucine, nor is there information available about the oxidative fate of isovalerate in these ciliates.

When [1-^{14}C] isovalerate was supplied, the labeling distribution found in the pentacyclic triterpene alcohol, tetrahymanol, and the other neutral lipids, except for the heavily labeled wax esters, was similar to that observed earlier with acetate (21). It seems reasonable to assume that a portion of the isovalerate is converted to β-hydroxy-β-methylglutaryl-CoA which could serve as a precursor for tetrahymanol and for acetyl-CoA which could be incorporated into the remainder of the lipids (22).

The glycerophosphatide saturated fatty acids of the polar lipids consist mainly of normal short chain components, lauric, myristic, palmitic acids, as well as isopentadecanoic and isoheptadecanoic acids, while the unsaturated fatty acids are predominantly C_{18} derivatives (81%) with smaller quantities of C_{16} (11%). All others are present in amounts of 2% or less.

The pattern of replacement of normal by iso acids in the polar lipids is of interest. In general, isovalerate supplementation leads to an increase in all members of the iso acid series with a corresponding reduction in normal acids. The changes observed in the unsaturated acids with the concentration of isovalerate employed are too restricted to allow interpretation. There is, however, a marked alteration in the composition of the saturated fatty acids in which a nearly equimolar replacement of C_{14} by iso C_{15}, of C_{16} by iso C_{17}, and C_{18} by iso C_{19} occurs. The preponderance of saturated acids is

found in the 1 position of the phosphoglycerides in these ciliates (unpublished data). Branched chain fatty acids possess a bulky terminal group which increases the cross-sectional area in monolayers, reduces van der Waals interactions, and, thus, restricts the packing of paraffin chains (23). Replacement of the normal series by iso fatty acids, as occurred with isovalerate supplementation, might, therefore, be expected to result in greater fluidity of the cellular membranes. The introduction of a *cis*-double bond into a fatty acid is believed to have the same effect which led to the speculation that an elevation in iso acids is comparable to an increase in unsaturation (24). Silbert, et al., demonstrated recently with fatty acid auxotrophs of *Escherichia coli* that iso fatty acids can replace completely the *cis*-monounsaturated fatty acid requirement (25). If the membrane permeability properties are influenced by the fatty acid composition, the observed inhibition of growth of *Tetrahymena* by isovaleric acid may be a reflection of an abnormal membrane due to an excess of long chain iso fatty acids. An analysis of the fatty acids of cells grown with concentrations of Tris isovalerate greater than 2.5 mM may provide additional evidence on this point if coupled with cellular permeability or other physiological measurements.

[1-^{14}C]isovaleric acid supplementation results in extensive labeling of two polar lipids containing mild alkali stable fatty acids. The first (IV) has been identified previously as ceramide aminoethylphosphonate (5,6) and ceramide monomethylaminoethylphosphonate (7). This investigation would confirm the observation that no α-hydroxy fatty acids are associated with either of these materials (7). Isovalerate labeling, however, reveals a second ninhydrin positive material that contains both mild alkali stable acids and long chain bases and demonstrates that the α-hydroxy fatty acid sphingolipids are cleanly separated from the nonhydroxy counterparts. In light of previous findings, it seems reasonable to assume that a mixture of ceramide aminoethylphosphonate and monomethylaminoethylphosphonate which contain α-hydroxy acids is present. The identity of the phosphorus containing bases associated with the α-hydroxy acid ceramide, however, remains to be established.

ACKNOWLEDGMENTS

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Lipids of Six Cultivated Barley (*Hordeum vulgare* L.) Varieties[1]

PHIL B. PRICE, Plant Science Department[2], and **JOHN G. PARSONS**,
Dairy Science Department, South Dakota State University, Brookings, South Dakota 57006

ABSTRACT

The lipids of representative varieties of 2-row spring, 6-row spring, and 6-row winter-type barleys were studied. Total barley lipids were classified by silicic acid gel column chromatography and separated by thin layer chromatography, and the fatty acid composition was determined by gas liquid chromatography. Total lipid content of the 6 barley varieties ranged from 3.12%-3.56% (dry wt basis). The average values for neutral lipids, glycolipids, and phospholipids were 71, 9, and 20%, respectively. The fatty acid composition of barley was rather typical of plant tissue. The neutral lipids and glycolipids from all the varieties contained a higher percent of linoleic and linolenic (C 18:2 and C 18:3) acids than the phospholipid fraction.

INTRODUCTION

Lipids in barley (*Hordeum vulgare* L.) have not been studied as extensively as the major biochemical constituents in this cereal. Their minor representation (ca. 3%) in the total dry wt of the barley kernel, their slight role in the malting and brewing process, and the absence of rancidity in storage have not prompted frequent research with this chemical group. Most studies on barley lipids have been concerned with the fatty acid composition of ripening grain (1) and mature grain (2-4), the distribution of lipids in the kernel (3,4), changes in lipid quality during the malting and brewing process (5-10), or with particular lipid fractions, such as lipoproteins (11) or waxes and sterols (8).

Barley has been downgraded as a livestock feed, because its energy content is lower than that of corn. When energy aspects are considered, barley lipids assume an importance out of proportion to their content in the barley kernel. The most efficient method of increasing the caloric energy level of barley with the least disturbance in proportions among the biochemical constituents would be to increase the barley lipid content.

The individual lipid classes, neutral lipids, glycolipids, and phospholipids, have not been examined previously in barley. This research was initiated to provide information on lipids as a basis for improvements in the nutritional value of this cereal.

MATERIALS AND METHODS

The six barley varieties used in this study were chosen because they are representative of the physiological and morphological types in production in North America. Firlbecks III and Zephyr are 2-row head-type spring varieties.

[1]South Dakota Experiment Station Paper 1248.
[2]ARS, USDA.

TABLE I

Total Lipid, Composition, and Lipid in Each Class for Six Barley Varieties

Variety	Percent total lipid (dry wt) basis)	Percent composition			Percent lipid by class		
		Neutral lipid	Glyco-lipid	Phospho-lipid	Neutral lipid	Glyco-lipid	Phospho-lipid
Two-row spring							
Firlbecks III	3.2	69.5	9.5	21.0	2.2	0.3	0.7
Zephyr	3.5	71.0	8.2	20.8	2.5	0.3	0.7
Six-row spring							
Paragon	3.3	72.8	8.2	19.0	2.4	0.3	0.6
Primus II	3.3	69.1	9.1	21.8	2.3	0.3	0.7
Six-row winter							
Harrison	3.1	68.7	11.8	19.5	2.1	0.4	0.6
Kearney	3.5	74.6	9.5	15.9	2.6	0.3	0.6
Means	3.4	71.0	9.4	19.7	2.4	0.3	0.7

TABLE II

Fatty Acid Composition in the Neutral Lipids of Six Barley Varieties[a]

Barley variety	Fatty acid					
	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3
Firlbecks III	0.6	26.4	1.1	13.1	52.1	6.7
Zephyr	0.4	23.0	1.0	15.2	54.0	6.4
Paragon	0.3	18.7	1.1	15.7	57.8	6.4
Primus II	0.4	23.1	1.2	15.9	53.9	5.5
Harrison	0.4	24.7	1.3	15.6	52.3	5.7
Kearney	0.5	23.9	1.1	17.6	51.0	5.9
Means	0.4	23.3	1.1	15.5	53.6	6.1

[a]Expressed as mole percent and calculated from peak areas of the gas chromatograms. Fatty acids are expressed as number of carbons:number of double bonds.

Paragon and Primus II are 6-row head-type spring varieties. Harrison and Kearney are 6-row head-type winter varieties. A survey of the quantitative and qualitative characteristics of barley lipids in several standard varieties with divergent genetic backgrounds was deemed a necessary first step leading to genetic improvement in the oil content of barley.

Whole grain samples of the 6 barley varieties were ground in a mill to pass a 0.024 in. screen, and extraction was begun immediately to minimize any oxidative and enzymatic activity. The ground sample (25 g) was homogenized in a Waring blender with 20 volumes of chloroform-methanol-water (1.0:1.0:0.9) for 5 min in a modification of the methods of Bligh and Dyer (12); Folch, et al. (13); Weber (14); and Atkinson, et al. (15). The homogenate was transferred to a 2,000 ml separatory funnel, swirled to affect further solvent action, and stirred with a glass rod to enhance solvent layering. After 48 hr, the lipid charged chloroform layer was drained off and replaced to the original volume with chloroform. After another 24 hr, the chloroform layer was drained and replaced again to volume for the final extraction of 24 hr. The lipid extracts were evaporated to dryness under vacuum, weighed, redissolved in diethyl ether, and refrigerated in tightly capped vials until analyzed.

The total lipid extracts were separated into classes by silicic acid column chromatography, according to the method of Rouser, et al. (16). The lipid extracts (0.5-1.0 g) were applied in 2 ml diethyl ether and washed into the column containing 10 g of silicic acid, activated by the method of Hirsch and Ahrens (17). Diethyl ether, acetone, and methanol (250 ml each) were used sequentially to elute the neutral lipids, glycolipids, and phospholipids, respectively. The solvents were removed from each fraction by a rotary vacuum evaporator at 35 C, then the lipids were weighed and transferred to vials.

The lipid classes were separated by thin layer chromatography (TLC), according to the techniques of Stahl (18). Neutral lipids were separated on activated Silica Gel G Plates (250μ thickness). Glycolipids and phospholipids were separated on Silica Gel H plates (500μ thickness. The solvent systems used were: neutral lipids, petroleum ether-diethyl ether-acetic acid (90:10:1 v/v); glycolipids, chloroform-methanol-water (75:25:4 v/v); and phospho-

TABLE III

Fatty Acid Composition in the Glycolipids of Six Barley Varieties[a]

Barley variety	Fatty acid					
	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3
Firlbecks III	1.7	22.8	2.5	5.5	62.4	5.1
Zephyr	0.9	26.5	1.7	4.4	60.3	6.2
Paragon	1.7	24.2	2.3	5.4	61.3	5.1
Primus II	0.5	21.2	1.9	5.5	65.1	5.8
Harrison	0.8	26.0	4.4	8.6	56.0	4.2
Kearney	2.3	24.4	2.9	7.4	57.2	5.8
Means	1.4	24.2	2.6	6.2	60.4	5.4

[a]Expressed as mole percent and calculated from peak areas of the gas chromatograms. Fatty acids are expressed as number of carbons:number of double bonds.

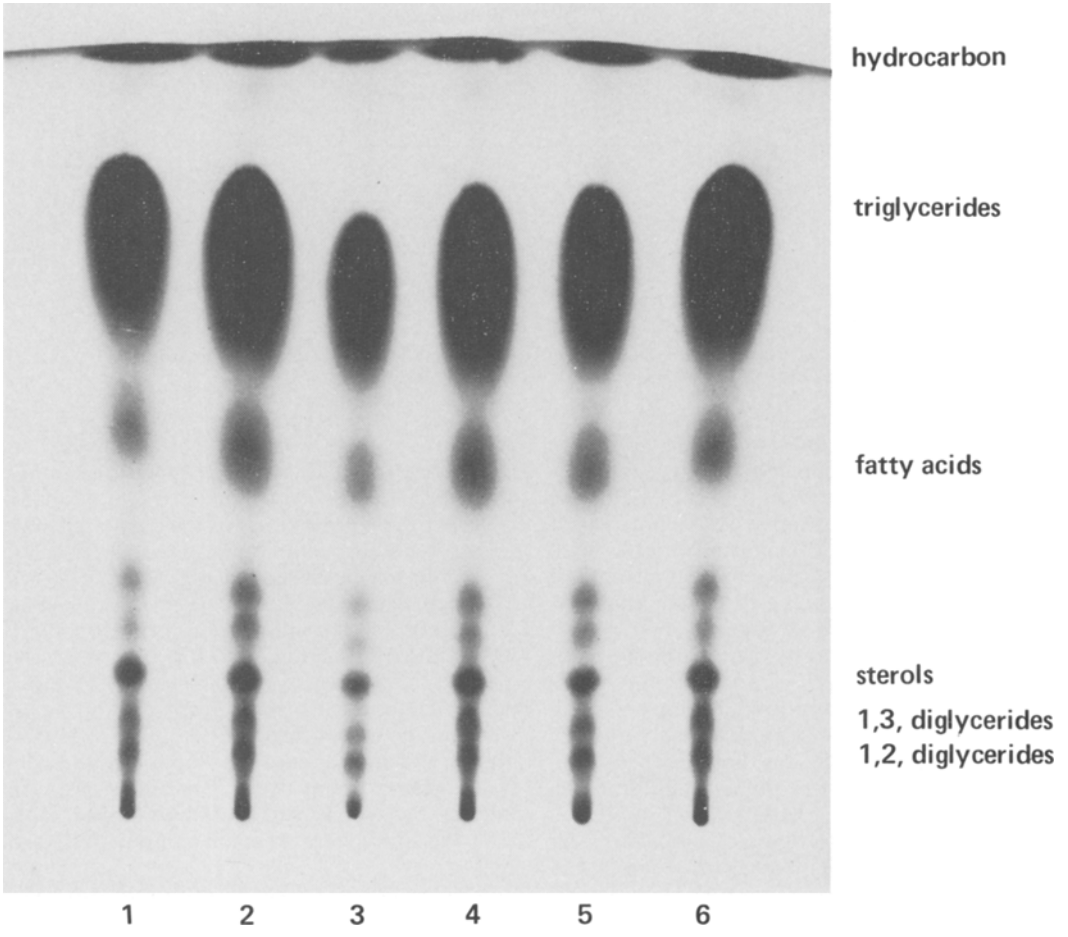

FIG. 1. Thin layer chromatographic separation of neutral lipids from six barley varieties: 1, Firlbecks III; 2, Kearney; 3, Primus II; 4, Paragon; 5, Harrison; and 6, Zephyr. Adsorbent: Silica Gel G; solvent; petroleum ether-diethyl ether-acetic acid (90:10:1); visualization: charring by heating with sulfuric acid-potassium dichromate (19).

lipids, chloroform-methanol-water-28% aqueous ammonia (64:35:4:0.2 v/v).

The individual lipids were detected on thin layer plates by the use of the following selective spray reagents: the sulfuric acid potassium dichromate reagent (Privett, et al, 19) to reveal the presence of all lipid material; ninhydrin reagent (0.2% in ethanol) for phospholipids containing free amino groups; and the specific phospholipid spray of Dittmer and Lester (20). The lipid components were identified by co-chromatography with authentic reference lipids (Applied Science Laboratories, State College, Pa.; Supelco, Bellefonte, Pa; and Analabs, North Haven, Conn.) and from published Rf values by Lepage (21) and Nichols (22).

Aliquots of the lipids were converted to methyl esters by the technique of Metcalf, et al. (23). The methyl esters were twice extracted from the water-salt saturated esterification mixture with petroleum ether. Then extracts were concentrated under a stream of nitrogen gas and analyzed by gas liquid chromatography (GLC). A Varian Aerograph Series 1440 gas chromatograph with a hydrogen-flame detector was used. This contained a 2.44 mm x 3.17 mm stainless steel column, containing 100-120 Gas Chrom P (Applied Science Laboratories, State College, Pa.), coated with 10% EGSS-X, organo-silicon polymer (Applied Science Laboratories). The fatty acids were identified by comparison with standard reference mixtures (Applied Science Laboratories). The triangulation method was used to quantify the fatty acids.

RESULTS

Total lipid content of the 6 barley varieties

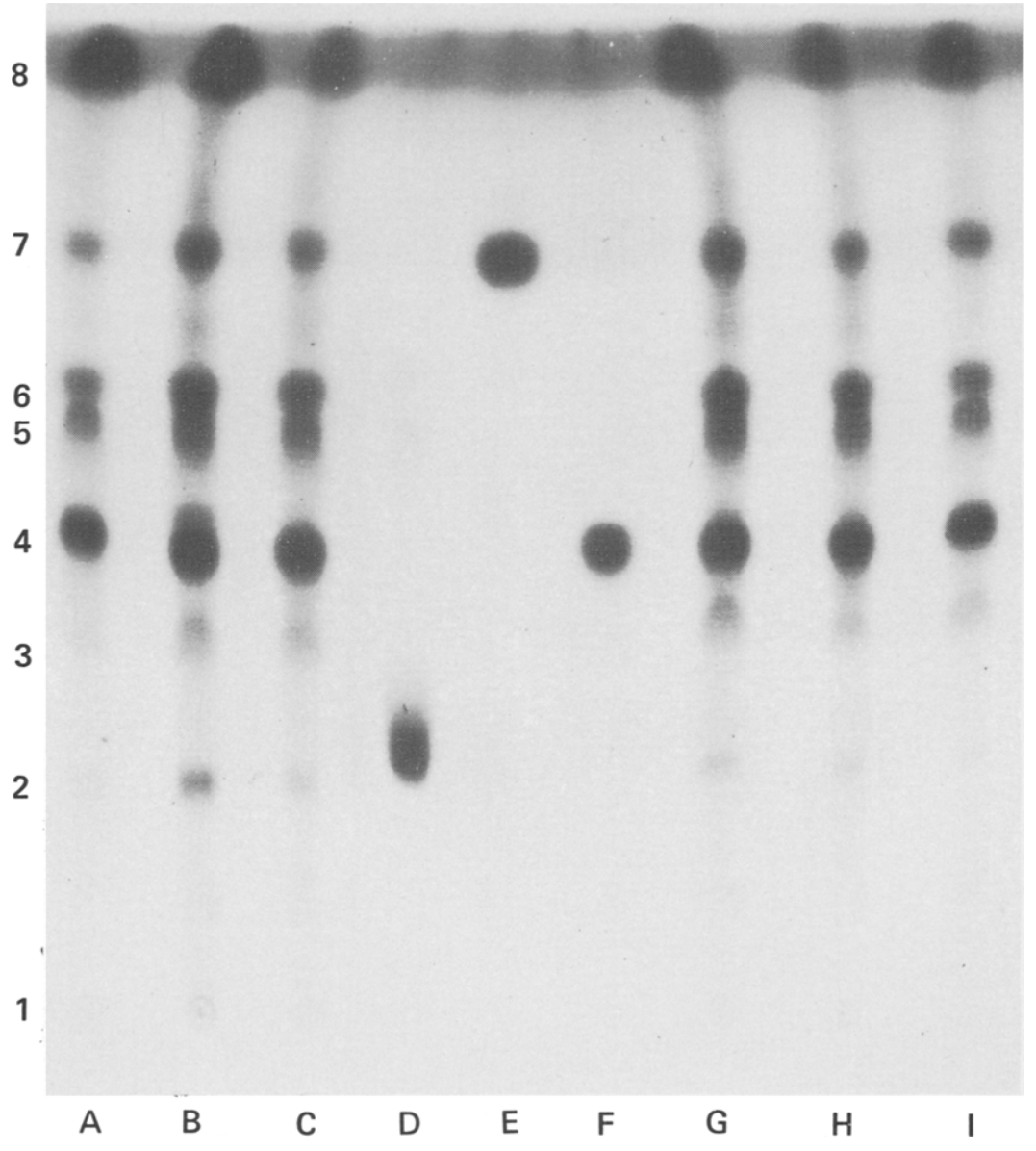

FIG. 2. Thin layer chromatographic separations of glycolipids from barley. Adsorbent: Silica Gel H; solvent: chloroform-methanol-water (75:25:4): visualization: charring by heating with sulfuric acid-potassium dichromate. A, Firlbecks III; B, Kearney; C, Primus II; G, Paragon; H, Harrison; I, Zephyr. (Standards: D, sulfatides; E, monogalactosyl diglyceride; F, digalactosyl diglyceride). The glycolipid spots were identified as follows: 1, origin; 2, sulfatides; 3, unknown; 4, digalactosyl diglyceride; 5 and 6, unknown (steryl glycoside, cerebrosides); 7, monogalactosyl diglyeride; 8, pigments.

used in this study ranged from 3.12-3.56% (dry wt basis). The lipid compositions, as determined by column chromatography, were remarkably similar (Table I). Neutral lipids, glycolipids, and phospholipids accounted for 71, 9, and 20% barley lipids, respectively. This also is presented in Table I as percent by lipid class.

The neutral lipids from the six barley varieties are shown on the TLC plate presented in Figure 1. There appeared to be no major differences among the neutral lipids of the barley varieties, and the triglyceride fraction comprised more than 50% of the total lipid extract. Two unknown spots migrated ahead of the sterol band.

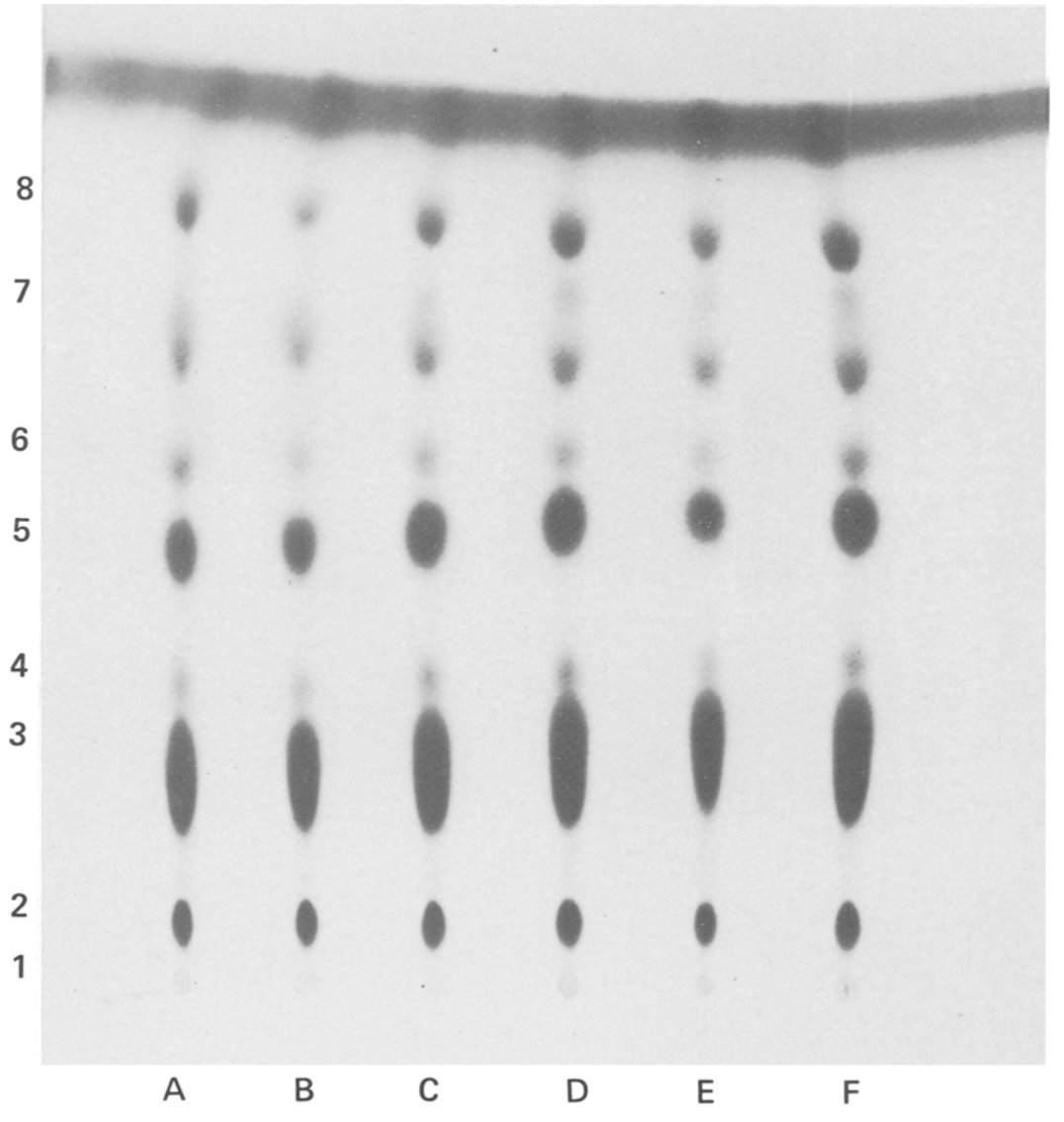

FIG. 3. Thin layer chromatographic separations of phospholipids from barley. Adsorbent: Silica Gel H; solvent: chloroform-methanol-water-28% aqueous ammonia (65:35:4:0.2); visualization: charring by heating with sulfuric acid-potassium dichromate. A, Firlbecks III; B, Kearney; C, Primus II; D, Paragon; E, Harrison; F, Zephyr. The spots were identified as follows: 1, origin; 2, lysophosphatidylcholine; 3, phosphatidyl choline and phosphatidyl serine; 4, phosphatidyl inositol; 5, phosphatidyl ethanolamine; 6, unknown; 7, unknown, (phosphatidyl glycerol), 8, unknown, (diphosphatidyl glycerol).

The glycolipid fraction from barley was separated into seven distinct spots by TLC (Fig. 2). The six varieties of barley averaged ca. 20% phospholipid, and the compositions of all varieties were quite similar (Fig. 3). All of the major phospholipids were present, in addition to an unknown phospholipid migrating ahead of phosphatidyl ethanolamine. Unknown spots, seven and eight, had R_f values similar to published values for phosphatidyl glycerol and diphosphatidyl glycerol.

The fatty acid composition of barley appears to be quite typical of plant tissue. The total fatty acid composition is represented by Table II, which shows the fatty acid composition of the neutral lipids. The neutral lipids (Table II) and the glycolipids (Table III) from all the barley varieties contained on the average more linoleic and linolenic (C 18:2 and C 18:3) acids than the phospholipid fractions did (Table IV).

TABLE IV

Fatty Acid Distribution in the Phospholipids of Six Barley Varieties[a]

Barley variety	Fatty acid					
	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3
Firlbecks III	1.1	27.9	2.1	9.2	54.5	5.2
Zephyr	1.2	28.4	1.2	9.9	54.8	4.5
Paragon	0.6	31.4	2.2	9.8	53.2	2.8
Primus II	0.8	28.0	1.8	11.1	54.9	3.4
Harrison	1.5	33.6	1.8	11.8	48.3	3.0
Kearney	1.3	28.5	1.6	11.2	53.5	3.9

[a]Expressed as mole percent and calculated from peak areas of the gas chromatograms. Fatty acids are expressed as number of carbons:number of double bonds.

Although the phospholipids from barley contained more palmitic acid (C 16:0), the compositions of all three fractions of barley were not greatly different.

DISCUSSION

The six barley varieties contained lipids that were qualitatively similar to those reported by Weber (24) in corn, Lepage (21) in turnips, and in plants generally by Allen and Good (25). The barley varieties revealed a rather narrow range in total lipid content, with only 0.42% separating Zephyr, the highest, from Harrison, the lowest (Table I). The composition and distribution of the neutral lipids, glycolipids, and phospholipids from the barley varieties were quite similar. The triglycerides constituted the major component of the total lipid extract, based upon the TLC separation (Fig. 1) and the predominance of neutral lipids in the barley seed (Table I).

The glycolipids of barley contained several major components (Fig. 2). These fractions were mono- and digalactosyl diglycerides, two unknown spots, five and six, and pigments. The distribution was similar for all varieties. Phosphatidyl choline was the major phospholipid (Fig. 3) in all six barley varieties. The other phospholipids were present in small but uniform quantities.

The fatty acids present in the lipid fractions of the barley varieties were those typical in plants. Alyward (26) reported a greater proportion of saturated fatty acids in the phospholipids. Our results indicate that only palmitic acid (C 16:0) (Table IV) was higher in the phospholipids than in the neutral lipids and glycolipids (Tables II and III).

Considerable intercrossing has been accomplished with these six barley varieties to progress toward several agronomical, pathological, and biochemical objectives. It does not appear, however, that significant genetic improvement in lipid characteristics will be possible with the rather narrow genetic base exemplified by these varieties.

A search among the more exotic barleys in the U.S. Department of Agriculture World Collection of barleys is now under way to find genetic sources for increased lipid content and a concomitant increase in caloric energy value.

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SYMPOSIUM: PHYTOSTEROLS

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JOHN S. LASETER, Chairman

Effects of Triparanol and AY-9944 upon Sterol Biosynthesis in *Chlorella*[1,2]

G.W. PATTERSON, P.J. DOYLE[3], L.G. DICKSON[3], and J.T. CHAN[3], Department of Botany, University of Maryland, College Park, Maryland 20742

ABSTRACT

The growth rates of three species of *Chlorella* were inhibited by triparanol and AY-9944. *Chlorella emersonii* was noticeably more resistant to both inhibitors than the other species. A large number of sterols were isolated and identified in inhibited cultures. Ca. 18 of these were identified from nature for the first time. Triparanol resulted in inhibition of the removal of the 14α methyl group. It also inhibited the second alkylation of the side chain and, in one species, strongly inhibited the $\Delta^8 \rightarrow \Delta^7$ isomerase reac-

[1]One of eight papers presented in the symposium "Phytosterols," AOCS Spring Meeting, New Orleans, April 1973.

[2]Scientific article A1896, contribution 4812, Maryland Agricultural Experiment Station.

[3]Present addresses: Department of Natural Sciences, University of Maryland, Princess Anne, Md; Department of Biology, Walla Walla College, College Place, Wash.; and Department of Biochemistry, Rice University, Houston, Tex., respectively.

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tion. In *C. ellipsoidea*, triparanol also inhibited Δ^{14}-reductase and Δ^{7}-reductase. The introduction of the Δ^{22} double bond was inhibited by both drugs. The effect of AY-9944 was similar to that of triparanol in *C. emersonii*, but it was an extremely effective Δ^{14}-reductase inhibitor in *C. ellipsoidea*. These various types of inhibition of sterol synthesis indicate a lack of specificity of both drugs in *Chlorella* and suggest that primitive plants such as these may be valuable as test organisms in an evaluation of the activity of potential inhibitors of sterol biosynthesis.

INTRODUCTION

The effects of triparanol and AY-9944 have been studied thoroughly in many animals. In vertebrates, triparanol inhibited the conversion of desmosterol to cholesterol (1). In an insect (2) and a nematode (3), triparanol inhibited conversion of sitosterol to cholesterol, resulting in an accumulation of desmosterol. Triparanol has been considered rather specific in its action, although some evidence has suggested that is also may affect the pathway prior to the formation of lanosterol (4). Clayton, et al., (5) found that triparanol caused a change in the ratio of Δ^{8}/Δ^{7}-sterol intermediates. Dempsey summarized some of the primary effects of triparanol and AY-9944 (6). The effect of triparanol upon sterol biosynthesis in plants previously had not been studied.

AY-9944 has been studied exhaustively by the Ayerst Research Labs, Montreal, Canada, and it has been shown to inhibit cholesterol biosynthesis at the Δ^{7}-reductase step (7). Until various effects of AY-9944 in *Chlorella* were described by Dickson (8) no other effects of this drug upon sterol biosynthesis had been reported. To our knowledge, AY-9944 had not been studied as an inhibitor of sterol biosynthesis in other plants. In view of the numerous studies with triparanol and AY-9944 in animals and the consistency of the results, it was of interest to us to determine whether these same effects would be observed in plants. These studies should be particularly significant in view of known differences in sterol biosynthesis in animals and plants (9).

The sterols of *Chlorella* have been studied extensively. The genus *Chlorella* has been divided into three groups on the basis of the types of sterols present (10). Each of these groups contain either Δ^{7}-, $\Delta^{5,7}$-, or Δ^{5}-sterols. *Chlorella emersonii, C. sorokiniana,* and *C. ellipsoidea* were chosen as representative species synthesizing predominately Δ^{7}, $\Delta^{5,7}$-, and Δ^{5}-sterols, respectively. The isolation and identification of these sterols have been reported previously (11-13).

MATERIALS AND METHODS

Each drug was added to the culture medium at a concentration which gave ca. a 50% reduction in the growth rate of the organism. Ca. five times the concentration of the drugs was required to produce this degree of growth inhibition in *C. emersonii*, as compared to the other species (AY-9944 level not yet determined for *C. sorokiniana*). Sterols were isolated by our usual methods, utilizing chromatography on alumina, silica gel, and $AgNO_3$ impregnated silica gel (9, 14). Identifications were made upon the basis of gas liquid chromatography (GLC) relative retention times on four columns (15), GLC-mass spectroscopy, and, where possible, by mp, optical rotations, UV and IR spectroscopies, and NMR.

RESULTS AND DISCUSSION

Effects of Triparanol

Application of triparanol to the nutrient medium resulted in the accumulation of a large number of sterols which had not been observed previously in these algae. Sterols identified from triparanol treated cultures which, in addition, had not been reported previously from nature were: 24-dihydroobtusifoliol; 4α,14α-dimethyl-5α-(24S)-stigmast-8-en-3β-ol; 24-methylene pollinastanol; 24-methyl pollinastanol; 14α-methyl-5α-ergost-8-en-3β-ol; 14α-methyl-5α-ergosta-8,24(28)-dien-3β-ol; 14α-methyl-5α-(24S)-stigmast-8-en-3β-ol; 5α-ergost-8(14)-en-3β-ol; 5α-ergosta-8(14),22-dien-3β-ol; 5α-ergosta-8,14,22-trien-3β-ol; and 5α-(24S)-stigmasta-7,25-dien-3β-ol. A total list of the sterols identified and their relative significances in the total sterol of the alga are shown in Table I.

The sterols accumulating in triparanol treated cultures were quite different from species to species. For instance, 24-methylene pollinastanol and 14α-methyl-ergosta-8,24(28)-dien-3β-ol accumulated in treated *C. emersonii* and were not detected in the other species. There was a large accumulation of $\Delta^{8,14}$-sterols in *C. ellipsoidea*, little in *C. sorokiniana*, and none detected in *C. emersonii*. A tremendous accumulation of 5α-ergost-8(9)-en-3β-ol was seen in *C. sorokiniana*, only a small amount was found in *C. ellipsoidea* and none was detected in *C. emersonii*. 5α-Ergost-8(14)-en-3β-ol and 24-methyl pollinastanol were detected only in treated *C. sorokiniana*, and a large accumula-

TABLE I

Sterols[a] of Control and Triparanol Treated[b] *Chlorella* Species

Sterols	*C. emersonii*		*C. ellipsoidea*		*C. sorokiniana*	
	Control	Treated	Control	Treated	Control	Treated
Cycloartenol	---	---	---	0.1	t[c]	0.2
24-Methylene cycloartenol	0.1	3.6	---	0.7	---	---
Cyclolaudenol	---	---	---	---	t	0.2
Cycloeucalenol	0.1	0.5	---	---	---	---
Obtusifoliol	0.8	3.4	---	7.5	---	---
24-Dihydroobtusifoliol	t	0.1	---	1.3	---	---
4α,14α-Dimethyl-5α(24S)-stigmast-8-en-3β-ol	t	t	---	1.2	---	---
24-Methylene pollinastanol	---	9.3	---	---	---	---
24-Methyl pollinastanol	---	---	---	---	t	2.1
Pollinastanol	---	---	---	---	---	t
14α-Methyl-5α-ergost-8-en-3β-ol	1.0	7.1	---	0.9	---	0.8
14α-Methyl-5α-ergosta-8,24(28)-dien-3β-ol	---	2.9	---	---	---	---
14α-Methyl-5α-(24S)-stigmast-8-en-3β-ol	---	0.4	---	0.1	---	---
5α-Ergost-8(14)-en-3β-ol	---	---	---	---	---	3.0
5α-Ergosta-8(14),22-dien-3β-ol	---	---	---	---	---	t
5α-Ergosta-8,14-dien-3β-ol	---	---	---	7.8	---	0.2
5α-Ergosta-8,14,22-trien-3β-ol	---	---	---	---	---	0.1
5α-(24S)-Stigmasta-8,14-dien-3β-ol	---	---	---	7.5	---	---
5α-Ergost-8(9),22-dien-	---	---	---	2.7	---	39.6
5α-Ergosta-8(9),22-dien-3β-ol	---	---	---	---	---	1.0
5α-(24S)Stigmast-8(9)-en-3β-ol	---	---	---	1.7	---	---
5α-Ergost-7-en-3β-ol	15.3	13.8	---	7.1	---	7.9
5α-Ergosta-7,22-dien-3β-ol	0.1	1.0	---	---	---	0.1
5α-(24S)-Stigmast-7-en-3β-ol	6.9	9.6	---	6.8	---	---
5α-(24S)-Stigmasta-7,25-dien-3β-ol	---	t	---	---	---	---
Chondrillasterol	75.5	47.0	---	29.3	15.3	6.2
Ergosta-5,7-dien-3β-ol	---	---	---	---	84.6	27.0
Ergosterol	---	---	---	2.0	---	---
(24S)-Stigmasta-5,7,22-trien-3β-ol	---	---	33.2	13.8	---	---
Ergost-5-en-3β-ol	---	---	4.5	2.8	---	---
Clionasterol	---	---	62.3	6.7	---	---
Poriferasterol	---	---	---	---	---	---
Unknown A	---	---	---	---	---	6.2
Unknown B	---	---	---	---	---	3.3

[a]As percentage of total sterol.

[b]Triparanol concentration: *C. emersonii*, 8.2 x 10^{-6}M; *C. ellipsoidea* and *C. sorokiniana*, 1.6 x 10^{-6}M.

[c]t = trace.

tion of ergosta-5,7-dien-3β-ol occurred in treated *C. ellipsoidea*.

Some of these differences may be attributed to the absence of certain biosynthetic steps in one or more of these algae. For example, triparanol cannot produce an accumulation of $\Delta^{5,7}$-sterols in *C. emersonii* as it does in *C. ellipsoidea*, because *C. emersonii* is incapable of metabolizing sterols beyond the Δ^7 stage (12). 24-Methylene pollinastanol did not accumulate in *C. sorokiniana*, because this organism apparently does not synthesize the 24-methylene group (16); but this explanation is not applicable to *C. ellipsoidea*. The absence of any accumulation of Δ^8- and $\Delta^{8,14}$-sterols in *C. emersonii*, even at a higher triparanol concentration, also is puzzling.

The situation is clarified somewhat by examining the different sterol structures or substituents, rather than specific sterols, which accumulate. This seems reasonable, since sterol biosynthesis is viewed best as a series of interconnected, parallel pathways, rather than a single, linear pathway (6). Looking at the effect of triparanol in this way (Table II), it is seen that in treated *C. emersonii*, sterols with 24-methylene groups and those with 14α-methyl groups accumulated compared to the control. An accumulation of sterols with the 9,19-cyclopropane ring also occurred. Doyle, et al., (17) suggested that, in *C. emersonii*, triparanol inhibited the removal of the 14α-methyl group and the second alkylation reaction leading to the formation of C-29 sterols. This can be seen in the large reduction in the percentage of sterols with 10-carbon side chains, although one of them (5α-24S-stigmast-7-en-3β-ol) increased. It is tempting to speculate that this sterol serves as a precursor of chondrillasterol and that the reaction is inhibited by triparanol. A similar introduction of the Δ^{22} bond has been reported in a higher plant (18).

TABLE II

Percentage[a] of Different Sterol Structures Occurring in Control and Triparanbl Treated[b] *Chlorella* Species

	C. emersonii		*C. ellipsoidea*		*C. sorokiniana*	
Sterol structure	Control	Treated	Control	Treated	Control	Treated
4,4',14-Trimethyl	0.1	3.6	---	0.8	t[c]	0.1
4,14-Dimethyl	0.9	4.0	---	9.0	---	---
14α-Methyl Δ^8	2.0	27.3	---	1.0	-	0.8
9,19-Cyclopropane	0.2	13.4	---	0.8	0.1	2.5
$\Delta^{8(14)}$	---	---	---	---	---	3.0
$\Delta^{8(9)}$(Desmethyl)	---	---	---	4.4	---	39.6
$\Delta^{8,14}$	---	---	---	15.3	---	0.3
Δ^7	97.8	71.4	---	13.9	---	8.0
$\Delta^{5,7}$	---	---	---	31.3	99.9	33.2
Δ^5	---	---	100.0	24.3	---	---
Δ^{22}	75.6	48.0	62.3	8.7	84.6	34.4
24-Methylene	1.0	19.7	---	8.2	---	---
10 Carbon side chain	82.4	57.0	66.8	28.8	---	---

[a]Percentage of total sterol.

[b]Triparanol succinate concentration: *C. emersonii*, 5 mg/liter (8.2×10^{-6}M); *C. ellipsoidea* and *C. sorokiniana*, 1 mg/liter (1.6×10^{-6}M).

[c]t = trace.

A total of 18 different sterols were identified in triparanol treated *C. ellipsoidea* (Table I). In this organism, we see a smaller accumulation of 24-methylene sterols and sterols with the 14α-methyl group—perhaps due to the lower drug concentration, which was one-fifth that used in *C. emersonii*. However, significant accumulations of $\Delta^{8(9)}$-, $\Delta^{8,14}$-, Δ^7-, and $\Delta^{5,7}$-sterols also were observed. In addition, a more effective inhibition of the synthesis of Δ^{22}-sterols and sterols with a 10 carbon side chain was observed than was obtained in *C. emersonii*.

The triparanol inhibition of *C. sorokiniana* is interesting for several reasons. We observed an inhibition of the synthesis of 10 carbon side chains by triparanol in the other two species, but *C. sorokiniana* normally does not synthesize these sterols. It seems to be more than a coincidence that 24-methylene sterols are absent also, even in inhibited cultures, while cyclolaudenol was identified in the trimethyl sterol fraction. This led to the suggestion (16) that the 25-methylene group replaces the 24-methylene group as the first product of side chain alkylation in *C. sorokiniana*; and, since the 24-methylene group is required for further alkylation (19), sterols with a 10 carbon side chain are not synthesized in this alga. The primary site of triparanol inhibition in this organism appears to be at the $\Delta^8 \rightarrow \Delta^7$ isomerase reaction. The accumulations of 5α-ergost-8(14)-en-3β-ol and 24-methyl pollinastanol also suggest roles for these sterols in the biosynthetic pathway. Schroepfer, et al., (20) recently suggested that 5α-cholest-8(14)-en-3β-ol is a precursor in the biosynthesis of cholesterol in the rat. As in the other species, a drastic reduction in the proportion of Δ^{22}-sterols was observed.

Triparanol treatment resulted in a 42-64% reduction in the concentration of total sterols (dry wt basis) in the algal species, also suggesting an additional point of inhibition prior to the formation of cycloartenol.

Effects of AY-9944

AY-9944 also resulted in accumulation of a number of sterols which had not been reported previously in the organism (Table III). The following sterols had not been reported previously from nature: 5α-ergosta-8,14-dien-3β-ol; 5α-(24S)-stigmasta-8,14-dien-3β-ol; 5α-ergost-8(9)-en-3β-ol; 5α-(24S)-stigmast-8(9)-en-3β-ol; 4α-methylergosta-8,14-dien-3β-ol; 4α-methyl-5α-(24S)-stigmasta-8,14-dien-3β-ol; and 4α-methyl-5α-(24S)-stigmast-8(9)-en-3β-ol.

The effects of AY-9944 are quite different in the two algae, compared with those reported in animals (7). In *C. emersonii*, although treated at the higher level of AY-9944, the only major effects were the accumulation of 14α-methyl sterols and the reduction of Δ^{22}-sterols and sterols with the 10-carbon side chain (21), all effects which were seen with triparanol in this organism. In contrast, the effect of AY-9944 in *C. ellipsoidea* was spectacular. Nearly 70% of the sterols in treated cultures were $\Delta^{8,14}$-

TABLE III

Sterols[a] of Control and AY-9944 Treated[b] *Chlorella* Species

Sterols	*C. emersonii*		*C. ellipsoidea*	
	Control	Treated	Control	Treated
24-Methylene cycloartanol	0.3	t	t	0.1
24-Dihydroobtusifoliol	0.8	0.2	---	0.5
4α,14α-Dimethyl (24S)-5α-stigmast-(8)-en-3β-ol	0.4	0.3	---	1.0
Cycloeucalenol	---	t	---	---
Obtusifoliol	0.2	t	---	---
14α-Methyl-5α-ergost-8-en-3β-ol	0.9	3.4	---	---
14α-Methyl-5α-(24S)-stigmast-8-en-3β-ol	0.4	9.8	---	---
14α-Methyl-5α-ergosta-8,24(28)-dien-3β-ol	0.8	3.6	---	---
4α-Methyl-5α-ergosta-8,14-dien-3β-ol	---	---	---	4.0
4α-Methyl-5α(24S)-stigmasta-8,14-dien-3β-ol	---	---	---	3.1
4α-Methyl-5α-ergost-8(9)-en-3β-ol	---	---	---	0.4
4α-Methyl-5α-(24S)-stigmast-8(9)-en-3β-ol	---	---	---	0.3
5α-Ergosta-8,14-dien-3β-ol	---	---	---	26.4
5α-(24S)-Stigmasta-8,14-dien-3β-ol	---	---	---	43.2
5α-Ergost-8(9)-en-3β-ol	---	---	---	6.1
5α-(24S)-Stigmast-8(9)-en-3β-ol	---	---	---	13.2
5α-Ergost-7-en-3β-ol	16.3	42.6	---	---
5α-Ergosta-7,22-dien-3β-ol	0.5	0.4	---	---
5α-(24S)-Stigmast-7-en-3β-ol	8.6	9.8	---	---
Chondrillasterol	70.8	29.2	---	---
Cholesterol	---	---	---	0.1
Brassicasterol	---	---	5.6	---
Ergost-5-en-3β-ol	---	---	21.9	0.4
Clionasterol	---	---	6.8	0.1
Poriferasterol	---	---	65.6	1.0

[a]As percentage of total sterol.

[b]AY-9944 concentration: *C. emersonii*, 4.3 x 10^{-5}M; *C. ellipsoidea*, 8.6 x 10^{-6}M.

[c]t = trace.

desmethyl sterols with another 7% being 4α-methyl $\Delta^{8,14}$-sterols (22). Another 19 and 0.7% of the total sterol were $\Delta^{8(9)}$-desmethyl sterols and 4α-methyl-$\Delta^{8(9)}$-sterols, respectively (Table IV). The naturally-occurring Δ^5-sterols were reduced from 99.9 to 1.6% of the total sterol and Δ^{22}-sterols were reduced from 70.2 to 1.0% of the total sterol. The proportion of sterols with 10-carbon side chains and the quantity of 24-methylene sterols were not affected significantly. The inhibition of Δ^5-sterol synthesis in this alga was essentially 100% effective, since the quantity of Δ^5-sterols in treated cells was ca. the amount present in the untreated cells used to inoculate the culture (14). We hope soon to have data on the effect of AY-9944 upon *C. sorokiniana*.

COMPARISON OF TRIPARANOL AND AY-9944 INHIBITION

In *C. emersonii*, triparanol (at a much lower concentration than AY-9944) resulted in a greater accumulation of 24-methylene sterols, although AY-9944 gave a greater reduction of sterols with 10 carbon side chains (Table V). Both drugs were effective in inhibiting the removal of the 14α-methyl group. In contrast to the effect of triparanol, there was no accumulation of trimethyl sterols or sterols with 9,19-cyclopropane rings due to AY-9944. The difference in the amount of inhibition seen in the introduction of the Δ^{22}-bond may have been due to the larger concentration of AY-9944 that was used.

In *C. ellipsoidea*, triparanol was much more effective than AY-9944 in causing an accumulation of 24-methylene sterols and 4,14-dimethyl sterols (Table VI). The accumulation of $\Delta^{8(9)}$- and $\Delta^{8,14}$-sterols in AY-9944 treated *C. ellipsoidea* cultures was striking, but the accumulation of these sterols in triparanol treated cultures was smaller only by the same factor as the concentration difference of the inhibitors. The accumulation of $\Delta^{8(9)}$- and $\Delta^{8,14}$-sterols in AY-9944 and triparanol treated *C. ellipsoidea* and in triparanol treated *C. sorokiniana* and their absence in all cultures of *C. emersonii* have prompted speculation that there may be an alternate pathway replacing the $\Delta^{8,14}$- and $\Delta^{8(9)}$-stage in this alga (21). Whether AY-9944 inhibits Δ^7-reductase in *C. ellipsoidea* that could result in accumulation of Δ^7- and $\Delta^{5,7}$-sterols, as seen in the triparanol treatment, cannot be answered at present, since, as previously mentioned, AY-9944 is essentially 100% effective in blocking sterol biosynthesis

TABLE IV

Percentage[a] of Different Sterol Structures Occurring in Control and AY-9944 Treated[b] *Chlorella* Species

	C. emersonii		*C. ellipsoidea*	
Sterol structure	Control	Treated	Control	Treated
24-Methylene	1.3	3.6	t[c]	0.1
4,4',14-Trimethyl	0.3	t	t	0.1
4,14-Dimethyl	1.4	0.5	---	1.5
14α-Methyl Δ^8	2.1	16.8	---	---
4α-Methyl $\Delta^{8,14}$	---	---	---	7.1
4α-Methyl $\Delta^{8(9)}$	---	---	---	0.7
9,19-Cyclopropane	0.3	t	t	0.1
$\Delta^{8,14}$(Desmethyl)	---	---	---	69.6
$\Delta^{8(9)}$(Desmethyl)	---	---	---	19.3
Δ^7	96.2	82.0	---	---
Δ^5	---	---	99.9	1.6
Δ^{22}	71.3	29.6	70.2	1.0
10 Carbon side chain	80.2	49.1	72.4	70.9

[a]Percentage of total sterol.

[b]AY-9944 concentration: *C. emersonii*, 20 mg/liter (4.3×10^{-5}M); *C. ellipsoidea*, 4 ppm (8.6×10^{-6}M).

[c]t = trace.

TABLE V

Comparison of Sterol Structures Occurring in Triparanol and AY-9944 Treated[a] *C. emersonii*

	Percent of total sterol		
Sterol structure	Control[b]	AY-9944 treated	Triparanol treated
4,4',14-Trimethyl	0.2	t[c]	3.6
4,14-Dimethyl	1.1	0.5	4.0
14α-Methyl Δ^8	2.0	16.8	27.3
9,19-Cyclopropane	0.2	t	13.4
Δ^7	97.0	82.0	71.4
Δ^{22}	73.4	29.6	48.0
24-Methylene	1.1	3.6	19.7
10 Carbon side chain	81.3	49.1	57.0

[a]Triparanol succinate concentration 5 mg/liter (8.2×10^{-6}M); AY-9944 concentration, 20 mg/liter (4.3×10^{-5}M).

[b]Average of the AY-9944 and triparanol controls.

[c]t = trace.

at the $\Delta^{8(9)}$- and $\Delta^{8,14}$-stages in this organism.

It is also of interest to note that, in triparanol treated *C. ellipsoidea*, the 14α-methyl was the last to be removed, while in AY-9944 treated cultures the 4α-methyl was the last to be removed (Table VI). The quantity of methyl sterols in the control culture was not sufficient to determine the sequence of demethylation. The 14α-methyl was the last to be removed in all other treatments in the three *Chlorella* species, although the reverse was apparently true in higher plants (23). The major points of triparanol and AY-9944 inhibition are summarized in Table VII. It is apparent that the effects of triparanol and AY-9944 upon sterol biosynthesis in *Chlorella* are numerous and complex. Triparanol caused an accumulation of 14α-methyl and 24-methylene sterols and a reduction of sterols with a 10-carbon side chain in the *Chlorella* species studied that have these structures as a part of their biosynthetic pathway. However, it appears that the accumulation of 14α-methyl sterols required a relatively large drug concentration and that little buildup of these sterols occurred in the species where smaller drug concentrations were used. The same was true of AY-9944, except in *C.*

TABLE VI

Comparison of Sterol Structures Occurring in Triparanol and AY-9944 Treated[a] *C. ellipsoidea*

Sterol structure	Percent of total sterol		
	Control[b]	AY-9944 treated	Triparanol treated
4,4',14-Trimethyl	t[c]	0.1	0.8
4,14-Dimethyl	---	1.5	9.0
14α-Methyl Δ^8	---	---	1.0
4α-Methyl $\Delta^{8,14}$	---	7.1	---
4α-Methyl $\Delta^{8(9)}$	---	0.7	---
9,19-Cyclopropane	t	0.1	0.8
$\Delta^{8,14}$(Desmethyl)	---	69.6	15.3
$\Delta^{8(9)}$(Desmethyl)	---	19.3	4.4
Δ^7	---	---	13.9
$\Delta^{5,7}$	---	---	31.3
Δ^5	99.9	1.6	24.3
Δ^{22}	66.3	1.0	8.7
24-Methylene	t	0.1	8.2
10 Carbon side chain	69.6	70.9	28.8

[a]Triparanol succinate concentration, 1 mg/liter (1.6×10^{-6}M); AY-9944 concentration, 4 mg/liter (8.6×10^{-6}M).

[b]Average of the triparanol and AY-9944 controls.

[c]t = trace.

TABLE VII

Major Points of Inhibition of Sterol Synthesis in *Chlorella* species by Triparanol and AY-9944[a]

Point of inhibition	*C. emersonii*	*C. ellipsoidea*	*C. sorokiniana*[b]
Second alkylation	TP[c], AY[d]	TP	
Removal of 14α-methyl	TP, AY		
$\Delta^8 \rightarrow \Delta^7$ isomerase		AY	TP
Δ^{14}-Reductase		TP, AY	
Δ^7-Reductase		TP	
Introduction of Δ^{22}	TP, AY	TP, AY	TP

[a]TP = triparanol and AY = AY-9944.

[b]Effect of AY-9944 upon *C. sorokiniana* was not studied.

[c]Major point of triparanol inhibition.

[d]Major point of AY-9944 inhibition.

ellipsoidea, where there was no effect upon 24-methylene sterols or upon sterols with 10 carbon side chains. It may be suggested that, in *C. ellipsoidea*, the Δ^{22}-bond is introduced at a very late stage in the pathway and that $\Delta^{8(9)}$- and $\Delta^{8,14}$-sterols are not normally substrates for the introduction of the Δ^{22}-bond. This would account for the effect of AY-9944 upon the introduction of the Δ^{22}-bond. However, in *C. sorokiniana*, $\Delta^{8(14),22}$-, $\Delta^{8(9),22}$-, and $\Delta^{8,14,22}$-sterols were identified in triparanol treated cultures and the inhibition of the introduction of the Δ^{22}-bond was still strong (24). Small accumulations of Δ^8- and Δ^7-sterols have been observed in triparanol inhibited animal systems (5), but these effects are much more apparent in *C. ellipsoidea* and *C. sorokiniana* at a concentration of only 1.6×10^{-6} M.

It appears that an organism like *Chlorella* would be useful to screen potential hypocholesterolemic drugs for possible secondary points of inhibition in sterol synthesis which are apparent in animal studies. A realization of the nonspecificity of certain drugs then may be obtained more readily.

The structures of the compounds isolated in these studies suggest that they are intermediates in sterol biosynthesis. Pathways of biosynthesis of sterols have been suggested for each species (14-17). Tracer studies are underway to determine which of the identified compounds are intermediates in sterol biosynthesis.

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Distribution of Sterols in the Fungi I. Fungal Spores[1]

JOHN D. WEETE, Department of Botany and Microbiology, Agricultural Experiment Station, Auburn University, Auburn, Alabama 36830, and **JOHN L. LASETER**, Department of Biological Sciences, University of New Orleans, New Orleans, Louisiana 70122

ABSTRACT

The freely extractable sterols of spores of *Linderina pennispora, Spicaria elegans, Penicillium claviforme, Aspergillus niger, Ustilago nuda, U. maydis, Puccinia graminis,* and *P. striiformis* were examined using mass spectrometric techniques. Each species contained at least 3-5 detectable sterol components in the 4-desmethyl sterol fraction, and, when present, ergosterol was generally the most abundant sterol produced by an individual species. Smaller relative concentrations of fungisterol (ergost-Δ^7-enol) di- and tetraunsaturated C_{28} sterols also were found. In some species, fungisterol was the most abundant sterol. In uredospores of rust fungi, stigmast-Δ^7-enol (C_{29}) was predominant and was accompanied by lower relative concentrations of a diunsaturated C_{29} sterol and fungisterol. Cholesterol was found only in the teliospores of the corn smut fungus (*U. maydis*). Application of glass capillary columns to the separation of yeast sterols by gas liquid chromatography is illustrated.

INTRODUCTION

The distribution (1-4) and biosynthesis (1) of fungal sterols have been reviewed by several investigators, and ergosterol has been reported as the predominant sterol constituent in more than half of the 100 or so fungi examined (1). From recent studies with more efficient and sensitive analytical instruments employed, it has become apparent that ergosterol often is accompanied by a number of closely related sterols, generally at very low relative concentrations.

More Phycomycete species have been studied for their sterol composition than any other fungal group. McCorkindale and his associates (5) carried out a systematic analysis of the sterol constituents of 22 Phycomycete species. Fungi belonging to the more primitive orders, Saprolegniales and Leptomitales, produced cholesterol, related C_{28} and C_{29} sterols having Δ^5 unsaturation, and desmosterol. Bean, et al., (6) also reported C_{28} and C_{29} sterols with Δ^5 unsaturation in other aquatic Phycomycetes, with several sterols having Δ^{22} unsaturation. Mucorales species, on the other hand, tend to accumulate ergosterol as their major sterol, with lesser relative concentrations of fungisterol and diunsaturated C_{28} sterols (5,7,8). Generally, the 4-desmethyl sterol components of imperfect fungi are similar to those identified in the Mucorales fungi (1).

Several fleshy (Homobasidiomycete) fungi have been analyzed for their 4-desmethyl sterol content, and it appears that ergosterol is also the most frequently encountered sterol in this group (9-11). In addition to ergosterol, fungisterol and 22-dihydroergosterol were identified in extracts of the mushroom *Agaricus campestris* (12). Ergosterol was absent from extracts of *Fomes applanatus*, and both fungisterol (13) and 5-dihydroergosterol (14) have been reported as the most abundant sterols in this species. Fungisterol was accompanied by lower concentrations of its diunsaturated isomer, ergosta-$\Delta^{7,16}$-dienol, which was the first report of a naturally occurring sterol with a double bond in the Δ^{16} position (13).

Few Heterobasidiomycetes have been examined, but it appears that the sterols produced by these fungi are qualitatively different from those of other fungi. Only the uredospores of *Puccinia graminis* and *Melamspora lini* have been examined previously, but stigmast-Δ^7-enol and related C_{29} sterols are predominant in these species (15-17).

This article concerns the identification of spore sterols of several fungal species selected from the major fungal classes. Also, the application of glass capillary columns (gas liquid chromatography [GLC]) to sterol analyses is presented.

EXPERIMENTAL PROCEDURES

Fungal Materials

Spores produced by the following fungi were examined: *Linderina pennispora* Raper and Fennell, Northern Illinois University (NIU) fungal stock culture collection, NIU I-100, *Spicaria elegans* (Corda) Hartz NIU I-134, *Penicillium claviforme* Bainier NIU-66, *Aspergillus niger* van Tiegh NIU-10, *Ustilago nuda, Ustilago maydis* (DC) Cda., *Puccinia graminis* var. *tritici*, and *Puccinia striiformis* (Table I shows classifi-

[1]One of eight papers presented in the symposium "Phytosterols," AOCS Spring Meeting, New Orleans, April 1973.

TABLE I

4-Desmethyl Sterols Isolated from Fungal Spores of Selected Genera

Fungal species	4-Desmethyl sterols[a]						
	I	II	III	IV	V	VI	VII
Phycomycete							
Linderina pennispora[b]	-	+[c]	+	-	-	+	-
Fungi imperfecti							
Spicaria (Paecilomyces) elegans	-	-	+	+[c]	+	-	-
Penicillium claviforme	-	+[c]	+	+	-	-	+
Aspergillus niger	-	-	+	+[c]	+	-	-
Basidiomycetes							
(Smuts)							
Ustilago nuda	-	+	+	+[c]	-	+	-
Ustilago maydis	+	+	-	+[c]	+	-	-
(Rusts)							
Puccinia graminis var. *tritici*	-	+	-	-	-	+[c]	+
Puccinia striiformis	-	+	-	-	-	+[c]	+

[a]I–Cholest-Δ^5-enol (cholesterol), II–ergost-Δ^7-enol (fungisterol), III–diunsaturated C_{28} sterol, IV–ergosta-$\Delta^{5,7,22}$-trienol (ergosterol), V–tetraunsaturated C_{28} sterol, VI–stigmast-Δ^7-enol, VII–diunsaturated C_{28} sterol.

[b]Contained evidence of saturated C_{28} (M^+ 402) and C_{29} (M^+ 416) sterols.

[c]Predominant sterol.

cation of these species). The Phycomycete and Deuteromycete fungi were grown in petri plates containing the media described below with agar added. Conidia of these fungi were harvested by suspending them in water and collected by low speed centrifugation. Smut and rust spores were collected as described previously (18).

Saccharomyces carlsbergensis (518) was cultured in 14 liter vats containing the following (g/liter): dextrose, 20, NH_4NO_3, 0.6, KH_2PO_4, 5, $MgSO_4 \cdot 7H_2O$; 1 ml/liter each of trace element solutions containing the following (g/liter): H_3BO_3, 0.114; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.484; $CuSO_4 \cdot 5H_2O$, 0.78; $MnCl_2 \cdot 4H_2O$, 0.144; $ZnSO_4 \cdot 7H_2O$, 16.72; and $FeCl_3$ solution, 1.92. The cultures were aerated constantly over a 4 day growth period. The cells then were harvested by low speed centrifugation and dried by lyophilization prior to extraction.

Extraction Procedures

Spores (1-5 g) were suspended in chloroform (50 ml) with sufficient neutral alumina (10-15 g) to make a thick paste and then ground in a Sorvall Omnimix homogenizer for 15 min. The reservoir containing the cell suspension was immersed in an ice-water bath to prevent overheating. The suspension was taken up in 100 ml $CHCl_3$:MeOH (2:1) and extracted by gentle heating, with constant stirring for 1 hr. The extract was centrifuged at low speeds to remove cell fragments and alumina, then taken to dryness under nitrogen. The total lipid residue was refluxed in 100 ml of 10% KOH in ethanol:H_2O (9:1) for 12-14 hr. The nonsaponifiable fraction was extracted with n-hexane and then taken to dryness as before. The yeast cells were treated in a similar manner.

Thin Layer Chromatography (TLC)

The 4-desmethyl sterols contained in the nonsaponifiable residue were isolated by TLC. A portion of the nonsaponifiable fraction was applied to a precoated silica gel plate (Eastman Kodak, Rochester, N.Y.) and allowed to develop in petroleum ether:diethylether:acetic acid (89:10:1). The 4-desmethyl sterols present were identified tentatively by cochromatography with ergosterol, which was located by spraying the plate with Rhodamine 6G.

Preparation and Operation of Glass Capillary Columns

Pyrex tubes of ca. 1.25 m, 7.8 mm outside diameter, and 3.3 mm inside diameter, were washed with acetone, methylene chloride, 1% aqueous KOH, and methanol, then dried under vacuum. The tubes then were drawn to capillary dimensions using a Hupe and Busch (Karlsruhe) drawing and coiling apparatus (19). Capillary columns were 43 m in length, with a 1.0 mm outside diameter, and 0.5 mm inside diameter. Silanization was accomplished by passing a 10-15% (v/v) solution of dimethyldichlorosilane in toluene through the column which was thoroughly washed with toluene. The column was given a final wash with methanol and dried with nitrogen gas. It then was coated using a method described by Merle d'Aubigne, et al. (20). The first coating consisted of GE SE-30 on a suspension of Silanox

(6-10 μ; grade 101), and a second coating was applied with a solution of a GE SE-30 (2%) in isooctane, as described by German and Horning (21). A silanized glass injection port was used with a splitter. Carrier gas flows to the column and splitter, as well as hydrogen to the flame, were controlled and monitored with Matheson Mass Flow Controllers. Chromatographic conditions are described in Figure 1.

GLC

Individual sterol components of the rust spores were separated using an F&M 400 gas chromatograph equipped with a 3 m x 4 mm glass column packed with 2% GE SE-30 on Chromosorb Q. The oven temperature was 240 C isothermal with the injection port and the detector at 250 C.

Mass Spectrometry

The components of 4-desmethyl sterols isolated from the TLC plates were analyzed (as mixtures) using a duPont 21-491 mass spectrometer. The sterol mixtures were introduced into the mass spectrometer by solid probe injector operated at 250 C. All spectra were obtained at 70 eV.

RESULTS

Fungal Spore Sterols

The 4-desmethyl sterols were isolated from fungal spores and analyzed collectively by mass spectrometry. Sterols identified from the eight species examined in this study are given in Table I. Each species contained three to five 4-desmethyl sterols, with one sterol always present in much higher relative concentrations than the others. The most abundant sterol in extracts of *L. pennispora*, a Phycomycete, had a molecular ion at M^+400, which is indicative of a monounsaturated C_{28} sterol. Prominent ion fragments at m/e 273 (M^+-side chain) and 255 (M^+-[side chain + H_2O]) suggest that this sterol has a saturated side chain and a single double bond in the ring structure. Additional ion fragments which appear to be associated with this sterol are m/e 385 (M^+-CH_3), 367 (M^+-[CH_3 + H_2O]), 231 (M^+-[side chain + C_{15}-C_{17}]), and 213 (M^+-[side chain + C_{15}-C_{17} + H_2O]) and are indicative of a Δ^7-C_{28} sterol. This sterol, ergost-Δ^7-enol, commonly is called fungisterol. The ion fragments which would be expected for a monounsaturated C_{28} sterol with a double bond in the Δ^5 position were not found.

The 4-desmethyl sterol fraction of *L. pennispora*, as in several other fungi discussed below, had in its mass spectrum a molecular ion at M^+398 which suggests the presence of a diunsaturated C_{28} sterol. For each of the fungi analyzed, a diunsaturated isomer was a minor component of the sterol function. Ca. nine C_{28} sterols containing two double bonds in their structure have been identified as fungal products (1). It is not possible to distinguish their structure, since they are present in such low relative concentrations and their mass spectra are similar to each other, as well as to that of the major sterol component. Additional separation techniques would have to be applied to the sample prior to further analysis to determine the double bond position. However, prominent ion fragments at m/e 271, 253, and 229 were present in the mass spectrum of sterols from *L. pennispora* which suggest that the double bonds may be located in the ring structure. No evidence of ergosterol was found in the spores of *L. pennispora*. However, other sterols seem to be produced by this fungus since low intensity molecular ions at M^+402 and M^+416 were present, indicative of saturated C_{28} and C_{29} sterols, respectively. Ion fragments expected for a fully saturated C_{29} sterol were detected at m/e 401 (M^+-CH_3), 387 (M^+-[CH_3 + H_2O]), 341, 316, 275, 257, 233, and 215. Many of these ion fragments also correspond to those expected for a C_{28} saturated sterol. A small molecular ion at M^+414 indicative of a monounsaturated C_{29} sterol also was present.

Conidia of *S. elegans* contained three sterol constituents: a major component having a molecular ion at M^+396 and two minor components with molecular ions at M^+398 and M^+394. The sterol corresponding to M^+396 appears to be ergosterol. The expected ion fragments for this sterol at m/e 363 (M^+-[CH_3 + H_2O]), 337 (M^+-[C_1-C_3 + H_2O]), 271, 251, 229, and 211 were present and correspond closely with those of an ergosterol standard. M^+394 is indicative of a tetraunsaturated C_{28} sterol. The exact location of the double bonds cannot be determined from these data; but, because of the presence of ion fragments at m/e 251 and 227, three of the double bonds may be in the ring structure of this sterol. A diunsaturated C_{28} sterol also appeared to be present in these spores (M^+398).

Conidia produced by *P. claviforme* contained C_{28} sterols having molecular wt of 396 and 398, but the predominant component appeared to be fungisterol with a mol wt of 400. The presence of a molecular ion at M^+400 and prominent ion fragments at m/e 385, 273, 255, 231, and 213 support this identification. The molecular ion at M^+396 and expected ion

fragments characteristic of ergosterol also were present in this fungus. A diunsaturated C_{28} sterol was suggested by an M^+398 molecular ion. In addition to the three C_{28} sterols, these conidia contained a component (M^+412) indicative of a diunsaturated C_{29} sterol.

The conidia of *A. niger* contained at least three C_{28} sterols. The most abundant of these (M^+396) appeared to be ergosterol, as described above. The second in abundance appeared to be a tetraunsaturated sterol (M^+394), but the location of the double bonds could not be determined by these methods. However, the ring structure appeared to contain three of the four double bonds. There was also evidence of a diunsaturated C_{28} sterol (M^+398) as a minor component in this fungus.

The teliospores of two smut fungi and uredospores of two rust fungi collected from their natural host tissues also were analyzed for their 4-desmethyl sterol constituents. In the teliospores of *U. nuda*, evidence for the presence of ergosterol and fungisterol was found, i.e. molecular ion and expected ion fragments. Ergosterol appeared to be the major sterol in these spores. Two minor components with molecular ions of M^+398 and M^+414 also were detected.

The sterol composition of teliospores of *U. maydis* differed from that of *U. nuda*. The most striking difference was the presence of cholesterol (cholest-Δ^5-enol) in *U. maydis* (indicated by a base peak at M^+386 and the expected ion fragments m/e 371, 368, 353, 301, 273, 255, and 247). However, the most abundant sterol found in the spores of *U. maydis* was ergosterol, with fungisterol as the minor component.

The sterols of the two rust species, *P. graminis* var. *tritici* and *P. striiformis*, were qualitatively and quantitatively similar to each other but differed considerably from those of other fungi examined in this study. The most significant difference between the rust spores and the other spores examined was the abundance of C_{29} sterols and the absence of ergosterol in the rusts. Preliminary gas chromatographic analysis revealed the presence of at least two sterols having a concentration ratio of 5:1 in *P. graminis* and ca. 9:1 in *P. striiformis*. Mass spectrometric analysis of the sterol fractions from these spores revealed that the predominant component had a molecular ion at M^+414, indicative of a monounsaturated C_{29} sterol. Ion fragments at m/e 399 (M^+-CH_3), 273, 255, 231, and 213 indicated a Δ^7-C_{29} sterol (stigmast-Δ^7-enol). The minor C_{29} component is thought to be its diunsaturated isomer having a mol wt of 412. The exact location of the double bonds could not be determined from these data, but no evidence indicating Δ^5 or $\Delta^{5,22}$ unsaturation was found when the mass spectrum was compared to that of a stigmasterol (stigmast-$\Delta^{5,22}$-dienol) standard. The minor peak indicated by gas chromatography may represent a mixture of two diunsaturated C_{29} sterols.

A molecular ion at M^+400 in the mass spectrum of the sterol fraction from each of the rust species suggests, as before, that a monounsaturated C_{28} sterol is a product of these fungi. The expected ion fragments for ergost-Δ^7-enol also were present.

Separation of Yeast Sterols

The total sterols isolated from *S. carlsbergensis* could not be resolved completely using conventional gas chromatographic glass columns. However, the presence of four compounds was indicated, as shown in the upper gas chromatogram of Figure 1. Mass spectrometric analysis of this sample confirmed the

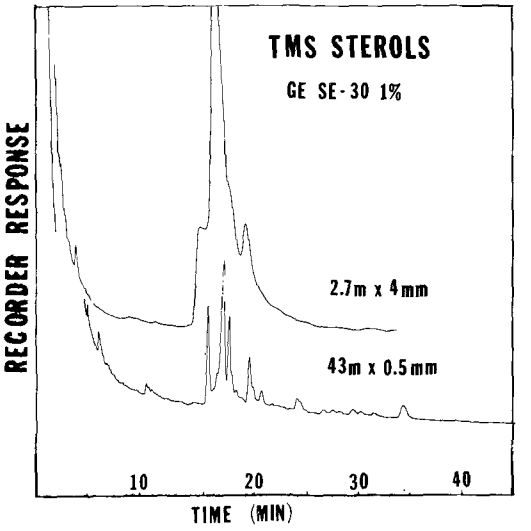

FIG. 1. Separation of the total sterols isolated from yeast (*S. carlsbergensis*) as trimethylsilyl (TMS) derivatives using packed glass (upper trace) and glass capillary (lower trace) columns. The packed column (1% GE SE-30 on 80-100 mesh Chromosorb Q) was operated isothermally at 240 C using a Hewlett-Packard 7620 gas chromatograph with hydrogen flame detectors. Helium carrier flow was 40 ml/min. Injection port temperature was 260 C and the detector 300 C. No split of the GLC effluent was used. The glass capillary column was coated with ca. 1% GE SE-30 and operated isothermally at 260 C. A Hewlett-Packard 5750 gas chromatograph with flame detectors were used. Carrier helium was maintained at a flow rate of 4.5 ml/min. Hydrogen flow was at 54 ml/min, and the splitter flow was 64 ml/min. The injection port was maintained at 250 C and the detector at 290 C. The major peak is the trimethylsilyl ether of ergosterol.

presence of at least four sterols: ergosterol, a diunsaturated C_{27} sterol, possibly a diunsaturated C_{28} sterol, and a compound having the same mol wt as lanosterol. The complexity of this sample is illustrated by the lower gas chromatogram of Figure 1, where resolution was obtained using a 43 m x 0.5 mm (inside diameter) glass capillary column. The four major constituents were resolved completely, and the presence of several additional minor components also was detected.

DISCUSSION

The sterols from conidia of *L. pennispora* were qualitatively different from those of related species belonging to the order Mucorales (class Zygomycete) in that no ergosterol was present. The predominant sterol found in spores from this species was fungisterol. Spore sterols have been examined in only one other member of the Mucorales. Sterols of sporangiospores of *Rhizopus arrhizus* Fischer were qualitatively identical and in the same relative proportions as those found in the mycelium, i.e. ergosterol, fungisterol, 5-dihydroergosterol, and ergosta-$\Delta^{5,7,14}$-trienol (8). It has been shown that quantitative changes in the sterol composition may accompany fungal spore germination (15,20,22).

Evidence for the presence of fully saturated C_{28} and C_{29} sterols also was found in the conidia of *L. pennispora*. Sterols of this type have not been reported previously as fungal products. The primitive parasitic fungus *Plasmodiophora brassicae* (Woronin) is the only other Phycomycete fungus whose resting spores have been analyzed, and no sterols considered to be fungal products were detected (23). It also has been reported that the Peronosporales studied do not produce sterols (5,24).

The sterols of *S. elegans* (mycelia) have not been reported previously, but its conidial sterols were similar to those produced by the related species, *A. niger*. A number of *A. niger* strains, cultured commercially for citric acid production, reportedly produce ergosterol as the sole sterol; but Barton and Bruun (25) have identified ergosta-$\Delta^{5,7,14,22}$-tetraenol as a minor sterol component produced by an *A. niger* isolate. This was the first recorded occurrence of a tetraunsaturated sterol as a natural product and the first containing the Δ^{14} double bond. This agrees with the results obtained in this study for the conidia of *A. niger*. Ergosterol was the predominant sterol, and there was evidence for a tetraunsaturated sterol as a minor component. Positive identification of this compound has not been made, but preliminary evidence that the ring structure contains three double bonds is consistent with the $\Delta^{5,7,14,22}$ configuration. Ergosterol has been reported as the principal sterol in extracts of *A. flavus*, and it was accompanied by two minor constituents believed to be artifacts of the experimental procedures (ergosterol peroxide and cerevisterol [26]). Rambo and Bean (27) reported ergosterol in higher concentrations than 22-dihydroergosterol in *A. flavus*.

The conidia produced by *P. claviforme* in this study differed from other imperfect fungi in having fungisterol as the most abundant sterol. The mycelia of *P. funiculosum* contained cholesterol as the sole sterol (28). No evidence of choesterol was present in the conidia of *P. claviforme*. Conidia of *P. claviforme* also appear to contain a C_{29} sterol, while the other imperfect fungi of this investigation contained an unidentified diunsaturated C_{28} sterol.

The basidiomycetous fungi selected for this study are of the subclass Heterobasidiomycetae and include the smut and rust fungi which generally are considered obligate plant pathogens. Techniques for the laboratory culture of these fungi have been developed recently, but these cultures do not produce spores in sufficient quantities for analysis. Hence, spores of these fungi must be collected from infected tissues. The evidence obtained in this and another investigation (15) suggests that no significant contamination with sterols of the host tissues occurs during spore formation or harvesting.

Sterol constituents of spores produced by fungi of the Ustilaginalés have not been reported previously. Ergosterol was the major sterol of teliospores of *U. maydis* and *U. nuda*, and fungisterol was present in lower relative proportions. These species could be distinguished from each other on the basis of their minor sterol constituents. For example, the corn smut spores contained cholesterol which appears to be of rare occurrence in most fungi (1).

The uredospores of *P. graminis* var. *tritici* and *P. striiformis* show remarkable similarity in sterol composition which was similar to those previously reported for rust spores (15-17). The most abundant sterol in these species was the C_{29} sterol stigmast-Δ^{7}-enol, as is also the case in wheat stem rust (15) and flax rust (*Melamspora lini* [PERS.]) Lev. (16) uredospores. The wheat stem rust spores used by Nowak, et al., (15) also contained, as minor components, cholesterol, an unknown sterol, and ergost-Δ^{7}-enol or stigmasterol (identified by GLC retention times only). In an earlier report (7), ergost-Δ^{7}-enol was identified as the major sterol of this

species. There was evidence of a diunsaturated C_{29} sterol in each of the rust species studied here. Its identity could not be determined from the data in this investigation, but it may be the same as that reported by Jackson and Frear (16) who identified stigmasta-$\Delta^{7,24(28)}$-dienol and possibly stigmasta-$\Delta^{5,7}$-dienol as minor constituents of *M. lini*. *Puccinia graminis* and *P. striiformis* also contained ergost-Δ^{7}-enol as a minor constituent, which is consistent with previous studies with *P. graminis* (15,17). This sterol was not reported in flax rust spores (16).

Too few fungal species have been examined to draw well defined conclusions concerning the distribution of sterols among the various taxonomic groups. However, now that a few species from each of the major fungal taxa have been examined using modern analytical techniques, certain generalizations can be made. It is evident that sterol extracts from fungi are much more complex than was once believed. C_{28} Sterols are produced by most species, and, when present, ergosterol tends to occur in higher relative concentrations than other sterols with the same number of carbon atoms. Although it is seldom the most abundant sterol, fungisterol seems to be encountered as frequently, if not more often, than ergosterol in extracts of species from various fungal taxa. Furthermore, Δ^{7} sterols are predominant in most fungi. However, species belonging to certain lower Phycomycete classes produce C_{27}, C_{28}, and C_{29} sterols with the predominant point of unsaturation in the Δ^{5} position. These data tend to support the proposal of Bartnicki-Garcia (29) that fungi belonging to the class Oomycetes evolved independently of the other Phycomycetes and more advanced fungi. This proposal is based primarily upon differences in the sedimentation properties of tryptophan synthesizing enzymes, pathways of lysine biosynthesis, and chemical composition of the cell walls.

C_{29} Sterols accumulate in the spores of rust fungi. The available evidence suggests that these sterols are not contaminants from the host tissues, but the extent to which the host influences sterol biosynthesis in the rust fungus is not known. It will be interesting to see the sterol distribution and biosynthesis in rust fungi presently being grown on synthetic media in the laboratory.

Perhaps the most significant point to be stressed is the absence of ergosterol from species belonging to two apparently distantly related groups of fungi; the aquatic Phycomycetes and the rust fungi. This could have taxonomic or phylogenetic implications which may become evident when more species are examined to determine their sterol composition. Also, species belonging to certain groups of the class Oomycete apparently do not produce sterols.

The problem of poor resolution of sterols obtained by conventional GLC techniques has been solved partially by a preliminary fractionation of the total sterol extract according into the 4,4-desmethyl, 4α-methyl, and 4-desmethyl classes and by degree of unsaturation, using alumina, or silica gel impregnated with silver ions. Nevertheless, without repeated GLC analysis using several liquid phases, complete resolution of the individual components of each fraction is often difficult. The use of glass capillary columns holds great potential for resolving complex mixtures of sterols.

ACKNOWLEDGMENTS

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Phytosterol Side Chain Biosynthesis[1]

L.J. GOAD, J.R. LENTON[2], F.F. KNAPP[3], and T.W. GOODWIN, Department of Biochemistry, University of Liverpool, Liverpool L69 3BX, England

ABSTRACT

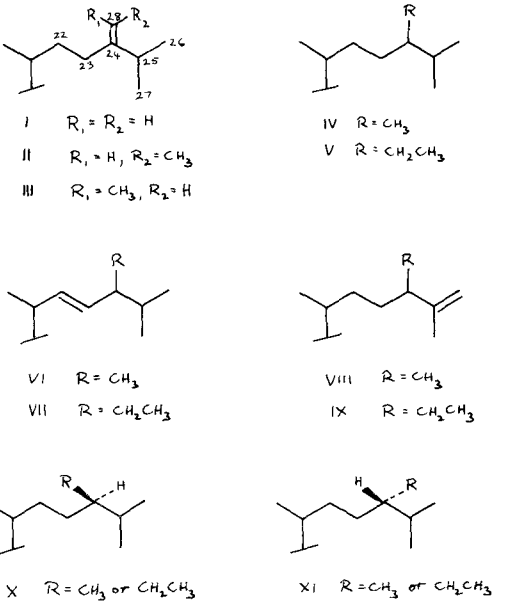
The typical plant sterols contain a substituent at C-24 of the side chain. This can be a methylene, ethylidene, methyl, or ethyl group; with the last three groups, all possible isomers have been reported in nature. The C-24 alkyl groups are derived by a transmethylation reaction from methionine. The details of several distinct alkylation mechanisms, which are now recognized in a range of lower and higher plants, have been reviewed. The operation of these different routes may have some phylogenetic significance.

INTRODUCTION

The biosynthesis of sterols in plants has attracted a great deal of attention in recent years (1-6). Among the most interesting facets of the problem to emerge have been the involvement of cycloartenol (4,6) and the C-24 alkylation mechanisms (2,6) operating in phytosterol elaboration. Investigations on the latter problem have proved particularly interesting, and our concepts of the C-24 alkylation mechanisms have needed continual revision and expansion as new experimental results have been obtained using a diverse array of organisms. Indeed, it now has become clear that several phytosterol side chain alkylation mechanisms have evolved in nature (6). The purpose of this article is to review our present state of knowledge in this field, emphasizing possible phylogenetic implications and including recent relevant observations from our laboratory.

PHYTOSTEROL SIDE CHAIN STRUCTURE

It is in the side chain structure that the most striking differences are apparent between the sterols produced by different classes of organisms. Four basic side chain types can be recognized in typical phytosterols. The first type comprises compounds with a 24-methylene (I) or 24-ethylidene (II and III) group. The second has a C-24 methyl (IV) or ethyl (V) substituent, while the third and fourth types, in addition to the C-24 alkyl group, have a double bond at the Δ^{22} (VI and VII) or Δ^{25} (VIII and IX) position, respectively. The introduction of these C-24 groups presents the possibility of isomerization. In the 24-ethylidene sterols both the Z (II) and E (III) configurations are naturally occurring (6-8). Differentiation between the E and Z configurations can be made by gas liquid chromatography (GLC) (9) and mass spectrometry (MS) (10), but the most satisfactory method, if sufficient pure sterol is available, is by NMR spectrometry (7,8,11).



Structures I-XI

The presence of a C-24 methyl or ethyl group produces chirality at the C-24 carbon, and both stereoisomers are known to occur naturally (6). Configurations at C-24 originally were designated (12) as 24α-(X) or 24β(XI), and this nomenclature still is preferred by some authors (13). However, the International Union of Pure and Applied Chemistry/International Union of Biochemistry (14) recommends the use of the 24R (X) and 24S (XI) conventions; but this has the disadvantage that introduction of a Δ^{22} bond reverses the assignment from

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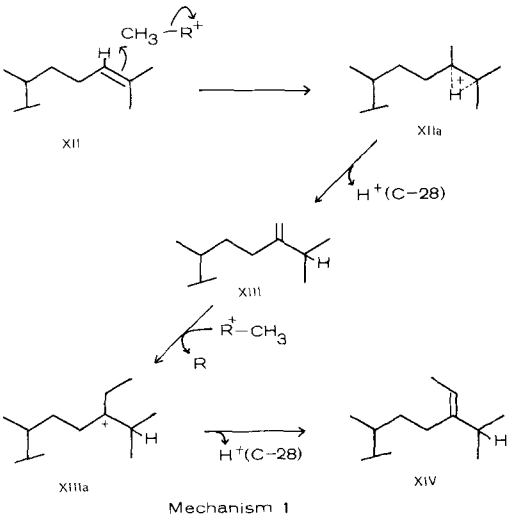
[2]Present address: Department of Botany, Rothamsted Experimental Station, Harpenden, Herts, England.

[3]Present address: Department of Biochemistry, Rice University, Houston, Texas, 77036.

24R to 24S or vice versa due to the change imposed by the Δ^{22} bond on the priorities of C-23 and C-25 (6,15). An examination of the phytosterol literature reveals that the C-24 configuration has not been established firmly in many cases, and attention has been drawn to this fact by other authors (13,16,17). Since a knowledge of the C-24 configuration is important to a complete understanding of the alkylation mechanism operative in an organism and also may be of phylogenetic significance, future phytosterol analysis should make the unambiguous determination of this point a major aim. However, it can be stated that, where C-24 configurations have been rigorously determined, the majority of fungal and algal sterols are 24β (or 24S—this applies to sterols with a saturated side chain; Δ^{22} sterols will be 24R, although still 24β- by the old nomenclature), while most higher plant sterols are 24α (or 24 R) (6,17). Such a division is attractive phylogenetically; but unfortunately far too few species, particularly algal, have had their sterols sufficiently well characterized to permit the above to be regarded as a general rule at present. Indeed, some apparent exceptions have been reported (3,6).

The C-24 alkyl isomers are difficult to differentiate and cannot, as yet, be separated by thin layer chromatography (TLC) or GLC. In the past, identifications have relied upon melting points and optical rotations, but for some isomeric pairs such differences are rather small and require absolutely pure sterol samples to be valid. NMR spectroscopy now has been demonstrated (13,18, and I. Rubinstein and L.J. Goad, unpublished results) as a reliable method for the differentiation of isomeric pairs of sterols (Table I). The 100 MHz (13) and particularly the 220 MHz (18 and I. Rubinstein and L.J. Goad, unpublished results) spectra reveal diagnostic differences in the resonances of the side chain methyl groups which permit the unambiguous assignment of C-24 configuration of an unknown sterol when its spectrum is compared with the spectrum of the appropriate authentic reference compound. The development of more refined silver nitrate-silica gel chromatography systems (19 and I. Rubinstein, Z.A. Wojciechowski, and L.J. Goad, unpublished results) for the separation of complex sterol mixtures coupled with NMR spectroscopy should now permit C-24 configurations of phytosterols from various sources to be more firmly established.



Mechanism 1

ALKYLATION MECHANISMS

The alkylation mechanisms first proposed (20) (Mechanism 1) postulated that methionine acted as the methyl group donor with a Δ^{24} sterol (XII) as the substrate. The 24-methylene compound (XIII) produced then could undergo a second transmethylation to give, for example, a 24-ethylidene side chain (XIV). While the essential features of this mechanism have been confirmed experimentally in a number of species (2,6), it is now clear that the stabilization of cations (XIIa) and (XIIIa) can follow various routes which are species-dependent (6).

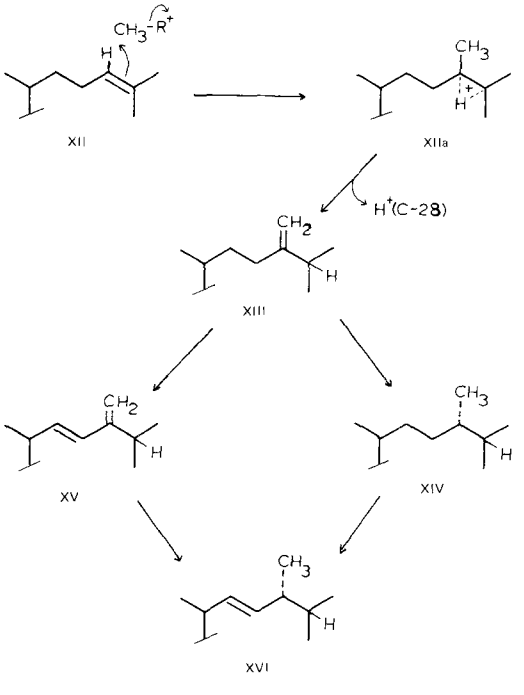
TABLE I

Isomeric Pairs of Sterols Which Can Be Differentiated by Their NMR Spectra

Sterols	Reference
Campesterol/22,23-dihydrobrassicasterol	13,18
22,23-Dehydrocampesterol/brassicasterol	18
(24R)-24-Methyl-5α-cholesta-7,22-dien-3β-ol/(24S)-24-methyl-5α-cholesta-7,22-dien-3β-ol	18
Stigmasterol/poriferasterol	13,18
Sitosterol/clionasterol	13,18
α-Spinasterol/chondrillasterol	13,18

EUMYCOTA

Using *Neurospora crassa*, it was established (21) that ergosterol biosynthesized in the presence of [CD_3]-methionine contained two deuteriums (Table II), thereby invoking a 24-methylene intermediate (Mechanism 2). In yeast, the isolation of 24-methylene sterols (22), the conversion of labeled 24-methylene sterols into ergosterol (23-25), and the demonstration (26) of a 1,2-hydride shift from C-24 to C-25 during ergosterol formation all support the operation of Mechanism 2. Also, a microsomal S-adenosyl methionine-Δ^{24} sterol methyl transferase has been prepared from yeast (27) and has permitted verification that a Δ^{24} sterol substrate (XII) is transmethylated to give a 24-methylene product (XIII).



Mechanism 2

The sterols of Ascomycetes, Basidiomycetes, and Deuteromycetes are typically C_{28} compounds (side chains: I; IV, 24β or 24S; VI, 24β or 24R), with ergosterol the most commonly encountered compound (6,28). It, thus, appears that, while these classes of fungi can perform the first transmethylation step to give C_{28} sterols, the second transmethylation required to produce C_{29} sterols (side chains II, III, V, VII, or IX) is of little significance and may not even be operative. Evidence favoring the general

TABLE II

Summary of Number of Deuterium Atoms from [CD_3]-Methionine Incorporated into C-24 Alkyl Groups of Phytosterols

Division[a]	Class	Order	Species	Sterol	Maximum number of deuterium atoms	Reference
Myxomycota	Myxomycetes	Physarales	*Physarum polycephalum*	stigmastan-3β-ol	5	2
	Acrasiomycetes	Acrasiales	*Dictyostelium discoideum*	stigmast-22-en-3β-ol	5	29
Eumycota	Ascomycetes	Xylariales	*Neurospora crassa*	ergosterol	2	21
			Gliocladium roseum	ergosterol	2	30
	Basidiomycetes	Polyporales	*Polyporus sulphureus*	eburicoic acid	2	31
			Daedalea quercina	pachymic acid	2	2
	Deuteromycetes	Moniliales	*Oospora virescens*	ergosterol	2	32

Lichens	Ascomycete (mycobiont)	Parmeliales	*Xanthoria parietina*	ergosterol	2	33
				lichesterol	2	33
Chrysophyta	Chrysophyceae	---	*Ochromonas malhamensis*	poriferasterol	4	34
				brassicasterol	2	b
			O. sociablis	poriferasterol	4	b
			O. danica	poriferasterol	4	
				clionasterol	4	2
				7-dehydro-poriferasterol	4	b
				brassicasterol	2	
				22,23-dihydro-brassicasterol	2	
	Bacillariophyceae		*Phaeodactylum tricornutum*	(24R)-24-methyl-cholesta-5,22-dien-3β-ol	2	35
Chlorophyta	Chlorophycea[c]	Chlorococcales	*Chlorella vulgaris*	chondrillasterol	5	36
				22,23-dihydro-chondrillasterol	5	
				ergost-7-en-3β-ol	3	
			Chlorella ellipsoidea	poriferasterol	5	37
				ergost-5-en-3β-ol	3	
			Scenedesmus obliquus	Chondrillasterol	5	
				22,23-dihydro-chondrillasterol	5	b
				ergost-7-en-3β-ol	3	
			Trebouxia sp. 213/3	poriferasterol	5	
				clionasterol	5	38
				ergost-5-en-3β-ol	3	
Anthophyta	Monocotyledonae	Graminales	*Hordeum vulgare*	stigmasterol	4	
				sitosterol	4	c
				campesterol	2	

[a]The classification used in this table and throughout the review is based upon that of Scagel, et al. (44).

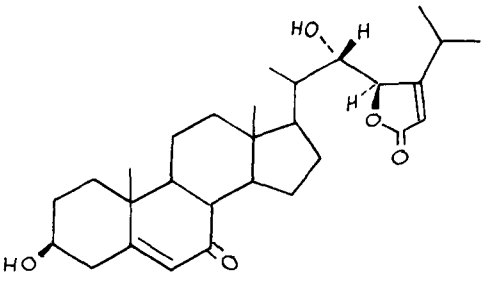
[b]W. Sach and L.J. Goad, unpublished results.

[c]J.R. Lenton, L.J. Goad, and T.W. Goodwin, unpublished results.

operation of Mechanism 2 in Ascomycetes, Basidiomycetes, and Deuteromycetes is provided by the occurrence in these classes of several 24-methylene compounds (6,39,40) and by limited labeling studies employing [CD_3]-methionine (Table II). Lichens, an algal-fungal symbiotic association, contain complex mixtures of both C_{28} and C_{29} sterols (41-43), and the question arises which of these sterols are produced by the fungal and algal symbionts. The lichen mycobiont is commonly an Ascomycete (44), and, in one case, *Xanthoria parietina*, the mycobiont has been cultured alone (33) and shown to produce only C_{28} sterols with ergosterol and lichesterol predominating. Moreover, 24-methylene sterols also were identified (33), and the incorporation of two deuteriums from [CD_3]-methionine into the ergosterol and lichesterol showed that Mechanism 2 was operative in this organism, bringing it into line with other Ascomycetes (Table II).

To produce ergosterol (side chain XVI), the reduction of the 24-methylene intermediate (XIII) must proceed stereospecifically to give the 24β-configuration and may precede (XIII → XVa → XVI) or follow (XIII → XVb → XVI) the introduction of the Δ^{22} bond (Mechanism 2). Both routes can operate in yeast since 24ξ-methyl-5α-lanost-8-en-3β-ol (45) and ergost-7-en-3β-ol (24) (side chain XVa) are converted into ergosterol, while sterols with side chain (XVb) have been isolated (22) from this Ascomycete and also shown to be metabolized (46) to give ergosterol. Recent evidence (47) suggests that the favored route in yeast is via the diene (XVb), and it may be that introduction of the Δ^{22} bond enhances $\Delta^{24(28)}$ reduction in a manner comparable to the sequence $\Delta^{7} \rightarrow \Delta^{5,7} \rightarrow \Delta^{5}$ observed in cholesterol biosynthesis (4).

The Phycomycetes are a diverse group of fungi in which only species belonging to the Order Mucorales produce ergosterol (48) which in the case of *Phycomyces blakesleeanus* probably is produced via a 24-methylene intermediate (49,50) by Mechanism 2. By contrast, members of the Peronosporales produce no sterol and are dependent upon a dietary source (51). Two fungi belonging to the Blastocladiales were found (52) to contain principally cholesterol but with the addition of low levels of 24-methyl- and 24-ethylcholesterol in one species. Similarly, two representatives from the Hyphochytriales contained 24-methyl- and 24-ethylcholesterol (52) (C-24 configuration unknown). Clearly, these two Orders contain the enzyme system required for the second transmethylation to produce C_{29} sterols, although the nature of the mechanism is unknown. Members of the Saprolegniales and Leptomitales also fail to produce ergosterol, but instead contain (48) cholesterol, desmosterol 24-methylenecholesterol, and 24-ethylidenecholesterol, again revealing that these fungi have evolved the second transmethylation step, which in this case proceeds by Mechanism 1. The configuration of the 24-ethylidenecholesterol was not firmly established (48); but, if it is a precursor, as seems likely, of the steroid hormone antheridiol (53) (XVII), then the E- configuration is favored. This would indicate a similarity between the second alkylation mechanism in the Saprolegniales and that found in the brown algae (Phaeophyta) which is the only Division at present known to produce a 24-ethylidene sterol with the E- configuration (17). Indeed, the production of C_{29} sterols in the Saprolegniales can be added to the other evidence, indicating a phylogenetic relationship between this class and some algae (54).

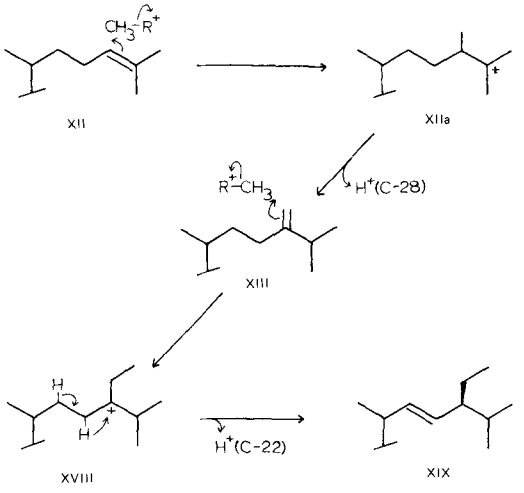


Structure XVII

MYXOMYCOTA

The organisms examined in this Division have developed alkylation mechanisms leading to both C_{28} and C_{29} sterols (55,56). *Physarum polycephalum* (Physarales) and *Dictyostelium discoideum* (Acrasiales) elaborate stigmast-22-en-3β-ol and stigmastan-3β-ol; and, when induced to ingest *E. coli* containing [CD_3]-methionine, both sterols contained five deuterium atoms (Table II). The formation of a 24-ethylidene side chain by Mechanism 1 followed by reduction to a 24-ethyl group, therefore, does not occur. An alternative mechanism for the production of the stigmast-22-en-3β-ol side chain (XIX) has been proposed (Mechanism 3) in which the cation (XVIII) is stabilized by hydride migration from C-23 to C-24 and expulsion of a proton from C-22 to produce the Δ^{22} bond as an integral part of the alkylation mechanism (55). Evidence supporting this route

in *D. discoideum* has been obtained (55), but it leaves the mode of biosynthesis of the stigmastan-3β-ol side chain unclear (55). Also, Mechanism 3 is apparently not a universal one for the introduction of the Δ^{22} bond into phytosterols in other organisms (6) and has been specifically eliminated for ergosterol production in yeast (57).



Mechanism 3

CYANOPHYTA

A number of blue-green algae now have been shown to produce small amounts of C_{27} and C_{29} sterols (6), but biosynthetic studies have not been performed, and no conclusions are possible regarding the C-24 alkylation mechanisms employed.

PYRROPHYTA AND XANTHOPHYTA

There appear to be no reports on the sterols present in species from these Divisions.

RHODOPHYTA

The most widespread sterols in this Division are C_{27} compounds, with cholesterol predominating in the majority of species examined (17) from the Orders Bangiales, Gelidiales, Cryptonemiales, Gigartinales, Rhodymeniales, and Ceramiales. Sterols with a C-24 alkyl group have only been reported in *Phormidium cruentum* (Porphyridiales) (58) and *Rytiphlea tinctoria* (Ceramiales) (59), but no studies on biosynthesis have been undertaken. At present it, therefore, appears that, for most species in this Division, sterol methylation is of little significance.

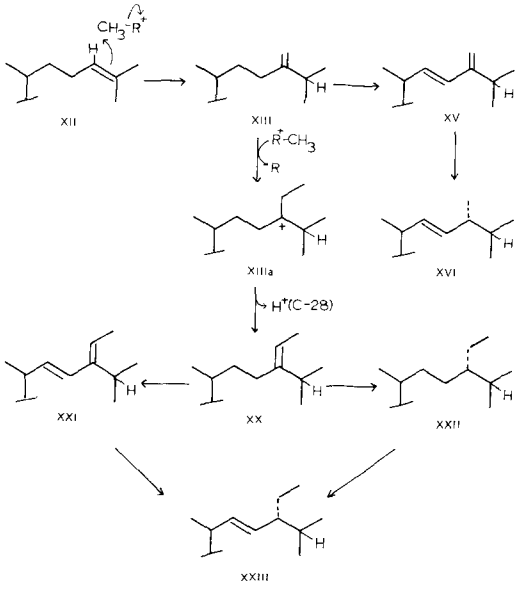
PHAEOPHYTA

In the brown algae, the 24-ethylidene compound, fucosterol, is the major sterol in all members examined of the Ectocarpales, Sphacelariales, Dictyotales, Chordariales, Dictyosiphonales, Laminariales, and Fucales (17). 24-Methylenecholesterol also has been reported in a few species (17). Biosynthesis of the fucosterol side chain can proceed by Mechanism 1 culminating in a C-28 proton elimination from cation (XIIIa) to produce a 24-ethylidene group (XIV) with the E- configuration found in fucosterol. A mechanism to explain the stereospecificity of this C-28 proton elimination has been proposed which envisages the formation of an enzyme-bound intermediate (11). The hydrogen migration from C-24 to C-25 which is required by Mechanism 1 has been demonstrated using *Fucus spiralis* (60).

CHRYSOPHYTA

The most studied alga (6) from the sterol biosynthetic view point is *Ochromonas malhamensis* (Chrysophyceae), which produces a mixture of poriferasterol (95%) and brassicasterol (5%) (61). When grown in the presence of [CD_3]-methionine, *O. malhamensis* incorporated a maximum of four deuterium atoms into poriferasterol (34) and two deuteriums into brassicasterol (W. Sach and L.J. Goad, unpublished results), and similar results have now been obtained with *O. sociablis* and *O. danica* (Table II). These observations are only consistent with the formation of 24-methylene (XIII) and 24-ethylidene (XX) intermediates, which are reduced to give the 24-methyl (XVI) and 24-ethyl (XXIII) sterols, respectively, with the required 24β (24R)- configurations (Mechanism 4). The C-24 configuration in the poriferasterol from *O. malhamensis* has been verified by NMR spectroscopy (I. Rubinstein and L.J. Goad, unpublished results) but remains to be confirmed in the brassicasterol.

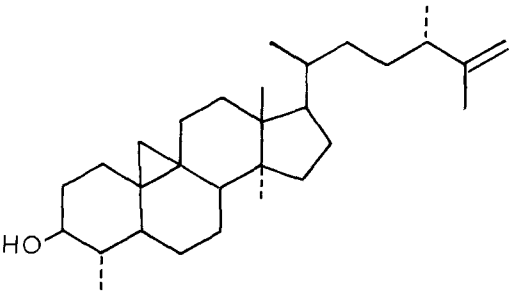
Further evidence for the precursor role of 24-ethylidene compounds in *O. malhamensis* was provided by the demonstration (62) of the hydrogen migration from C-24 to C-25 which is required by Mechanism 4. Also the incorporation of several labeled 24-ethylidene sterols into poriferasterol has been demonstrated using *O. malhamensis* (63,64). These incorporation studies (64) indicated that 28-isofucosterol (Z-configuration, side chain II) appeared to be more readily utilized for poriferasterol formation than fucosterol (E- configuration, side chain III). While this result may be a reflection of the relative stabilities of these sterols while in the growth medium or of the rates at which they



Mechanism 4

are absorbed by *O. malhamensis*, it also may represent a real specificity of the 24-ethylidene reductase for the 24(Z)-ethylidene compound. However, a second possibility exists and is suggested by an earlier observation (65) with the protozoan *Tetrahymena pyriformis*. Although unable to synthesize sterols, this organism can introduce a Δ^{22} bond into 28-isofucosterol but cannot perform this desaturation on fucosterol, presumably because of the C-29 methyl group hindering the C-22 to C-23 bond. In yeast, there is evidence (47) that 24-methylene reduction proceeds by the sequence $\Delta^{24(28)} \rightarrow \Delta^{22,24(28)} \rightarrow \Delta^{22}$ (Mechanism 2). A similar introduction of a Δ^{22} bond to facilitate 24-ethylidene reduction (XX→XXI→ XXIII, Mechanism 4) by *O. malhamensis* conceivably might explain the apparently faster rate of utilization of 28-isofucosterol (Z-) compared to fucosterol (E-), since, on the basis of the *T. pyriformis* results, the former would be more amenable to desaturation. However, even if this is the preferential route, the enzymes must be sufficiently nonspecific to allow the observed conversion of fucosterol into poriferasterol and to explain the production of clionasterol (side chain XXII) in *O. danica* (61). No 24-ethylidene sterol has been obtained from *Ochromonas* spp., and any final conclusions regarding the alkylation mechanism must await the isolation of such a compound. It is worth noting at this point that the stereochemistry of hydrogen elimination for Δ^{22} bond introduction into poriferasterol by *O. malhamensis* is opposite to that observed during ergosterol production in some fungi (6).

The incorporation of two deuterium atoms from [CD_3]-methionine into brassicasterol by the *Ochromonas* spp. (Table II) is evidence that a 24-methylene intermediate is produced. This also could be converted into the 24-methyl compound (XVI) via a $\Delta^{22,24(28)}$ (XV) intermediate (Mechanism 4). The following apparently anomalous result might then be explained on this basis. Tritium labeled 31-norcycloaudenol (XXIV) was administered to an *O. malhamensis* culture (F.F. Knapp and L.J. Goad, unpublished results) and the sterols isolated and analyzed by preparative gas liquid chromatography (Fig. 1). As expected, there was negligible radioactivity associated with the C_{29} sterols while tritium was present in a C_{28} compound. However, this had the retention time of 22,23-dihydrobrassicasterol and not of brassicasterol, the only detectable C_{28} sterol in *O. malhamensis*. Silver nitrate-silica gel thin layer chromatography also confirmed that the radioactivity was in a saturated side chain sterol. Consequently, *O. malhamensis* must have an enzyme capable of Δ^{25} reduction, although such a compound is not normally encountered by this alga. The subsequent failure of the organism to introduce the Δ^{22} bond to give brassicasterol prompts the tentative suggestion that route XIII → XV → XVI (Mechanism 4) is normally operative and that the 24-methylene group facilitates Δ^{22} bond introduction.



Structure XXIV

Few diatoms (Bacillariophyceae) have been analyzed for their sterol content. *Navicula pelliculosa* was reported (66) to contain the C_{29} compound, chondrillasterol, but this warrants reinvestigation, particularly with regard to the C-24 configuration. More recently, a C_{28} sterol has been isolated from *Cyclotella nana* and *Nitzschia closterium* and recorded as brassicasterol (67). In this case, the assignment of

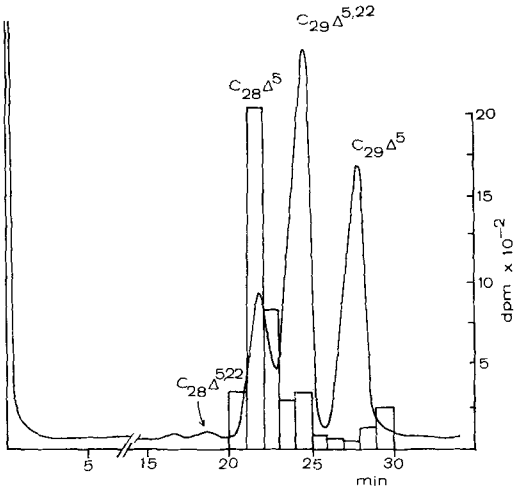


FIG. 1. Incorporation of [2,2,4-$^3H_3$]-31-norcycloaudenol into the sterols of *Ochromonas malhamensis*. A culture of *O. malhamensis* was grown for 7 days in the presence of 4.2 μCi of the tritiated 31-norcycloaudenol, and the sterols were isolated by the usual methods (1.62 x 10^6 dpm). Carrier campesterol ($C_{28}\ \Delta^5$) and sitosterol ($C_{29}\ \Delta^5$) were added to the *O. malhamensis* sterols (brassicasterol, $C_{28}\ \Delta^{5,22}$ and poriferasterol, $C_{29}\ \Delta^{5,22}$) and the mixture analyzed by preparative gas liquid chromatography (3% OV-17) with sample trapping at 1 min intervals.

C-24 configuration again probably warrants reassessment since the 24-methylcholesta-5,22-dien-3β-ol obtained from *Phaeodactylum tricornutum* has been established by NMR spectroscopy to have the 24α (24S)-configuration (35). Growth of *P. tricornutum* in the presence of [CD_3]-methionine gave a low incorporation of deuterium into the sterol but mass spectrometry showed the presence of only two deuteriums so establishing a 24-methylene intermediate (35). If sterols with the 24α-configuration can be demonstrated in other diatoms, this will be in marked contrast to the Chrysophyceae (24β-sterols) and may show that these two classes have evolved stereospecifically opposite $\Delta^{24(28)}$ reductases.

CHLOROPHYTA

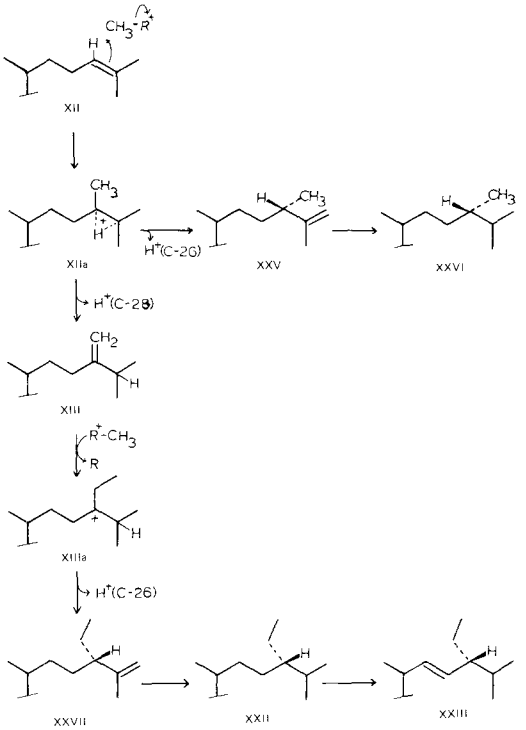
The Chlorophyta is a large diversified Division, and detailed information on sterol composition is fragmentary (17), while sterol biosynthetic studies have been limited to a few members of the Order Chlorococcales.

The Class Chlorophyceae has been the most widely studied (17). The predominant sterol in species from the Ulotrichales (11,17) is 28-isofucosterol (Z-, side chain II) so revealing an opposite stereospecificity in cation (XIIIa) stabilization from that observed in the Phaeophyta, which produce fucosterol (E-, side chain III). In view of the possible ancestral role of the Ulotrichales in higher plant development (54), this may be significant since 24-ethylidene sterols of the Z- configuration appear to be fairly widespread in vascular plants (6).

A range of 24-methyl- and 24-ethyl- sterols have been characterized from a number of species of the Chlorococcales, and all have the 24β- configuration (17). Culture of *Chlorella* spp. with [CD_3]-methionine resulted (36,37) in the incorporation of three and five deuterium atoms into the 24-methyl- and 24-ethyl sterols, respectively. This contrasts with the results observed with the Chrysophyta (loc cit) and eliminates 24-methylene and 24-ethylidene sterol precursors for the C_{28} and C_{29} sterols respectively of these Chlorococcales. This observation has been extended to other Chlorococcales species (Table II). Moreover, it has been shown that when [CD_3]-methionine is utilized by both *Scenedesmus obliquus* and *Trebouxia*, sp. 213/3, a strong hydrogen-isotope effect is apparent (38). This results in a change in the composition of the mixture. For example, in *Trebouxia*, sp. 213/3, grown with normal [CD_3]-methionine, the sterol mixture contained 77% of C_{29} sterols and 23% of C_{28} sterol, but when [CD_3]-methionine was present the mixture contained only 60% of C_{29} sterols with a corresponding increase to 40% of C_{28} sterol (38). These results can be rationalized by assuming that alkylation Mechanism 5 is functioning in these algae. Here stabilization of cation (XIIa) can proceed in two directions. Elimination of a proton from C-26 produces a 25-methylene side chain (XXV) which then can be reduced to produce the C-24 methyl sterol (XXVI). Alternatively, loss of a C-28 proton gives a 24-methylene sterol (XIII) which is then available for a second transmethylation step that can proceed via cation (XIIIa) to yield another 25-methylene intermediate (XXVII) and then give the C_{29} sterol side chains (XXII and XXIII).

Clearly the operation of Mechanism 5 will explain the retention of three and five deuteriums from [CD_3]-methionine in the C_{28} and C_{29} sterols, respectively. Also, if the loss of the C_{28} proton is the rate limiting step, then the presence of a strong deuterium isotope effect will tend to divert stabilization of cation (XIIa) toward the production of XXV); and, hence, with less 24-methylene sterol (XIII) available for the second transmethylation, this will lead to the experimentally observed increase in C_{28} sterol.

Compelling evidence for the operation of Mechanism 5 in *Trebouxia* sp. is provided by



Mechanism 5

other work. When this alga was grown in the presence of labeled 31-norcyclolaudenol (side chain XXV), only radioactive C_{28} sterol (side chain XXVI) was obtained (38) showing that Δ^{25} compounds (XXV) cannot act as precursors of C_{29} sterols. On the other hand, when tritiated cycloeucalenol (side chain XIII) was administered to a *Trebouxia* culture C_{29} labeled, sterols (side chains XXII and XXIII) were obtained, but the C_{28} sterol was devoid of radioactivity (38). This established that in this organism 24-methylene compounds (XIII) act solely as a substrate for the second transmethylation step. The actual production of 24-methylene (XIII) and 25-methylene (XXV) sterols by *Trebouxia* sp. 213/3 and *S. obliquus* has been demonstrated (68) using cell-free preparations. With cycloartenol as substrate, the products of the S-adenosyl methionine-sterol methyltransferase reaction were identified (68) as 24-methylene cycloartanol and cyclolaudenol in the ratio 76:24 which roughly corresponds to the C_{29}:C_{28} sterol proportions in these organisms (38). The intriguing question of whether the production of these two compounds is mediated by one enzyme or by two separate enzymes remains to be answered by the isolation and purification of the enzyme(s) involved. However, the isotope effect observed with [CD_3]-methionine provides limited, but not conclusive, evidence in favor of a one enzyme system. The involvement of a 24-methylene (XXVII) intermediate in C_{29} sterol (XXII and XXIII) production in the Chlorococcales gains support from the identification of 5α-stigmasta-7,25-dien-3β-ol in a triparanol-treated culture of *Chlorella emersonii* (69).

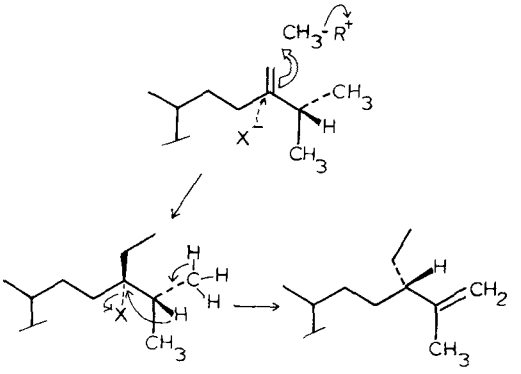


FIG. 2. Suggested mechanism for the elaboration of the 24-ethyl-25-methylene type of side chain.

One important aspect of the alkylation mechanisms discussed so far requires emphasis at this point. Both *Ochromonas* spp. (Chrysophyceae) and *Trebouxia* and *Chlorella* spp. (Chlorophyceae, Chlorococcales) produce porifersterol (side chain XXIII) with a 24β- (24R) configuration. However, the C-24 configuration apparently arises by fundamentally different mechanisms. In the *Ochromonas* spp. it is by stereospecific reduction of a 24-ethylidene group (Mechanism 4), whereas in *Trebouxia* and *Chlorella* spp. it must be regarded as a consequence of the actual transmethylation mechanism as indicated in Figure 2 which envisages active participation of the enzyme or some other X-group (6). A similar mechanism (Fig. 3) has been suggested (6) for the production of the 25-methylene side chain (XXV) with the C-24 configuration (24β or 24S) required for production of the C_{28} sterol of *Trebouxia* and *Chlorella* spp. By contrast, the 24β-configuration of the C_{28} sterols typical of many fungi arises by the stereospecific reduction of a 24-methylene sterol (XIII), as shown in Mechanism 2.

The sterol composition of species belonging to other Orders of the Chlorophyceae has received little attention (17). A member of the Cladophorales (*Chaetomorpha crassa*) contains a mixture of C_{27}, C_{28}, and C_{29} sterols (70), but information indicating the C-24 alkylation configurations or mode of biosynthesis is not

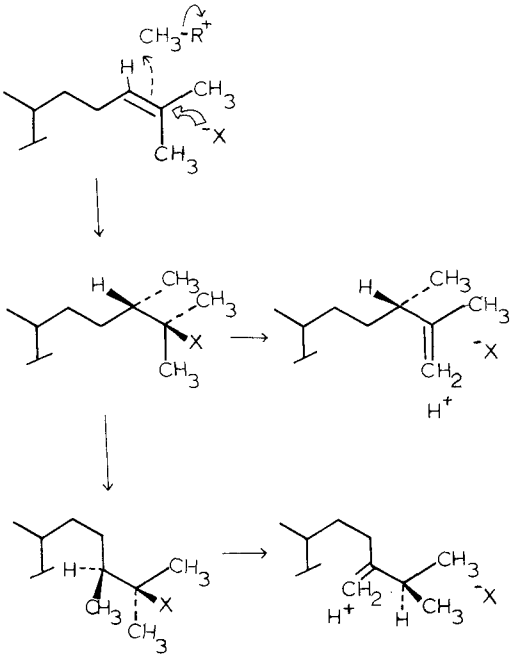


FIG. 3. Postulated mechanisms for the formation of the 24-methylene and 25-methylene sterol side chains.

available. One alga, belonging to the Siphonales (*Codium fragilis*), has been found (35,71) to contain, principally, (24S)-24-ethylcholesta-5,25-dien-3β-ol (side chain XXVII), with a small proportion (∿5%) of the hitherto unknown sterol (24S)-24-methylcholesta-5,25-dien-3β-ol (side chain XXV). These compounds probably arise by Mechanism 5 and their presence in a member of the Siphonales may be phylogenetically significant in view of the postulated decendency of this Order from the Chlorococcales (54).

Only two species from the Charophyceae have been examined, but, in both *Nitella flexilis* and *Chara vulgaris*, the major sterols were 28-isofucosterol (Z-) and clionasterol (24β or 24S) with a small quantity of 24-methylenecholesterol (72). Thus, it appears likely that Mechanism 4 has evolved in this Class of algae.

EUGLENOPHYTA

Earlier reports (17) indicated the presence of ergosterol in *Euglena gracilis*, but a more recent investigation has shown this alga to contain a more complex mixture of sterols (73). A preliminary study has shown that *E. gracilis* incorporates only four deuterium atoms from [CD_3]-methionine into a 24-ethyl sterol (D.R. Threlfall, J.R. Lenton, and L.J. Goad, unpublished results), indicating a 24-ethylidene intermediate (Mechanism 4).

BRYOPHYTA, PSILOPHYTA, LYCOPODOPHYTA, ARTHROPHYTA, AND PTEROPHYTA

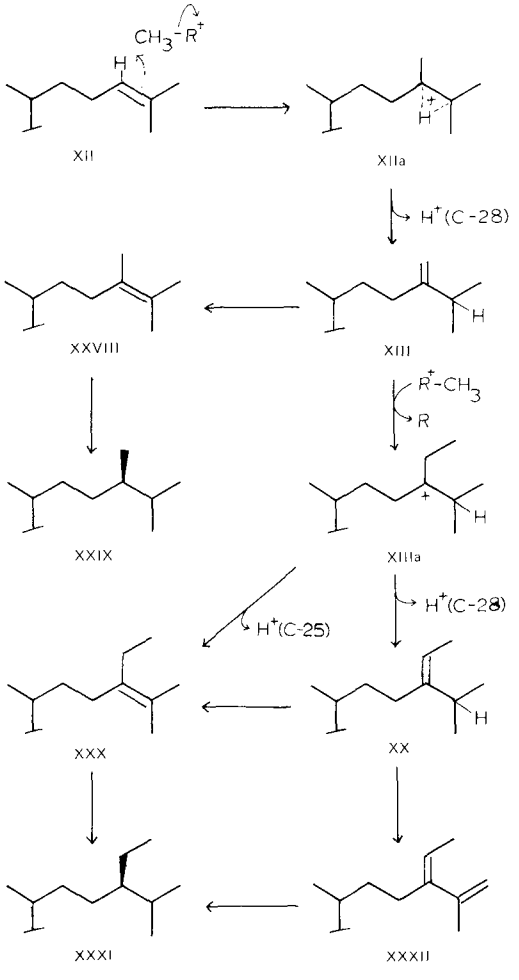
These Divisions have not been extensively examined for their sterol content, but C_{28} and C_{29} sterols have been identified in some species (6,74). Biosynthetic studies have been restricted to one member of the Pterophyta. The fern *Polypodium vulgare* (Filicales) contains cyclolaudenol and 31-norcyclolaudenol (side chain XXV), and their formation has been shown (75) to comply with Mechanism 5, with the added observation that the 25-methylene group was derived from the methyl group, which was *cis-* to the rest of the side chain in the Δ^{24} precursor (XII). There is no information on the mode of biosynthesis of the 24-ethyl sterol in this plant.

PTERIDOSPERMOPHYTA, CYCADOPHYTA, GINKGOPHYTA, CONIFEROPHYTA, AND GNETOPHYTA

For three of these Gymnosperm Divisions, the information on sterols is again scanty. The exceptions are the Ginkgophyta whose sole extant representive, *Ginkgo biloba*, contains sitosterol (76) and the Coniferophyta in which sitosterol again predominates in the plants so far examined (77,78). Biosynthetic studies have been undertaken with *Larix decidua* and a number of 24-methylene and 24(Z)-ethylidene sterols identified (78,79) which are regarded as phytosterol precursors in higher plants (6). The occurrence of these $\Delta^{24(28)}$ sterols reveals the operation of alkylation Mechanism 4 up to the production of (XX), and the required hydride migration from C-24 to C-25 has been proved for the formation of 28-isofucosterol in *Pinus pinea* (80). Evidence for the transformation of the 24-ethylidene side chain into a 24-ethyl compound was provided by the conversion of labeled 28-isofucosterol into sitosterol by *P. pinea* (81) and by the tentative evidence for the retention of only four hydrogens from the methionine methyl groups in the 24-ethyl group of sitosterol produced by *L. decidua* (3,82). However, in *L. decidua* this cannot result from a simple reduction of the 24-ethylidene side chain, since it has been indicated that the C-25 hydrogen of the 24-methylene precursor (XIII) is lost from the 24-ethyl sterol (sitosterol) produced (83). This observation can perhaps be explained by Mechanism 6 which is discussed below.

ANTHOPHYTA

No attempt will be made here to catalogue the sterols identified in the many Orders of the Dicotyledonae and Monocotyledonae, since



Mechanism 6

such a list would assume encyclopaedic proportions. The typical sterols encountered are C_{28} and C_{29} compounds with campesterol, sitosterol, and stigmasterol the most commonly reported (6). Most higher plant sterols are documented as the 24α (24R)-isomers but some 25-methylene sterols with the 24β (24S)- configuration have been reported in species from the Cucurbitaceae and Verbenaceae (6). The identification of several 24(Z)- ethylidene sterols and the incorporation of acetate and mevalonate into these compounds in higher plants (6) suggested the involvement of 24-ethylidene sterols (e.g. by Mechanism 4) in phytosterol elaboration. However, proof that the C-25 hydrogen of the 24-methylene intermediate (XIII) was lost in stigmasterol biosynthesis in *Nicotinia tabacum* and *Dioscorea tokoro* led to the suggestion (84) that a 24-ethylidene sterol is not produced, but instead cation (XIIIa) is stabilized by loss of the C-25 hydrogen to give a Δ^{24} side chain (XXX) which then is reduced to the saturated side chain (XXXI), as shown in Mechanism 6.

The loss of the C-25 hydrogen of XIII) has been confirmed for the biosynthesis of 24-methyl-5α-cholest-7-en-3β-ol, stigmast-7-en-3β-ol, and stigmasta-7,22-dien-3β-ol by *Spinacea oleracea* and *Medicago sativa* (85) and for sitosterol and stigmasterol produced by *Hordeum vulgare* (J.R. Lenton, L.J. Goad, and T.W. Goodwin, unpublished results). If route XIIIa → XXX → XXXI operates, then 24-ethylidene sterols (XX) in higher plants would have to be considered as by-products of the main biosynthetic pathway to 24-ethyl sterols (XXXI). It also would leave unexplained the high labeling of 24-ethylidene sterols from [^{14}C]-acetate and mevalonate and the demonstrated conversion of 28-isofucosterol into sitosterol by higher plants (6). Clearly, the involvement, or otherwise, of a 24-ethylidene sterol can only be decided unambiguously by determining how many of the hydrogens in the ethyl group of a 24-ethyl sterol are derived from the two methionine methyl groups utilized. Tentative evidence (82) that only four hydrogens were retained in the ethyl group of the C_{29} sterols of *Zea mays* was obtained using [CT_3]-methionine, but this result is open to the serious objection that a strong tritium isotope effect could render the results inconclusive (2). An alternative, more satisfactory, approach is to allow the organism to incorporate [CD_3]-methionine into the sterol followed by mass spectrometry (2). However, with most higher plant tissues, the relatively poor incorporation of [CD_3]-methionine into sterol, coupled with the high level of unlabeled endogenous sterol, has made impracticable the detection of the newly synthesized deuterated sterols (F.F. Knapp, J.R. Lenton, and L.J. Goad, unpublished results). To overcome this problem, isolated barley (*Hordeum vulgare*) embryos have been utilized (J.R. Lenton, L.J. Goad, and T.W. Goodwin, unpublished results), since these will develop into seedlings if cultured in a nutrient medium. Because the seedlings increase greatly in size during the culture period they presumably synthesize a relatively large quantity of new sterol which will not be diluted excessively by the endogenous sterol present in the undeveloped embryo. Moreover, the isolated embryos retain the scutellum which is responsible for the absorption of amino acids and other nutrients released in the endosperm during germination (86). Consequently, this offers an opportunity for maximal uptake of [CD_3]-methionine added to the amino acid-

nutrient medium in which the embryos are cultured. When the sterols from such an experiment were analyzed (J.R. Lenton, L.J. Goad, and T.W. Goodwin, unpublished results), the campesterol contained only two deuterium atoms proving the intermediate role of a 24-methylene (XIII) compound, while the stigmasterol and sitosterol contained species with one, three, and four deuteriums which are consistent with a 24-ethylidene (XX) precursor (Table II).

Further evidence for the importance of a 24-ethylidene (XX) intermediate was provided by the demonstration that the isolated barley embryos could absorb labeled 24(Z)-ethylidenelophenol (citrostadienol), a constituent of the barley endosperm (W. Sach and L.J. Goad, unpublished results), and convert it into sitosterol (J.R. Lenton, L.J. Goad, and T.W. Goodwin, unpublished results). In a complementary experiment (J.R. Lenton, L.J. Goad, and T.W. Goodwin, unpublished results), the fate of the C-25 hydrogen of (XIII) was examined using [2-^{14}C,(4R)-4-$^{3}H_1$] mevalonate (6). This hydrogen was retained in the isolated 28-isofucosterol (side chain XX), but, as noted in other higher plants, it had been eliminated from sitosterol (side chain XXXI) and stigmasterol. These results, therefore, indicate that in *Hordeum vulgare* and, possibly other higher plants, the biosynthesis of the sterol side chains may proceed as shown in Mechanism 6. The observations that a 24-ethylidene intermediate (XX) is involved but that the C-25 hydrogen of this compound is lost from the product 24-ethyl sterol (XXXI) are best accommodated by an isomerization of (XX) to a Δ^{24} side chain (XXX) which then can be reduced to (XXXI) with the 24α (24R)- configuration. A similar route also can give the C_{28} sterol (XIII → XXVIII → XXIX). This should be compared to the Chrysophyceae where the evidence (loc cit) suggests that direct reduction of the 24-ethylidene group, without prior isomerization, occurs to give the 24β (24S)-isomer. Sterols with the Δ^{24} side chain types (XXVIII) and (XXX) have not been reported in nature, and further consideration of Mechanism 6 must await their detection and the demonstration that they can be metabolized by plant tissues to produce the saturated side chain sterols (XXIX and XXXI). The production of a $\Delta^{24(28),25}$ diene side chain (XXXII) could also explain the experimental results obtained with *H. vulgare*, but again there is no evidence for the natural occurrence of such compounds.

With the limited information available, it is not possible to conclude how widespread in the Anthophyta are alkylation mechanisms based upon 24-methylene or 24-ethylidene intermediates. Also, it is known that Mechanism 5 is functional in at least a few cases, since 25-methylene sterols (side chains VIII and IX, 24β [24S]- configuration) have been reported in isolated species from apparently unrelated orders of vascular plants (6). Thus, evidence substantiating the operation of Mechanism 5 is reported (87) for the production of the cycloladenol side chain (XXV) in *Musa sapientum* (Monocotyledonae, Zingibarales). Also, during the biosynthesis of (24S)-24-ethylcholesta-5,22,25-trien-3β-ol by *Clerodendrum campbellii* (Dicotyledonae, Verbenales), it was established (88) that the C-24 hydrogen of the Δ^{24} precursor (XII) first moves to C-25 in the 24-methylene intermediate (XIII) and then subsequently remigrates back to C-24 at the second transmethylation step to give (XXVII), in accord with Mechanism 5 and as shown in Figure 2.

The cooccurrence (13) of the 24-methyl sterols, brassicasterol (24β-), and campesterol (24α-), in the same plant (*Brassica rapa*, Cruciales) has been discussed elsewhere (16), and, although not readily explained, it serves to illustrate that intriguing problems of phytosterol alkylation remain to be resolved.

The above discussion reveals that several phytosterol alkylation mechanisms have evolved in nature and that a particular mechanism may be preferred in some classes or orders. However, it must be emphasized that, with the limited evidence available (often none or only one species examined in a class), any conclusions can be no more then speculative and should be regarded more as an indication of the direction in which future work should proceed in this field. From the foregoing, it seems that the following points in particular should receive attention if studies on phytosterols are to have any possible phylogenetic value.

In the analysis of plant sterols, particular attention should be paid to the determination, with certainty, of the configurations of 24-ethylidene, 24-methyl-, and 24-ethyl-sterols.

Since it is now clear that the same sterol side chain may be produced by fundamentally different mechanisms, it is important that some evidence is obtained concerning the actual alkylation mechanism operating in the organism under study. This information can be obtained by: (A) examining minor sterols which in many cases probably can be regarded as precursors of the major sterol; (B) using [CD_3]-methionine to determine the status of the 24-methyl and ethyl hydrogens; and (C) determining the fate of the C-24 hydrogen of the precursor Δ^{24}

sterol by use of either (2-^{14}C,(4R)4-$^{3}H_1$)-mevalonate or a 24-tritiated Δ^{24} sterol.

The pursuit of the above could be augmented by examining the stereospecificity of C-4 demethylation and double bond introduction in phytosterol biosynthesis, since species variations in these steps also are becoming apparent (4,6).

Such studies possibly may prove tedious with repetition and could be difficult in some cases because of the intransigence of some plants to experimentation, but the results obtained hopefully might add to the other biochemical, physiological, and morphological evidence being used (54) to elucidate plant phylogeny.

APPENDIX

Trivial name	Systematic name
brassicasterol	(22E)-ergosta-5,22-dien-3β-ol
campesterol	(24R)-24-methylcholest-5-en-3β-ol
cholesterol	Cholest-5-en-3β-ol
chondrillasterol	(22E,24R)-24-ethyl-5α-cholesta-7,22-dien-3β-ol
clionasterol	(24S)-24-ethylcholest-5-en-3β-ol
cycloartenol	9,19-cyclo-5α,9β-lanost-24-en-3β-ol
cycloeucalenol	4α,14α-dimethyl-9,19-cyclo-5α,9β-ergost-24(28)-en-3β-ol
cycloläudenol	(24S)-24-methyl-9,19-cyclo-5α,9β-lanost-25-en-3β-ol
22,23-dehydrocampesterol	(22E, 24S)-24-methylcholesta-5,22-dien-3β-ol
7-dehydroporiferasterol	(22E, 24R)-24-ethylcholesta-5,7,22-trien-3β-ol
desmosterol	cholesta-5,24-dien-3β-ol
22,23-dihydrobrassicasterol	ergost-5-en-3β-ol
ergosterol	(22E)-ergosta-5,7,22-trien-3β-ol
fucosterol	(24E)-stigmasta-5,24(28)-dien-3β-ol
28-isofucosterol	(24Z)-stigmasta-5,24(28)-dien-3β-ol
lichesterol	(22E)-ergpsta-5,8,22-trien-3β-ol
24-methylenedihydrolanosterol	24-methylene-5α-lanost-8-en-3β-ol
24-methylenecycloartanol	24-methylene-9,19-cyclo-5α,9β-lanostan-3β-ol
31-norcycloläudenol	4α,14α-dimethyl-9,19-cyclo-5α,9β-ergost-25-en-3β-ol
poriferasterol	(22E, 24R)-24-ethylcholesta-5,22-dien-3β-ol
α-spinasterol	(22E)-5α-stigmasta-7,22-dien-3β-ol
stigmastanol	5α-stigmastan-3β-ol
stigmasterol	(22E)-stigmasta-5,22-dien-3β-ol
sitosterol	stigmast-5-en-3β-ol

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Role of Sterols in Membranes[1]

WILLIAM R. NES, Department of Biological Sciences, Drexel University, Philadelphia, Pennsylvania 19104

ABSTRACT

Sterols, or in rare cases structurally similar molecules, are biosynthesized or at least required by all eucaryotic organisms, as well as by many procaryotic ones, regardless of their status as plants, animals, or protista. This information, together with quantitative, structural, metabolic, and other data is reviewed. It is interpreted to mean that the primary role sterols play in nature is a nonmetabolic one as architectural components of membranes and that this role can be played, but less well, by other molecules which approximate the steroidal structure. The biosynthetic process should, therefore, and actually does appear to be correlatable with this role, which, in turn, is correlatable with phylogenesis. The Δ^{24}-reduction-alkylation bifurcation, for instance, appears to be interrelated profoundly with the evolutionary differentiation of the animal from the plant kingdom.

INTRODUCTION

Much attention has been given to the metabolic role of sterols as intermediates in the biosynthesis of hormones and other substances (1-4). The purpose of this paper is to examine their nonmetabolic significance. The existing evidence indicates that a chemical structure represented best, but not exclusively, by cholesterol or its 24-methyl and 24-ethyl derivatives, is necessary to the life of all biological forms, with the possible exception of some bacteria. The structural requirements, which also can be met, though less well, by nonsteroidal substances, appear to be determined by the fit of the molecule into a multicomponent architectural system (a membrane) which plays the role of spatially limiting, biochemically segregating, and in other ways physiologically regulating cellular phenomena. Since this function tends to be a role which is not itself dynamic but rather one which encapsulates and regulates dynamism, membranes and, hence, their components are relatively stable metabolically. Information supporting the membraneous role of sterols is reviewed immediately following. The phylogenetic significance then is examined; and finally the apparent anomaly represented by bacterial membranes is considered. Not discussed, but nevertheless interesting, is the possibility that the nonmetabolic role of sterols implies a very long existence for some molecules as they pass in and out of various life forms. Since most of the sterol in nature appears to function nonmetabolically, the total amount of sterol may be increasing in the world and some, actually present in current organisms, could conceivably be as old (10^9 years) as the origin of the biosynthetic pathway (5). While most, but by no means all, mammalian sterol is converted to bile acid (4), the fate of sterol in the majority of plants, lower animals, and microbes is not well understood. Some bacteria which can utilize hydrocarbons as their source of carbon are known to oxygenate sterols (6). This may represent a first step in degradation, but the question remains open whether these or other organisms reconvert enough of the world's sterol to CO_2 to balance its biosynthesis.

DISCUSSION

The Membraneous Role

Organismic occurrence of sterols: A generalized membraneous role for sterols would require that sterols be present in all organisms. Unfortunately, there are several million different species, not to mention the different types of tissue, etc., within a given species, most of which have not been studied. In a survey, we first have to perceive what major groupings there are among the various forms of life. This is best done, as shown in Figure 1, with some sort of evolutionary hierarchy. Exact parallelism in evolutionary development is not intended to be depicted in Figure 1 from left to right as the scale is ascended. This needs particular emphasis in the case of the slime molds, fungi and sponges, and algae. It might be more appropriate, for instance, to place some of the fungi (especially phycomycetes) and algae with the lower eucaryots, but this then would require a more detailed consideration of the evolution of individual groups of organisms than is possible in the scope of this paper. We shall return to certain aspects of the manner of grouping subsequently. What is important at this juncture is to recognize the following points.

[1]One of eight papers presented in the symposium "Phytosterols," AOCS Spring Meeting, New Orleans, April 1973.

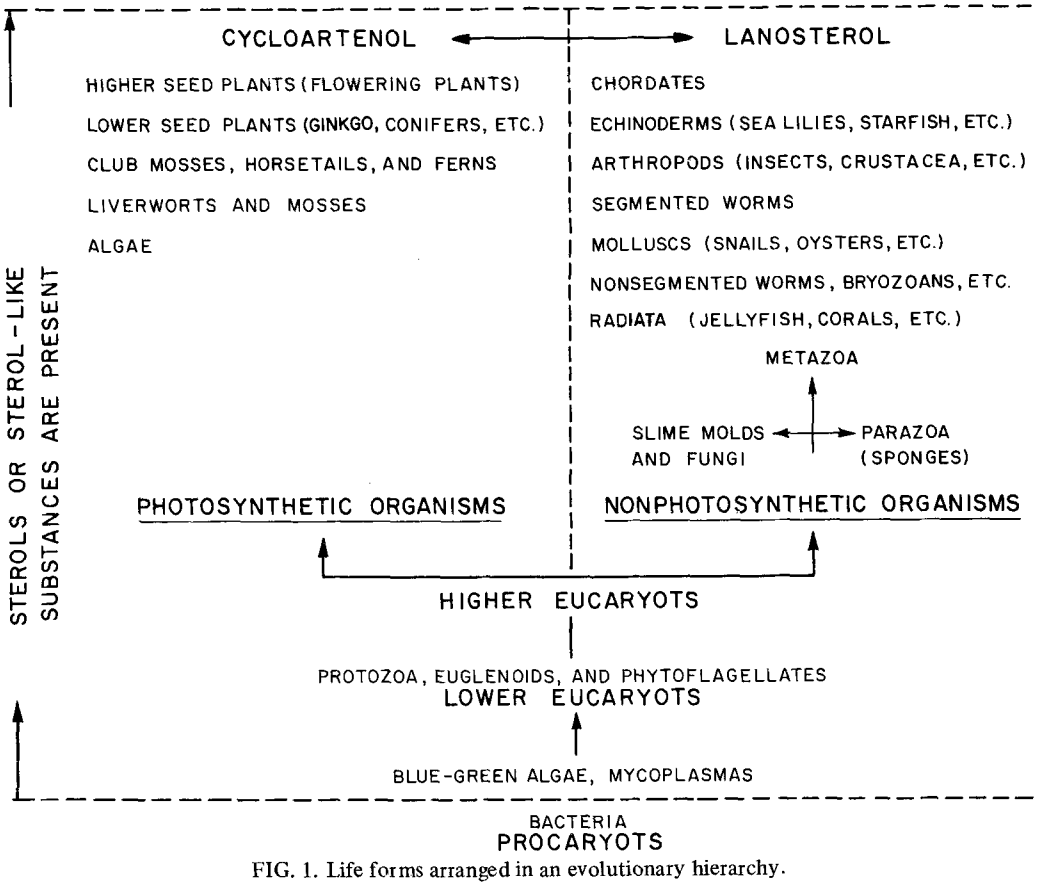


FIG. 1. Life forms arranged in an evolutionary hierarchy.

There are several kinds of organism collectively known as procaryots and represented by the bacteria, mycoplasmas, and blue-green algae, which lack a nuclear membrane and clearly defined membrane-bounded subcellular organelles. Their membraneous entity is believed to be restricted to a limiting envelope around the cell, possibly in the case of bacteria with an internal reticulum to which ribosomes are attached. The gram-positive bacteria seem to be unique in having a rigid external wall which has recognizable shape and is composed of polysaccharide and polypeptide (the so-called peptidoglycan, mucopeptide, glycopeptide, or murein) layered over a smaller cytoplasmic membrane (7). The gram-negative bacteria are similar except that outside of the peptidoglycan wall is a final membrane composed of lipopolysaccharide, phospholipid, and protein (7). The mycoplasmas, which are similar to the bacterial L-phase in which the wall has been discarded, have only a cytoplasmic membrane (8). The blue-green algae have an external sheath thought to consist of cellulose fibrils in a matrix of proteinaceous material with addditional but smaller amount of membraneous material underneath (9). In addition, blue-green algae possess a well developed organelle in their photosynthetic lamellae (9).

Above the procaryots are all the rest of the biological systems in one great group which consists of eucaryotic cells possessing not only a limiting cellular envelope but subcellular particles with their own membraneous envelopes. In addition, one finds an extension of the cellular envelope into the cell itself, forming the so-called endoplasmic reticulum.

The lower eucaryots comprise various unicelled organisms with as yet only partially understood direction toward the higher forms.

Among the higher eucaryots, one finds two major groups, the photosynthetic and nonphotosynthetic. The nonphotosynthetic group may be subdivided into organisms with and without a nervous system. There is a relatively well defined hierarchy in these higher forms, depending not so much upon cellular characteristics per se as on the higher level of order and specialization found in intercellular organization, e.g. vascularization, nature of reproduc-

tion, and development of the nervous system and its control of other processes.

In examining this array of life, let us start from the top and work down. Sterols have been found in all examined phyla of the section Deuterostomia (subkingdom Metazoa) from the chordates, including man (10), as first found in 1815 (11), through the bony fish (12-15) to echinoderms (most thoroughly investigated in the class Asteroidea, the starfish, etc. [16-18]). Below this is the section Protostomia in which the two phyla comprised by arthropods (crustacea, insects, etc.) and molluscs (snails, oysters, etc.) have been especially well examined (16, 19-28). In every case, sterols have been found. Below them are nonsegmented worms represented by nematodes, e.g. *Caenorhabditis briggsae*, in the phylum Aschelminthes, which have been found to possess sterols (29), as do the lowest of the Protostomia phyla (Platyhelminthes, the flatworms), represented by the blood fluke, *Schistosoma mansoni* (30). In the next lower section (Radiata), the main phylum (Coelenterata) consisting of jellyfishes, sea anemones, corals, etc., also has yielded sterols in every case (16), but below this level in the animal kingdom questions of a severe taxonomic nature arise. Thus, the subkingdom Parazoa, consisting of the single phylum Porifera, the sponges, is constituted by nonphotosynthetic organisms which lack a clearly defined nervous system (31), and the subkingdom Protozoa is still less well defined, since it is by no means clear upon what basis a first animal will be classified as such. Locomotion has been one criterion. What then will be said of the phytoflagellates which are independently moving, photosynthesizing organisms? To this we shall return, but now it is sufficient to note that sponges all contain sterols (16), and protozoa either biosynthesize or require sterols or a sterol-like molecule (32-41).

If one now notes the position in the evolutionary scale which we have reached, it will be seen we are among the lower eucaryots and are approaching quite primitive systems. Before discussing them, let us reascend to the top of evolution and return to our present place via the photosynthetic kingdom. All photosynthetic systems studied contain sterols without exception. Vegetable oils have been especially well surveyed (42). Examples of whole organisms are peas (43), illustrative of the highest (the angiosperms), pines (44), illustrative of the next highest (the gymnosperms), on down to the algae (45). In addition, many studies have shown that leaves, roots, fruit, seeds, pollen, embryos, chloroplasts, and other parts all contain sterols. Among the higher eucaryots this leaves us only with the slime molds and fungi, and every single one examined biosynthesizes or requires sterols (46-49). The fungal genera *Phytophthora* and *Pythium* are unusual in that their biosynthetic pathway is blocked (50-52), and they must assimilate sterol from their medium, especially for reproduction (52-57). While it is reported that growth of the mycelium can occur without sterol (52) and that the mycelium does not contain sterol (52), growth is definitely enhanced by sterol (58-60). This sort of behavior is strongly reminiscent of well studied cases among somewhat more primitive systems, to be discussed below, in which a sterol-like molecule is present; and judgment upon the significance of these two divergent fungal genera should be withheld until their chemical constituents are more thoroughly examined. Above the bacterial stage of evolution, the vegetative stage of life of these organisms at the moment represents the only cellular material which is reported to lack a sterol and at the same time is not known to contain another isopentenoid which mimics the steroidal structure.

We are now in a position to discuss the lower eucaryots. Of those that are photosynthetic, all biosynthesize sterols (36,41) as do both the light and the dark grown phases of the heterotrophic Euglena (36,61). The purely nonphotosynthetic cases are more complicated and in consequence more enlightening. They have been studied most thoroughly in the case of classically defined protozoa, of which *Tetrahymena pyriformis* has been the subject of greatest attention. It is a ciliated creature with all the common characteristics of a very primitive animal, except it lacks and fails to biosynthesize sterol (62,63) and, while sterol enhances growth, (64) the organism does not require sterol in its medium for normal growth and general vitality (65). However, the block in the sterol pathway is by no means thorough, for enzymes are present to induce the formation of a Δ^7- and a Δ^{22}- bond (65), the latter commonly being found in the plant kingdom where stigmasterol, ergosterol, etc., are encountered frequently. Of more crucial significance to our present discussion, is the fact that the sterol pathway in *T. pyriformis* has taken an alternative lane at the polyfurcation represented by the various modes of metabolism of squalene. Instead of proceeding to squalene oxide and then into the tetracyclic sterol series, squalene is protonated immediately and pentacyclized with an input of electrons by the inclusion of oxygen from water, which replaces the neutralization of the cation by deprotonation that occurs in sterol formation (66-69).

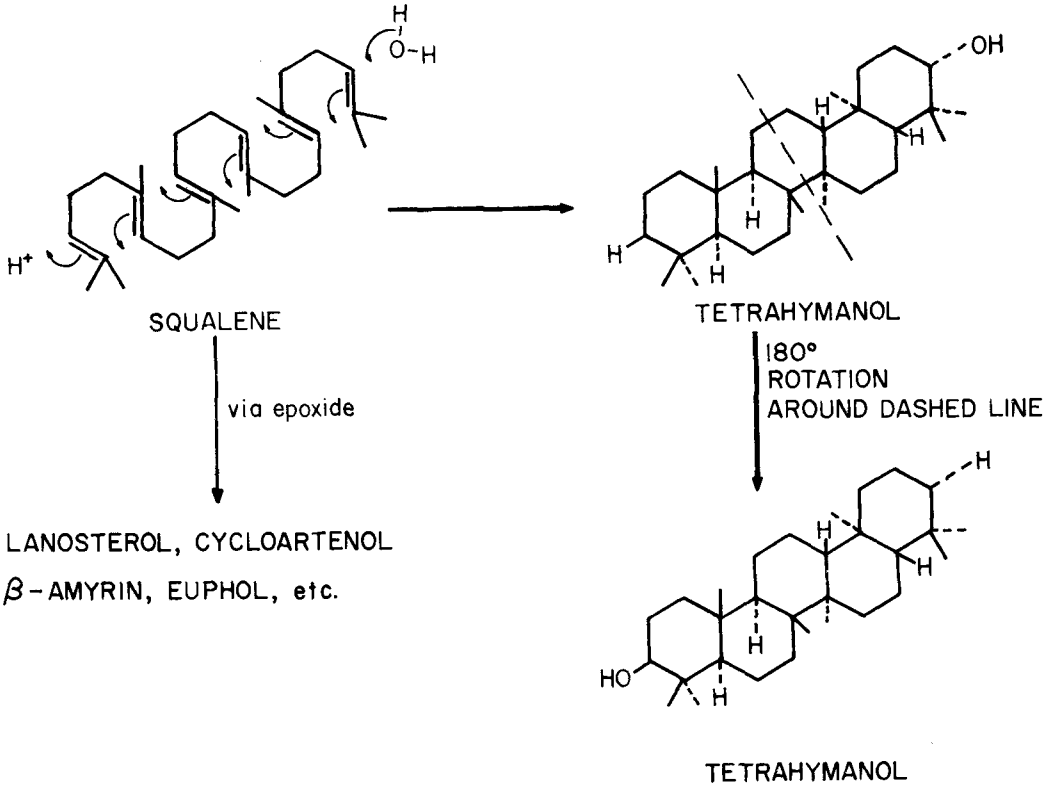
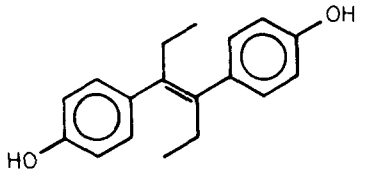


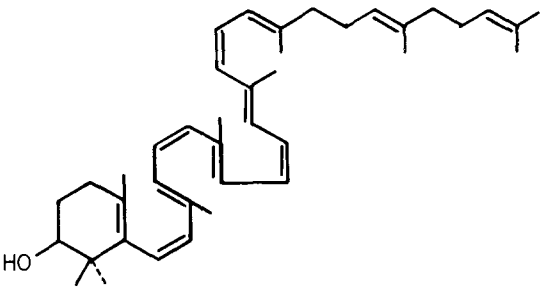
FIG. 2. The cyclization of squalene and an illustration of the structural resemblance of tetrahymanol to sterols.

This remarkable process (Fig. 2) gives a precise pentacyclic analogue of lanosterol, since the structure and stereochemistry (32,70,71) is such that the true, biosynthetic ring-D-end of the product, tetrahymanol, corresponds exactly to the ring-A-end of the sterol. Thus, this apparently sterol-less organism is not quite so simply sterol-less. It does indeed lack an actual sterol but contains a molecule with similar though not identical structural characteristics. The absence of cellular sterol, instead of contravening the general idea that sterols play a basic role in life, actually only raises the more sophisticated questions of what structural features in sterols make them important, to what extent the structure can be modified and still confer vitality on the cell, and what still more detailed correlations there may be between structure and function.

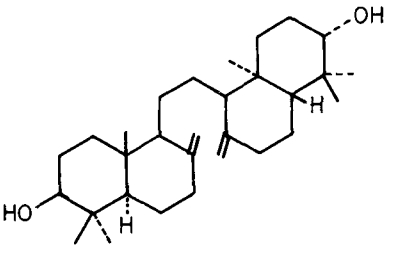
If what we see in *T. pyriformis* were unique, it could be passed over as an accidental and meaningless correlation. However, the same sort of phenomenon is observed in mycoplasmas. We now have descended to the realm of the very primitive procaryots inhabited by the apparently sterol-less bacteria (72,73). The very closely related mycoplasmas which, with one possible exception (*Mycoplasma* sp.), do not biosynthesize sterols (74), are divided into two groups: those which require sterol in their media, e.g. *M. gallinarum*, and those which do not, e.g. *M. laidlawii*. It is the latter which are interesting, for as with *T. pyriformis* the sterol pathway is by no means totally absent. Again at a polyfurcation, in this case at the farnesyl stage, an alternative lane is taken to yield the so-called carotenols. Two things make it clear that these compounds play the role of sterols. In the first place their presence confers a nonrequirement for sterol (8); and, secondly, when carotenol biosynthesis is inhibited in *M. laidlawii*, retarding or preventing growth, administered sterol is incorporated, restoring growth (75). The structure of the carotenols is not yet completely understood, but they definitely are hydroxylated carotenoids bearing a system of ca. eight conjugated double bonds, with the hydroxyl group probably at position 3 (8). By analogy to the diethylstilbestrol-estrogen relationship, in which a compound capable of assuming a conformation approximating the steroidal structure serves the steroidal role



trans-Diethylstilbestrol
(Mimics estrogens biologically)



Possible Carotenol Structure
(Mimics sterols biologically)



α-Onocerin
(Possibly mimics sterols biologically)

FIG. 3. Nonsteroidal molecules with conformations which mimic steroids.

biologically (76), one can write a possible structure for the carotenols, as shown in Figure 3. It will be seen that if the double bonds introduced between the second and third, third and fourth, fourth and fifth, and fifth and sixth isopentenoidal units are *cis*-oriented, a conformation can be written which mimics the sterol structure.

Sterol structures: To understand the relationship between sterols and sterol-like molecules, we need to examine the structures of the naturally occurring sterols. It is now unequivocal that, in the vast majority of systems, only a few structures constitute what we shall call the dominant sterols, meaning the quantitatively most important compounds. Only two or three sterols in a given organism are found to comprise 90% or more of the mixture. These dominant sterols (Fig. 4), presumably representing the most important functional structures, are related to one another by the following characteristics: (A) they possess a Δ^5-bond, (B) they have alternating *trans*-antistereochemistry at the ring junctures, (C) they possess an uncyclized side chain at C-17 retaining all of the original carbon atoms of the terminal two isopentenoid units of squalene, (D) they may or may not possess an extra 1 or 2 carbon atoms at C-24 (in plants the C_2-system is more common, except in fungi), (E) they may or may not possess a *trans*-Δ^{22}-bond, (F) they commonly do not possess a $\Delta^{24(28)}$-bond, (G) they have either the S- or the R-configuration at C-24 in the 24-alkylated cases, the former common in lower and the latter in higher organisms, and (H) they have a single hydroxyl group which is at C-3 and equatorially oriented. Exceptions to these generalizations are exceedingly rate, regardless of the position of the organism in the evolutionary hierarchy. Examples of compliance are in pea and pine seeds where the dominant sterols are 24-methyl and 24-ethylcholesterol (43,44). In both cases, as well as in practically all other

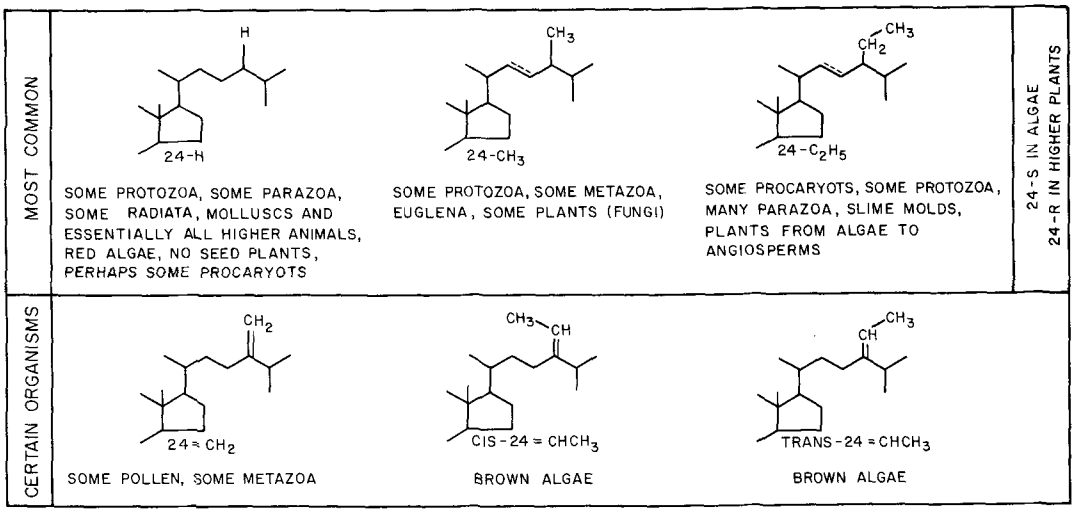


FIG. 4. Side chains of the dominant sterols and examples of their occurrence. The dashed line indicates with or without a Δ^{22}-bond.

higher plants studied, 24-C_2-sterols and 24-C_1-sterols are dominant, with the former nearly always in larger amount (80% or more of total sterol) (42). 24-Ethylcholesterols (sitosterol and clionasterol) and their *trans*-Δ^{22}-derivatives (stigmasterol and poriferasterol) are the two most frequently encountered types of dominant 24-C_2-sterols. The analogous 24-C_1-sterols are campesterol, 5,6-dehydroergostanol, *trans*-22,23-dehydrocampesterol, and brassicasterol, respectively. Except in some $\Delta^{5,7}$- and Δ^7-sterol-containing fungi and algae (45,48) in which the Δ^7-reductase and Δ^5-dehydrogenase apparently are lacking, in the brown algae (45) which apparently lack the $\Delta^{24(28)}$-reductase and in some rare but similar cases, the dominant sterols of plants generally consist of some combination of these 8 compounds, represented collectively along with cholesterol by Figure 5. In the primate counterpart at the top of the scale the dominant sterol is cholesterol. In fish (12-15) and the lower animals (16-18, 21-30), one similarly finds that the dominant sterols are either cholesterol and (dietary) 24-C_1- and 24-C_2-derivatives, with or without a Δ^{22}- or $\Delta^{24(28)}$-bond.

Among the procaryotic mycoplasmas, one is tentatively believed to biosynthesize cholesterol (77), but, in the remainder, the pathway is blocked prior to squalene. In those species in which the pathway to carotenols also is blocked, an opportunity is afforded to study structure-activity relationships. Such experiments have been done (78-81), and it is found that only sterols bearing a long side chain and alternating *trans*-antistereochemistry in the ring

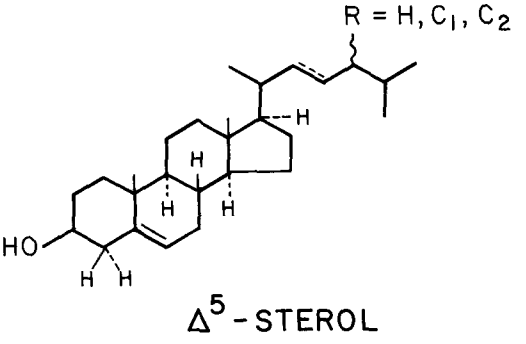


FIG. 5. Full structure of the dominant sterol of most organisms. C_1 and C_2 imply methyl and ethyl groups. In rare cases, e.g. brown algae, they are methylene and ethylidene groups. The 24-C_2-sterols are usually dominant over the 24-C_1-sterols. In animals 24-H is dominant. A Δ^7-bond with or without the Δ^5-bond is dominant in a few cases, e.g. yeasts, some *Chlorella* species, and the family *Curcurbitacea*.

TABLE I

Sterols and Growth of *Mycoplasma arthritidis*[a]

Growth promoting	Not growth promoting
Cholesterol	3-Epicholesterol
5α-Cholestanol	3-Epi-5αcholestanol
Ergosterol	5β-Cholestanol
Sitosterol	3-Epi-5β-cholestanol
Stigmasterol	Solanesol
Carotenol	Cholestane
	Squalene
	Fatty acid esters of cholesterol

[a]See ref. 8.

system will support growth (Table I). Furthermore, sterols with or without a Δ^{22}-bond or with or without 24-alkylation support growth, but sterols in which the hydroxyl group is masked by a long acyl chain (fatty acid esters) are ineffective. Esters of shorter chain acids are growth supporting. However, the organism possesses an esterase with substrate specificity for the shorter chains which catalyzes hydrolysis to give the free sterol. Similar correlations have been made between structure and ability to induce sexual reproduction of the eucaryotic fungus *Phytophthora cactorum*, where the requirements are an hydroxyl group at C-3, a ring-B with one double bond, and a side chain of at least 5 carbon atoms (58, 82,83). In the animal kingdom, similar findings have been made regarding the ability of sterols to support insect pupation (84). At the protozoan level, such sterols have been found to support the growth of *T. pyriformis* and also to inhibit the biosynthesis of the endogenous tetrahymanol (33,85).

Since the procaryotic blue-green algae biosynthesize the same sterols (86,87) as described for other organisms, it is obvious that across the whole of the evolutionary hierarchy, with the possible exception of the bacteria, both a functional requirement for sterol and the same structure-activity correlation exists, whether one perceives it observationally in the dominant sterols or experimentally in the effect of administered sterols. The only reasonable interpretation of this is that sterols play a common role in the various organisms.

Structural requirements: It follows from the foregoing section that the minimal requirements for functionality in a sterol are flatness (seen in the alternating *trans*-antistereochemistry) and a free, equatorial hydroxyl group at C-3. The latter is seen in the mycoplasma studies, the occurrence of dominant sterols, and, not yet mentioned, the fact that by and large the dominant sterols are in the free form. There is, however, much sterol which occurs in other forms. One can only guess that the frequently encountered esters represent either storage forms or metabolic pools. While definitive proof is lacking, much suggestive evidence is available which is too diffuse to review here (for examples, see 88 and 89). In the case of sterol glycosides especially, good evidence for a metabolic role as carriers for glucose oxidation is available. Variations in double bonds and the nature and extent of substitution at C-24 imply a fine tuning of the structure-function phenomenon. Dominance of the 24-ethyl over the 24-methyl group indicates slightly different roles for each. The general failure to have significant steady state concentrations of the 4,4-dimethyl and 4,4,14-trimethyl sterols suggests that the functional capacity of the sterol is best met by a molecule which has a strictly unhindered hydroxyl group and is not only flat but possesses preferred dimensions. The absence of the 14α-methyl group in dominant sterols seems to reflect the latter, since this group is axially oriented and would extend the dimension of thickness. The problem with substitution at C-4 probably has more to do with steric hindrance of the 3-hydroxyl group. This phenomenon is well known in the increasing order of elution of 4,4-dimethyl-, 4-monomethyl-, and 4-desmethylsterols in adsorption chromatography, where H-bonding is inhibited by substitution which, therefore, decreases the retention time. In gas liquid chromatography where the mass effect dominates, one finds the reverse order; and, unlike adsorption chromatography, the effect is essentially position independent. It also should be noted from the adsorption chromatographic data, that the effect of substitution is quantitative rather than qualitative (all or nothing). This means that in the biological function of sterols, if H-bonding (or reactions with similar spatial requirements) to the hydroxyl group were important, one might expect a 4,4-dimethyl or 4-monomethyl sterol to act positively, albeit not as well as a 4-desmethyl sterol. Substitution at position 14 could or could not be more critical than at position 4. No quantitative evidence is available biologically. Structurally, the molecule would have to be ca. 2 Å thicker at the C/D-ring juncture but would have no alteration in any other obvious characteristic. Insofar as the dimension of length is concerned, we can only surmise from the occurrence data that it is best represented functionally by the calculable dimensions of the tetracyclic sterol system, together with the dimensions of whatever the physiological conformation of the side chain might be. Grossly, the latter will have to be at least the size of a cyclohexane ring, since one could not fit all of the eight or more atoms of the side chain into a smaller volume in any conformation. The studies on reproduction of fungi indicate a minimal requirement of five carbon atoms in the side chain (58, 82,83) which indicates that the cyclohexane volume is a reasonable first approximation of the amount of space beyond ring-D involved in conferring functionality on a molecule. No data are available on how much more bulk could be added in this direction before functionality is destroyed.

There are few, if any, proteins with absolute specificity for a single substrate, because at a

molecular level the ability of biomolecular complexes to form between any two substances is a quantitative, rather than simply qualitative, matter arising out of structural detail. This idea of integration of quantity with quality is central to an examination of the place sterols have in biology. Equally important is a separation of our notions on the meaning of sterol based upon arbitrary criteria (arising out of biosynthesis, organic chemistry, or simply out of one's own incomplete knowledge) and the criteria imposed by biological systems. The criteria imposed by biological systems are going to be, among other things, functionally oriented, which means that a sterol for membrane purposes is not necessarily a sterol for the purposes of hormone biosynthesis. The relationship between cholesterol and tetrahymanol is an exceedingly good example of these principles. Cholesterol is the classic chemically defined sterol, tetrahymanol fulfills all the similar definitions of a pentacyclic triterpenoid, and neither crosses over into the other's categorical domain. However, while in *Tetrahymena pyriformis*, tetrahymanol clearly plays the sterol's role, it would strain credulity to suppose that tetrahymanol could play cholesterol's role in mammalian hormone production. In the case of hormones, structure-activity correlations (76) unequivocally require certain kinds of oxygenation, etc., which would require the most extensive kind of enzyme system to achieve from tetrahymanol; and such processes have never been observed in any organism. The difference in the two cases is that, in the first, tetrahymanol and sterol are presumably directly competing functionally, while in the second they are not, additional metabolism being required before either could reach the functional level. There are, of course, good reasons for retaining the older definitions, but the thoughts expressed allow us to add a new parameter, function, and, for instance, to definte the term sterol-like as a molecule which, to a greater or lesser degree, can play a particular biological role which a classic sterol can play. Tetrahymanol, being structurally similar to sterols might then be functionally similar, although one would guess that the presence of the 4,4,14-trimethyl groups and perhaps cyclization of the side chain might reduce its effectiveness. Since the biological data are in accord, tetrahymanol would be an example of a sterol-like molecule. Parenthetically, as previously alluded to, this implies a distinction between a functional and a biosynthetic nomenclature for the rings. In tetrahymanol they are in inverse order, functional ring-A being identical with biosynthetic ring-E, etc.

The idea of sterol-like molecules suggests that other pentacyclic triterpenoids also may play the same role as sterols. Many of them are flat and differ from tetrahymanol only in such things as a 5-membered instead of a 6-membered ring at the hydrocarbon end of the molecule. One might expect to find them instead of or along with sterols in some organisms. They do, in fact, accompany sterols in many plants (90) and perhaps play membraneous roles, too. On the other hand, those which are not flat, e.g. the widely distributed β-amyrin with a *cis*-juncture between rings-D and E, may play some other role. Similarly, while the tetracyclic lanosterol and cycloartenol are both precursors of 4,4,14-trisdesmethyl-Δ^5-sterols (in different organisms), lanosterol might reasonably play less well, but nevertheless play, the normal end-product's (architectural) role, while cycloartenol (having the shape of a butterlfy instead of a table top) presumably could not. Thus, it would not be surprising to find a viable organism with a biosynthetic pathway blocked after cyclization of epoxysqualene, but only in nonphotosynthetic systems or in those photosynthetic organisms in which the block occurred after opening of the 9,19-cyclo grouping (which is what confers the butterfly shape on cycloartenol and its relatives). Further, the tetracyclic euphol series of triterpenoids found in some plants may be (very poor and probably primitive) sterol-like molecules, since they mimic the lanosterol structure, differing only in an enantiomeric junction between rings-C and D and an opposite configuration at C-17. The configuration at C-17 is probably much more detrimental than at the ring juncture, since the latter allows retention of flatness and only alters thickness by ca. 2 Å (a methyl group) toward the α-face at the expense of the β-face, which seems not to prohibit activity in tetrahymanol. The inversion of C-17, however, throws the side chain into a pseudoaxial position on the α-face and might seriously interfere with flatness.

Many other molecules can be assessed for sterol-like qualities at a partially quantitative level in the manner outlined. An extreme, but important, further example is the structure with less than four rings. Two cases are worthy of mention. They are the carotenols and α-onocerin with one and two rings, respectively, in a region of the molecule which could be compared to the tetracyclic portion of sterols. As shown in Figure 3, these compounds, both naturally occurring (90,91), can assume conformations which mimic the flat, tetracyclic sterol with its space-filling group at C-17; and one class, the carotenols, is found in organisms

which lack sterols and do not require them (8).

It certainly appears, therefore, that some organisms have a perfect or, at least, nearly perfect structure. The occurrence of cholesterol and its 24-methyl and 24-ethyl derivatives of the 24-R-configuration as the dominant sterols at the top of the evolutionary hierarchy indicates that these three closely related molecules represent this perfect mating of structure with function. Imperfect structures, whether in the form of other sterols, tetracyclic triterpenoids, or mono or bicyclic molecules, which can approximate the perfect structures then should be able to confer vitality but not at the same level of sophistication. These ideas are tantamount to an extension of Bergmann's famous thought on "the survival of the fittest sterol" (16). To paraphrase him, the sterol structure itself appears to represent the survival of the fittest isopentenoid.

Organismic amounts of sterols: The amount of sterol found in a whole organism is remarkably constant (Table II). It amounts to ca. 0.1% wet wt usually well within one order of magnitude, except in rare cases, e.g. the adrenal gland, where the sterol appears to be largely esterified (89). There appear to be no reports of a eucaryotic cell with less than 0.01%, except in the case of two species of phycomycetes (92). They may bear reexamination, especially since other species of phycomycetes (92), are in the normal range. The only other organismic types with a very low content are the blue-green algae (86), which are procaryotic. In this group, one would expect a low content, if sterols are membraneous components. Their procaryotic relatives, the bacteria, are believed to have none. Whether it really is none remains to be seen, but probably it is less than 0.01%. As a rough first approximation, there appears to be a correlation between the amount of sterol and the extent of membrane in the cell; the procaryots with a polysaccharide cell wall and a thin inner membrane having the least and the eucaryots the most. In the procaryotic mycoplasmas which lack the polysaccharide cell wall, all the sterol (and carotenoid) is in the cytoplasmic membrane which constitutes ca. a third of the dry wt of the organism. The relative constancy of the amounts in eucaryots would, in any event, be exceedingly difficult to explain on any other than a common basis. It should be noted that this constancy is all the more remarkable when it is realized that part or all of the sterol in some of the organisms, e.g. arthropods, is ingested. Much of the variation reported may well disappear when a survey is made which discriminates between free and other forms (esterified, etc.) of sterol. Such information is not yet available, except in isolated cases.

TABLE II

Combined Amounts of Free and Esterified Sterol in Various Organisms and Tissues

Organism	Percentage	Basis
Blue-green algae	0.003	Wet wt
Brown algae	0.1	Wet wt
Mycoplasma	2.4	Dry wt
Fungi (yeasts)	0.1-0.3	Wet wt
Phytoflagellates	0.7	Freeze dried
Pea seeds	0.07	No water
Pea seeds	0.03	Germinating
Pine seeds	0.13	No water
Pumpkin leaves	0.02	Wet wt
Pollen (Saquaro)	1.0	As collected
Protozoa	0.3-1.5	Freeze dried
Sea anemone	0.07	Wet wt
Snails	0.03-0.13	Wet wt, no shells
Crustacea	0.03-0.5	Wet wt
Earthworm	0.1	Wet wt
Marine annelids	0.3-0.5	Wet wt
Fish (carp)	0.07	Whole animal
Frog	0.04	Whole animal
Rat	0.1	Whole animal
Human being	0.3-0.6	Whole person
Brain cortex	1.2	Wet wt
Heart	0.2	Wet wt
Liver	0.3-0.6	Wet wt
Erythrocytes	0.1	Wet wt
Adrenal	3-10	Wet wt

Subcellular distribution of sterols: The subcellular distribution of sterols has not been extensively investigated; but, where information is available, free sterols have been associated with organelles. This has been shown for plants (3, 93,94), mycoplasmas (8,95), and often for higher animals. In addition, in a few cases extensively reviewed (96-98), isolated membranes have been shown to contain sterols (Table III). It is particularly interesting that carotenoids synthesized by *M. laidlawii*, and cholesterol added to the medium, are both found in the membrane (95). It appears that the only membranes examined which do not contain sterols are those in organisms possessing

TABLE III

Cholesterol in Membranes[a]

Membrane	Amount (percent membrane lipid)
Myelin	25
Erythrocyte	25
Liver cell	14-17
Endoplasmic reticulum	6-8
Mitochondria	2-5
Mycoplasma laidlawii	2
Azotobacter agilis	0
Escherichia coli	0
Bacillus megaterum	0

[a]See ref. 95-98.

a sterol-like substance when no sterol is supplied and those in bacteria. In the nonbacterial cases where the organism is completely and normally viable without sterol (certain protozoa and mycoplasmas), the sterol-like substances (tetrahymanol and carotenols) are found to be associated with particulate structures. When sterol is supplied, the sterol becomes particulate. The amount of sterol in isolated membranes of eucaryots varies from ca. 2-25% membrane lipid. This tenfold variation, incidentally, is of the same order of magnitude as the variation in the organismic amounts of sterol and, probably in part, is also responsible for the quantitative variations observed in Table II, since the amount and kind of particulate material in different cells varies considerably.

Metabolic stability of sterols: The turnover rate for membranes, in general, is quite slow. If sterols primarily were acting as architectural components of membranes, one might expect a slow turnover rate. This was perhaps first observed in the late 1950's in the case of the brains of chickens and rabbits. Labeled cholesterol was not removed greatly after 1 year (99-101). More recent studies (102) on whole human beings have shown that cholesterol turnover is only ca. 1 g/person/day, which is ca. 0.3% whole body sterol/day. This is to be contrasted with, for instance, hormonal turnover where amounts approaching 100% are metabolized in hr. In 1959 Clark and Bloch (84,103,104), however, published decisive metabolic work. They showed that nearly all of the sterol which insects require retain their gross structure. Subsequent studies (105) have confirmed and extended the original findings. The sterols actually may be metabolized, but it is clear now that this is related to fine tuning of the structure. In particular, 24-alkylsterols are dealkylated. Sterols required by protozoa, as well as by mycoplasmas, also are not destroyed (8, 33), although again some are dealkylated (65 and W.R. Nes, A. Alcaide, F.B. Mallory, J.R. Landrey, and R.L. Conner, unpublished observations). Appropriate experiments have not yet been done with photosynthetic plants, but one would expect from the other data that a similar slow turnover exists for similar reasons. Experiments that do not prove, but are at least in accord with, the prediction are already available. Peas have the same content and distribution of sterols before germination and after roots and leaves have appeared (43). Since the amount of newly biosynthesized sterol represents only a small fraction of the total sterol (43), the sterol present must turn over slowly, despite rapid organismic development. By contrast, rapid biosynthesis of β-amyrin occurs during germination (43) which, together with its smaller concentration and D/E-*cis*-structure, suggests a metabolic role.

Biosynthesis in autotrophs: All eucaryotic autotrophs (organisms utilizing only CO_2 as a carbon source) contain and, therefore, must biosynthesize sterols. Content has been examined in many and biosynthesis in several lower and higher cases. Retention of the genetics for sterol biosynthesis throughtout 10^9 years (5) as a general phenomenon would be hard to explain other than by a vital and probably common role for the end-products. In a number of organisms, it is clear that mutations must, indeed, have occurred, proving that there is no essential protection to the pathway. One such case is in the *Cucurbitaceae*, which are angiosperms standing at the top of evolution, below which are organisms generally biosynthesizing Δ^5-sterols. However, as proven by an examination of leaves and fruit of the pumpkin (W.R. Nes, B. Harris, and G.F. Gibbons, unpublished observations), as well as by studies of pumpkin seeds and of similar parts of other species of *Cucurbitaceae* (106,107), no Δ^5-sterols are present. Only Δ^7-sterols are observed, the principal one being 24-ethyl-22-dehydrolathosterol. This almost certainly must mean that mutational deletion of the Δ^5-dehydrogenase (and perhaps also the Δ^7-reductase) has occurred. There is no other family of angiosperms, to the author's knowledge, which lacks Δ^5-sterols. An A/B-*trans*-juncture and a Δ^7-bond, however, in the *Cucurbitaceae* sterols ensure the required flat stereochemistry. Similar Δ^7-sterols, incidentally, are acceptable to insects which cannot biosynthesize sterols (105), and occur (rarely) as the exclusive sterol among some algae (45).

Other evidence: The role of sterols in membranes is suggested by several other kinds of evidence, two of which deserve mention. If mycoplasmas are grown in the presence of the sterol-complexing agent, digitonin, lysis occurs (81,108), but only with those species which require sterol. Thus, the carotenol containing species, *M. laidlawii*, is not only refractory to digitonin in the absence of sterol but becomes susceptible to lysis by this agent when grown in the presence of sterol. Secondly, studies (109 110) with precise molecular models of myelin have shown that interdigitation of cholesterol with the other lipids present can account for the dimensions of the membrane determined experimentally from X-ray data (111, 112).

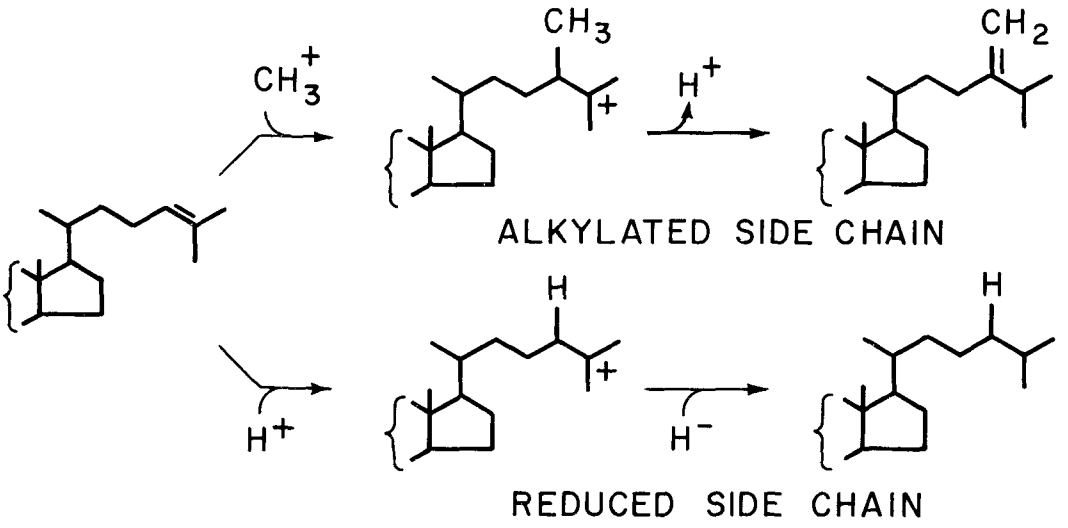


FIG. 6. Δ^{24}-Reduction-alkylation bifurcation in sterol biosynthesis. In animals, only reduction occurs. In plants, both reduction and alkylation occur.

Phylogenetic Implications

Cholesterol and its 24-desalkyl relatives (desmosterol, lathosterol, etc.) have long been thought to represent the characteristic sterols of animals and 24-alkyl-sterols those of plants, giving rise to the terms zoosterol and phytosterol. This implies that there is a correlation between structure, function, and evolutionary development. The recent discovery that cholesterol and other 24-desalkyl precursors constitute the only sterols of some eucaryotic algae (45,113,114) and are present in higher plants (42,115,116), as well as in the procaryotic blue-green algae (86,87) compromised the simple exclusivity which was thought to exist. The problem further is complicated by the occurrence of 24-alkylsterols in lower animals (16). Nevertheless, the confusing situation partly can be resolved through a consideration of several additional factors. After all these are considered, one is still left with phylogenetic implications, although the terms zoosterol and phytosterol probably should not be retained. Preferable terms would simply be the structural ones, 24-alkyl- and 24-desalkylsterol. With them one can make the following kind of meaningful statements. Since phytophagous arthropods do not biosynthesize sterols de novo, they must metabolize dietary 24-alkylsterols to 24-desalkylsterols to a greater or lesser extent to satisfy certain functional requirements. Carnivorous arthropods, on the other hand, feeding on animals with an intact biosynthetic pathway, would not require such metabolic processes.

The origin of the two types of sterol resides in a biosynthetic bifurcation in which the $\Delta^{24(25)}$-bond alternatively is reduced (117,118) or attacked by a C_1- group (119-121), and the two mechanisms are similar (122) (Fig. 6.). From the occurrence of both types of end-product in eucaryotic, as well as in procaryotic algae, it is clear that the alkylation process must have arisen prior to the evolution of true animals. Yet, one of two things clearly is seen to be characteristic of animals, if we define them as organisms with a definite nervous system. They either biosynthesize only unalkylated sterols (commonly cholesterol) or, if biosynthesis is blocked, they convert ingested 24-alkylsterol to the desalkyl variety. With the possible exception of some individual species, cholesterol is unquestionably the principal sterol of the animal phyla which have been examined. These include the well known mammalian cases, the molluscs (27, 123), arthropods (21,22), and echinoderms (16), even though some 24-alkylsterol may be present. Whether or not a 24-alkylsterol is present clearly depends upon the animals's evolutionary sophistication, its feeding habits, its digestive tract, its metabolic capacity to remove 24-alkylsterol once absorbed, and its capacity to biosynthesize sterols de novo. At the top of evolution, in man and other mammals, these phenomena are all controlled quite highly so that no alkylation occurs (124, 125, and W.R. Nes and P.A.G. Malya, unpublished observations); 24-alkylsterol is turned over much faster than cholesterol (126, 127); absorption of sterol, especially 24-alkylsterol, by the intestinal walls is limited (128); and de novo biosynthesis of cholesterol occurs in high yield from mevalonic

acid (129). This indicates that the absence of extra carbon atoms at C-24 is quite important. Although there appears to be less control of these phenomena at lower levels of the evolutionary scale, the importance of the unalkylated sterol to animals is clearly seen in an evolutionary adaptation of lower species which have lost part or all of the sterol pathway. Several decades ago, Bergmann (20) found that, in the silkworm, cholesterol is the dominant sterol despite a diet of 24-alkylsterols in mulberry leaves. This and similar information (130) indicated to him (16) that dealkylation had occurred, and this has since been unequivocally demonstrated in phytophagous arthropods (105), as well as in *Tetrahymena pyriformis* (W.R. Nes, A. Alcaide, F.B. Mallory, J.R. Landrey, and R.L. Conner, unpublished observations). In both of these cases, de novo biosynthesis of sterols is blocked (33, 103) at or before squalene. Terrestrial annelids also have a blocked pathway beyond squalene (131), but again their principal sterol is cholesterol (131, 132).

Thus, a crucial structure-function relationship between the 24-desalkylsterol and the presence of a nervous system appears to exist. The only exception to the proposition that all differentiated animal phyla possess cholesterol as the dominant sterol is found in the sponges (16), and the sponges fail to posess a well developed or even a well defined nervous system, and they have still other characteristics of a plant (31). It is, therefore, probably best not to regard them as true animals and simply to place them, as is done in Figure 1, in the nonphotosynthetic kingdom along with slime molds, etc., below animals but above the simpler cellularly undifferentiated lower eucaryots.

The clear association between cholesterol and the nervous system, which is manifest not only in the tanoxomic distribution and in studies of myelin structure (109-112), but also in the fact that brain biosynthesizes cholesterol (133) and that brain abnormalities are correlatable with anomalous brain biosynthesis of sterol (134), makes the discrimination against 24-alkylsterols in animals as a whole satisfying, but only if we make an evolutionary assumption which would explain why nonnervous system tissue of animals make only cholesterol. The assumption is that the 24-alkylation process was deleted or masked early in evolution, probably at the level of the lower eucaryots, in one or more organisms and that it is out of the resultant gene pool that animals arose.

This does not mean that all nonphotosynthetic organisms came from this same pool, because there is a clear dichotomy in the world relative to the presence or absence of photosynthesis, which is associated with the cyclization of squalene oxide to cycloartenol in the former case and to lanosterol in the latter (135) (Fig. 7). Furthermore, the cycloartenol pathway operates in the nonphotosynthetic, as well as photosynthetic, tissue of a photosynthetic organism (136), indicating common genetics of the two types of tissue. This bifurcation at the cyclase level lacks functional significance, since either pathway proceeding from it yields 24-alkyl- or 24-desalkylsterols (135, 137-139). What it has, though, is phylogenetic significance. When it is coupled with the truly functional bifurcation represented by the metabolism of the $\Delta^{24(25)}$-bond, several possible types of gene pool emerge; one with the cycloartenol-alkylation codes leading to certain plants; one with the triple cycloartenol-alkylation-reduction codes leading to other plants (perhaps most higher examples); one with only the cycloartenol-reduction codes leading to still other plants (presumably represented by the cholesterol containing red algae); one with the lanosterol-alkylation codes leading to organisms, such as the fungi; one with the lanosterol-alkylation-reduction codes yielding perhaps other nonphotosynthetic organisms; and, finally, one with the lanotsterol-reduction codes leading to animals.

One should be able to assess whether a lower eucaryot was or was not on a particular evolutionary path from such information. Euglena, for instance, despite both light and dark grown aspects, utilizes cycloartenol in both phases (140) and is, thus, phylogenetically photosynthetic. It presumably could not have been in the gene pool out of which animals and other nonphotosynthetic organisms arose, but may be a precursor to angiosperms. Similarly, if photosynthesis in the red algae can be taken to imply the cycloartenol pathway, despite their reductive pathway to give cholesterol, they must not have participated in the gene pool out of which animals arose, and due to the absence of alkylation may not be on the line to higher plants. One can probably also eliminate the protozoa, e.g. *T. pyriformis*, which fail to make sterols at all. They may represent an evolutionary path which did not go much, if at all, further. Protozoa, in the sense that they are first animals, presumably must minimally possess the lanosterol-reduction genetics where reduction is at least meant to imply the absence of alkylation; but, in the minimal case, they probably would better be described as prezoa. True protozoa should have undergone additional mutations leading to the use of cholesterol in the actual development of a nervous

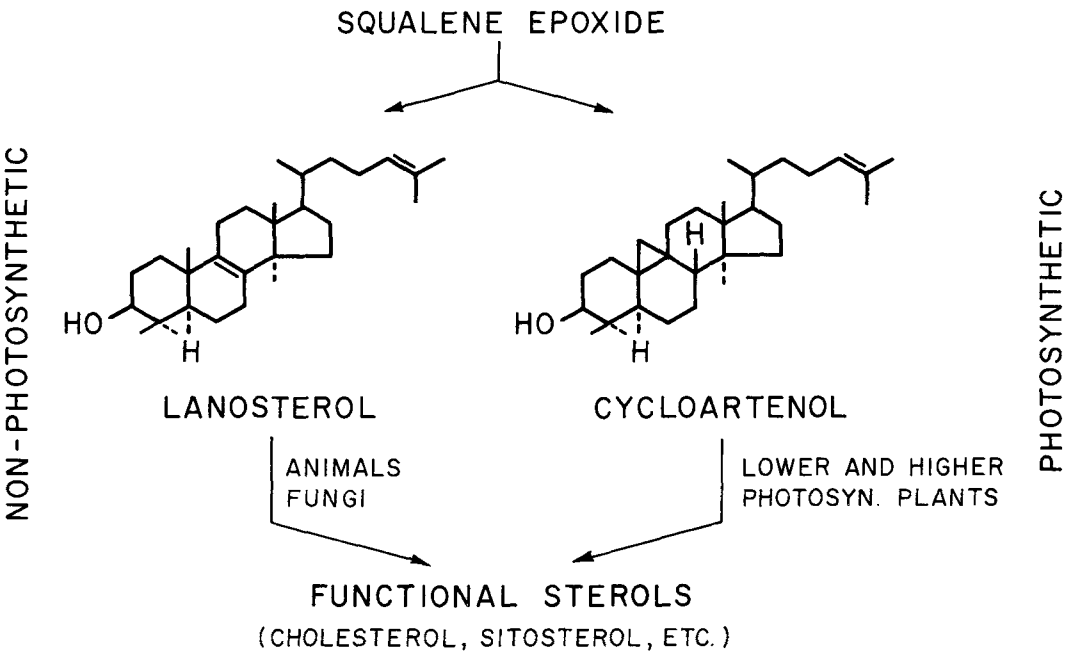


FIG. 7. The lanosterol-cycloartenol bifurcation in sterol biosynthesis and its relationship to photosynthesis.

system. Certain tissues in sponges conceivably may represent the true protozoan state.

These ideas can be carried over into other aspects of sterol biosynthesis. An intriguing example has to do with the common occurrence of the Δ^5-bond. The apparent absence of the Δ^5-dehydrogenase in the *Cucurbitaceae*, discussed above, conceivably could have been due to a separate but parallel evolutionary line proceeding all the way from the procaryotic level; but this seems unlikely. The improbability and the apparent functional importance of the Δ^5-bond (from occurrence data) are given credence by the presence in *Cucurbitaceae* of the cucurbitacins (141). These are tetracyclic triterpenoids with an unusual feature. The methyl group (C-19) which is normally between rings- C/D is at C-9 instead of C-10, and a Δ^5-bond is present! This almost certainly arises from a modified epoxysqualene cyclase leading to the processes shown in Figure 8. Moreover, in cucurbitacin-A one sees hydroxylation of C-19 which would be a prelude to its removal or rearrangement back to C-10 (via a 3-membered ring). The organism appears to be trying to correct for its Δ^5-sterol deficiency. This would be a biochemical feed-back, but of quite a different nature to any known heretofore. The presence of a teleological principle in biology is implied. Although far from fashionable, it may not be dismissed too easily, since the same principle seems to be operating in the arthropoda, in which a block in cholesterol biosynthesis is corrected for by the development of a dealkylation process of ingested 24-alkylsterols (105). Similarly, the inhibition of tetrahymanol biosynthesis by administered sterol, not to mention the fact that sterols are absorbed by *T. pyriformis* (33), mycoplasmas (8), and by the bacterial L-phase (142), may be part of phenomena that are not simply the result of random mutation. In the case of dealkylation, the process used by insects (23, 143-145) is essentially the reverse of the alkylation process used by plants (44, 119). It is quite possible that all higher forms of life came from a common procaryotic gene pool possessing alkylation genes and, therefore, that arthropoda simply unmask and reverse these genes. Even if this were so, how and why do arthropoda know the genes should be unmasked after they have suffered a block in de novo sterol biosynthesis? Is the absorption of sterol by the bacterial L-phase only a random process, or is it also trying to alleviate a steroidal deficiency? Do people have masked or have they lost alkylation genes?

The Bacterial Anomaly

In 1930, von Behring (73), confirming earlier observations of Hammerschlag (72) on the absence of sterols in bacteria, concluded that sterols are not fundamentally important compounds and also that sterols are connected with

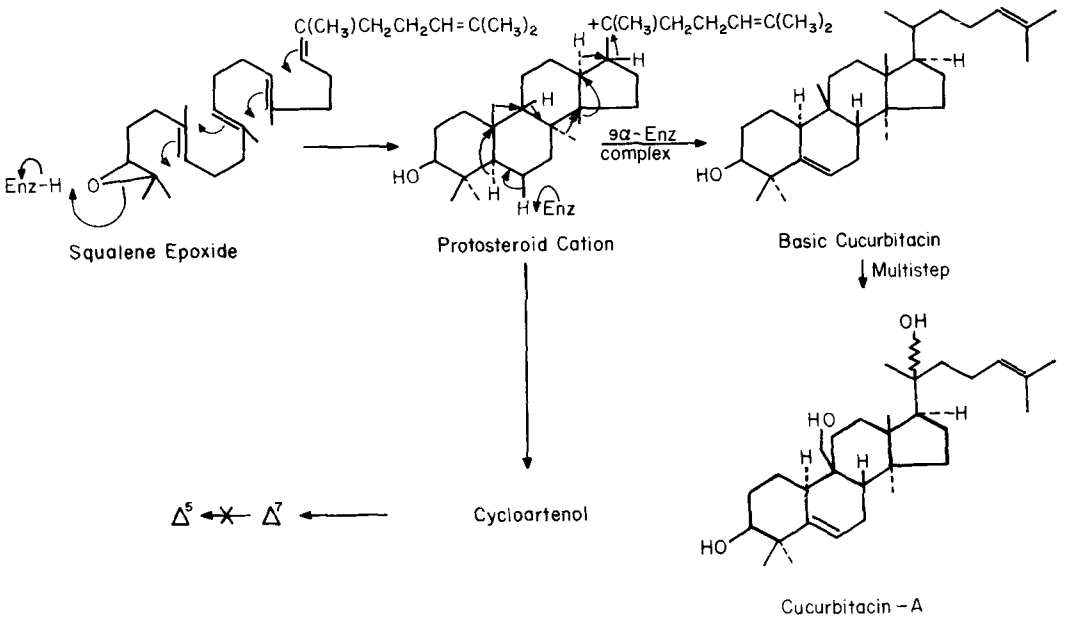


FIG. 8. The cyclization of squalene in the *Cucurbitaceae* which contain Δ^7-sterols and Δ^5-cucurbitacins.

functions which are acquired. More recent investigators have taken similar views. In part, the current view also is derived from biosynthetic information which seems at first sight to corroborate von Behring's conclusions. True sterols almost certainly could not be biosynthesized by anaerobic bacteria, because the formation of epoxysqualene is an aerobic process in which molecular oxygen is introduced into the molecule. It should not be forgotten, though, that anaerobes do possess the isopentenoid pathway. Mevalonic acid was, in fact, discovered in one, only later being shown to arise in higher forms of life (129); and the pathway in bacteria is known to proceed to a polymer which is used, interestingly enough, as a carbohydrate carrier in cell wall biosynthesis (7). Does the bacterial work then really imply that sterols are not involved as basic components of the life process?

Nothing we know would require that any and all membranes have to be constructed with a true sterol. The data reviewed here only suggest strongly that sterols act biologically in membranes as their primary role and that the sterol structure is better than any other, while not being the only one which can function in this way. Consequently, the absence of sterols in anaerobic bacteria probably only means that they have a less functional cytoplasmic envelope, which, in any event, may well utilize an appropriate conformation of a fatty acid as an approximation to a sterol. The utilization of an isopentenoidal alcohol, e.g. the C_{30}-geranylgeranylgeranyl alcohol, as a sterol-like material is similarly conceivable. Both it and a fatty acid should be able to take conformations analogous to that shown for the carotenols (Fig. 3). Bacteria contain fatty acids possessing as many as 17 and 18 carbon atoms (146). The former figure is for g positive organisms and the latter for g negative ones. Both sizes are adequate to form conformations mimicing all four rings of the steroidal structure.

In further work a distinction between anaerobes and aerobes will have to be made. Since the isopentenoid pathway per se is present, (some) aerobes may well biosynthesize sterols or a related material. Sterols and pentacyclic triterpenoids actually have been reported recently in certain bacterial species (147, 148), which emphasizes the danger of an absolute view. Similarly, when no sterols are found, the experimental work will have to show absence below some critical quantitative level, which perhaps would have to be less than the very small quantity of 0.001% of wet wt, to be meaningful. In isolated membranes, it would have to be a few percentages of the lipids present. Future investigations of really sterol-less bacteria would then also have to involve a search for sterol-like substances, including even acyclic ones, to struggle successfully with the significance of the absence. Until this kind of information is available, bacteria probably should best be regarded only as a challenge to

our insight. The clue to an understanding of the problem may well prove to be the quantitative phenomenon of bad, poor, good, better, and best structures in terms of a particular function, without regard to our preconceived notions about what sterols, triterpenoids, etc., are. Nature, of course, is only interestered in what molecules do.

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Function of Steryl Esters in Plants: A Hypothesis That Liquid Crystalline Properties of Some Steryl Esters May Be Significant in Plant Sterol Metabolism[1]

A.M. ATALLAH and **H.J. NICHOLAS,** Institute of Medical Education and Research, and Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri 63104

ABSTRACT

The synthesis of a wide variety of desmethyl, 4α- and 4,4-dimethyl steryl esters, whose physical properties have been described elsewhere, has indicated that side chain structure and the position of a double bond in the nucleus are critical in determining whether or not a steryl ester can form a mesophase (liquid crystalline state). Since the capability of mesophase formation seems rather specifically distributed in the biosynthetic steps leading to the formation of animal, plant, and fungal sterols, the hypothesis is presented that such mesophase formation, or lack of it, may control the biosynthetic sequence by virtue of the viscosity changes associated with the formation of liquid crystals. Mesophase formation of thermotropic liquid crystals is influenced by a number of physical factors, including heat, electrical current, pressure, and impurities, e.g. solvents. Some preliminary experiments with incubation of cell-free extracts of *Phaseolus vulgaris* are reported, with heat as the physical parameter studied, following the addition of cholesteryl-1,2-^{3}H, sitosteryl-22,23-^{3}H(N) palmitates, and the respective free compounds to the cell-free extracts. Although some possible influence of heat during the incubation of the compounds upon their distribution in subcellular fractions was indicated, no consistent pattern was found. However, some observations on the capability of the subcellular organelles to hydrolyze the added esters and esterify the free compounds are presented. The data suggest that hydrolysis of steryl esters and their synthesis by plant cell-free extracts are both of more than coincidental occurrence.

[1]One of eight papers presented in the symposium "Phytosterols," AOCS Spring Meeting, New Orleans, April 1973.

INTRODUCTION

Steryl Esters

The function of cholesterol as an integral part of the mammalian membrane seems to have been established firmly, its most likely role being to serve as a binding or condensing agent for the classical membrane lipid-protein bilayer (1,2). The role of cholesteryl esters is less well established. Indeed, only a few suggestions have been made with regard to the function of steryl esters in general, and these suggestions are supported by only minimal experimental data. For example, as Frank and Byrd (3) state: "their wide distribution in the body suggests that they are important in many normal or pathological functions." Brady and Gaylor (4) have suggested from their data obtained by using broken cell preparations of rat liver and skin that the "very significant competition between esterification and demethylation of methyl sterol intermediates of skin suggests that sterol ester accumulates in rat skin because of rapid formation of esters that may not be further metabolized." Cholesteryl esters accumulate in atheromatous plaques (5) and in plaques from the brains of multiple sclerosis patients (6). Possibly relevant to this are the observations of Ramsey, et al., (7) who has shown that the classical cholesteryl ester fraction of rat brain formed during the period of active brain myelination consists of ca. 50% cholesteryl esters, the remaining constituents of this fraction being esters of intermediates required for the biosynthesis of cholesterol itself.

Finally, two additional observations from this laboratory have tended to concentrate our interests and efforts to determine the role of steryl esters in plants and animal tissues. First, we recently have reported that incubation of cholesteryl palmitate, but not free cholesterol, with various rat brain preparations in vitro results in the formation of cholesterol α- and β-epoxides and, on more prolonged incubation, in the conversion of the latter to cholestane-3β,5α,6β-triol (8). Secondly, during an examination of banana skin, it was found that the lipids of this tissue consist largely of the sterols and methylated sterols shown in Figure 1. The

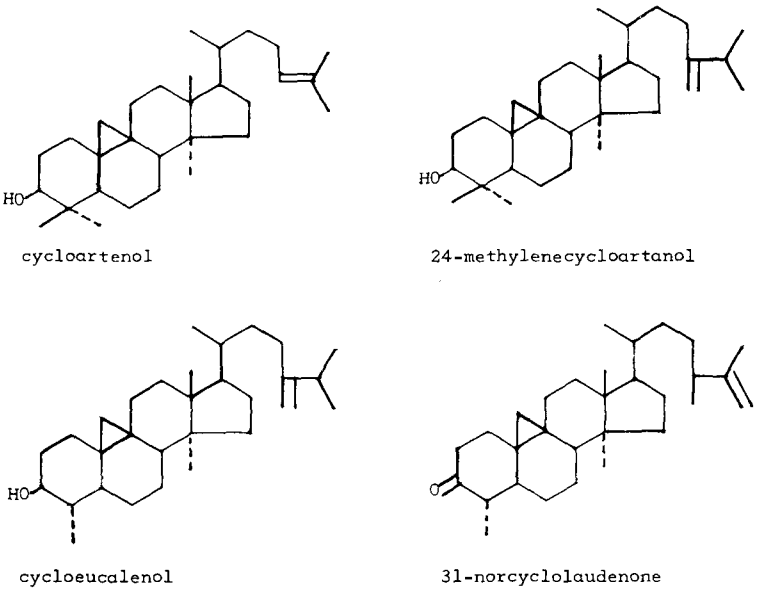

FIG. 1. Triterpenes and sterols of the banana peel (9,10).

methylated sterols, except the ketone 31-norcyclolaudenone, of course, exist almost entirely in the esterified form; the desmethyl sterols, on the other hand, exist almost completely in the free form (9,10). Coincident to this, one of the methylated sterols in banana skin was determined to be cycloartenyl palmitate (Fig. 1), which subsequently proved to be a new type of liquid crystal, previously not recognized in the group of sterol liquid crystals (11). Cycloartenol itself recently has been established as a key intermediate in plant sterol biosynthesis (12). As a result of these observations, we have instituted a detailed study of the properties of many methylated and nonmethylated steryl esters, a number of them established or suspected intermediates in the biosynthetic sequence leading to the formation of plant sterols, such as sitosterol or stigmasterol, and have been attempting to relate the liquid crystalline properties of these compounds to some specific biochemical role in plant and animal tissues. Examples of such methylated sterols which we found to form liquid crystals are 31-norcycloartanol (13), lophenol (14), and pollinastanol (15). This article has been directed largely toward this area.

Liquid Crystals

Many excellent reviews exist on the nature of liquid crystals and their properties (16,17). (Our comments and experimental observations have been limited to the broad class of liquid crystals classified as those of the thermotropic type [13-20].) It would be inappropriate to elaborate on this complex state at this time. However, several relevant comments are necessary.

First, they do not undergo the usual crystal-isotropic liquid transition exhibited by most organic substances on subjection to thermal treatment, but instead exhibit mesophases usually extending over a period of several degrees before becoming completely isotropic. Liquid crystals, for example, are classified upon the basis of their textures and molecular packings as discussed below.

Smectic structures: This is a turbid, viscous state, with certain properties reminiscent of those found for soaps. There are several modifications of the smectic type which can be differentiated by X-ray diffraction methods.

Nematic structures: There are two types of nematic structures, normal nematic and twisted

nematic. Normal nematic structures are turbid but mobile states. On surfaces, such as glass, this mesophase adopts a characteristic threaded texture. The twisted nematic structure (commonly called the cholesteric structure) is a turbid, mobile mesophase exhibiting distinct optical properties.

The older, outdated classifications divided liquid crystals into three different classes and regarded the cholesteric mesophase as an individual class, as in Fergason's review (21). For further details of the properties of liquid crystals, reference should be made to this primary review for classical information and to others (16,17) for more modern aspects. It should be mentioned that such mesophases can sometimes be distinguished by the naked eye (22), but the finer distinctions are best determined under crossed polarizers through a thin film of the substance heated between cover slips under a microscope at low power. All smectic mesophases described throughout this article were of the smectic A type, since they showed resemblance to the type already described (Fig. 8, ref. 20). Among the many parameters that influence mesophase formation, the chain length of the fatty acid moiety is critical (16,17). This is indicated in Table I, which shows for several methylated sterol esters (from our own observations) that these substances may exhibit cholesteric or smectic mesophases or both or none, depending upon chain length of the fatty acid.

A general summary of some key plant and animal sterols and methyl sterols and their capacity to form smectic and cholesteric mesophases is given in Table II. Of more potential from the biosynthetic standpoint, it appears that a considerable degree of selectivity for mesophase formation exists within the biosynthetic sequence indicated from presently established data for cholesterol biosynthesis (Fig. 2), for plant sterol biosynthesis (Figs. 3 and 4), and biosynthesis of the fungal sterol, ergosterol (Fig. 5). Figure 6 shows a possible biosynthetic pathway leading to cholesterol from the yeast sterol zymosterol and the mesophase forming capacity of each intermediate. It will be noted

TABLE I

Mesophase Types and Transition Temperatures Exhibited by Some Animal and Plant Steryl Esters[a]

Saturated fatty acid	Cholesterol[b]		31-Norcycloartanol (13)		Ergosterol (23)		Lophenol (14)	
	CHO	SM	CHO	SM	CHO	SM	CHO	SM
C_2	113	---	---	72.5	---	---	---	96
C_4	112.5	---	---	103.5	---	142	---	127
C_6	98.5	---	---	97	---	141.5	---	---
C_8	92.5	---	---	87.5	---	137.5	---	---
C_{10}	91	68.5	57	87	---	132	---	59
C_{12}	87.5	80.5	65	83.5	---	127.5	---	74
C_{14}	73.5	80	68	80.5	---	118	---	68
C_{16}	70	64	68	78	---	110	---	75

[a]Numbers indicate temperatures in C at which the mesophase appears either on heating or cooling.
[b]CHO = cholesteric and SM = smectic.

TABLE II

Plant and Animal Sterols Exhibiting Liquid Crystalline States When Esterified with Fatty Acids

Plant sterols[a]		Animal sterols	
Cycloartenol	(CHO-SM)	Cholesterol	(CHO-SM)
Cycloartanol	(CHO)	Cholestanol	(CHO-SM)
Cycloeucalenol	(CHO-SM)	Lathosterol	(SM)
Cycloeucalanol	(CHO-SM)	7-Dehydrocholesterol	(SM)
24-Methylene cycloartanol	(CHO)	4,4-Dimethyl-cholest-7-en-3β-ol	(SM)
24-Methyl cycloartanol	(CHO-SM)	Lophenol	(SM)
31-Norcycloartanol	(CHO-SM)		
Lophenol	(SM)		
Pollinastanol	(CHO-SM)		
Campesterol	(SM)		
Ergosterol	(SM)		

[a]CHO = cholesteric, and SM = smectic.

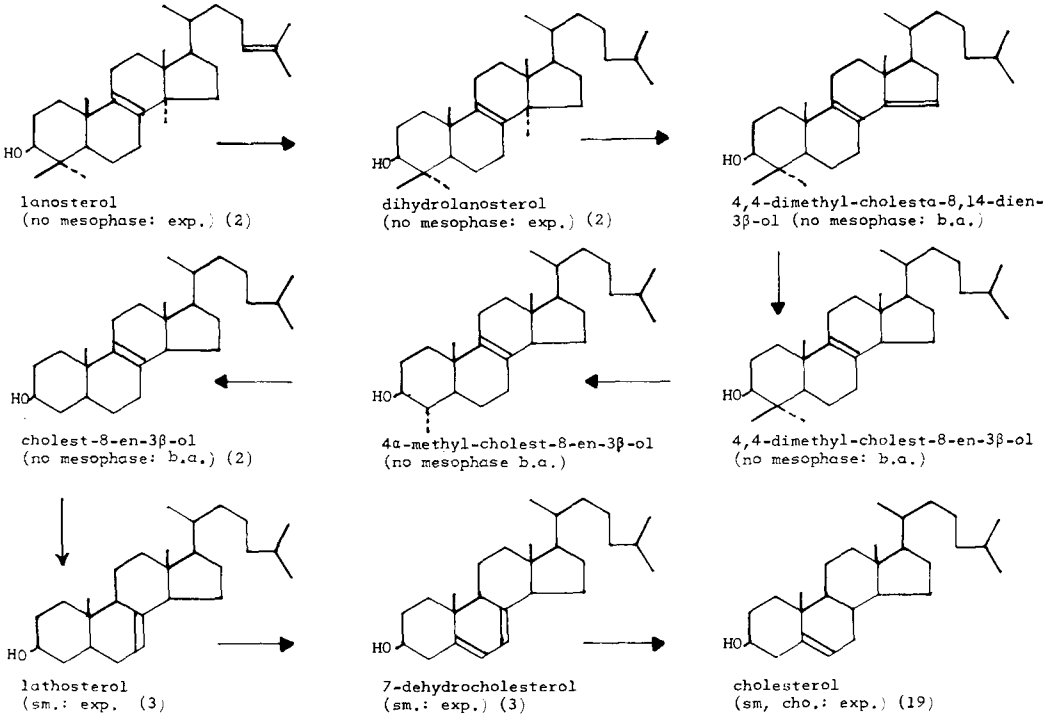

FIG. 2. Possible biosynthetic conversion of lanosterol into cholesterol (24). sm = Smectic, cho = cholesteric, exp = experimental, and b.a. = by analogy.

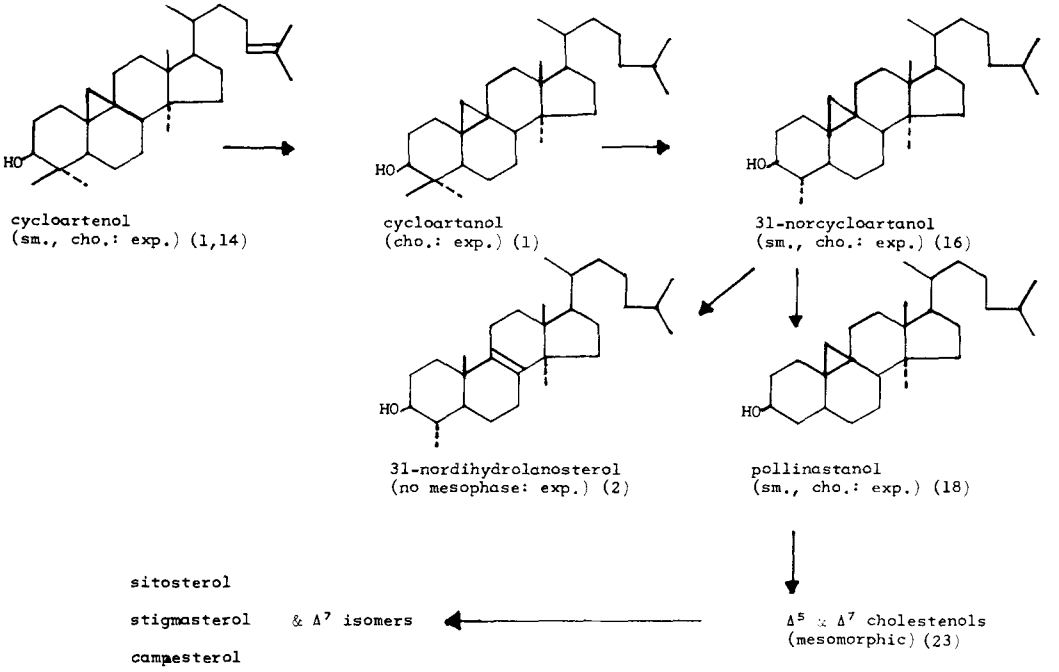

FIG. 3. Possible biosynthetic conversion of cycloartenol into phytosterols. sm = Smectic, cho = cholesteric, and exp = experimental.

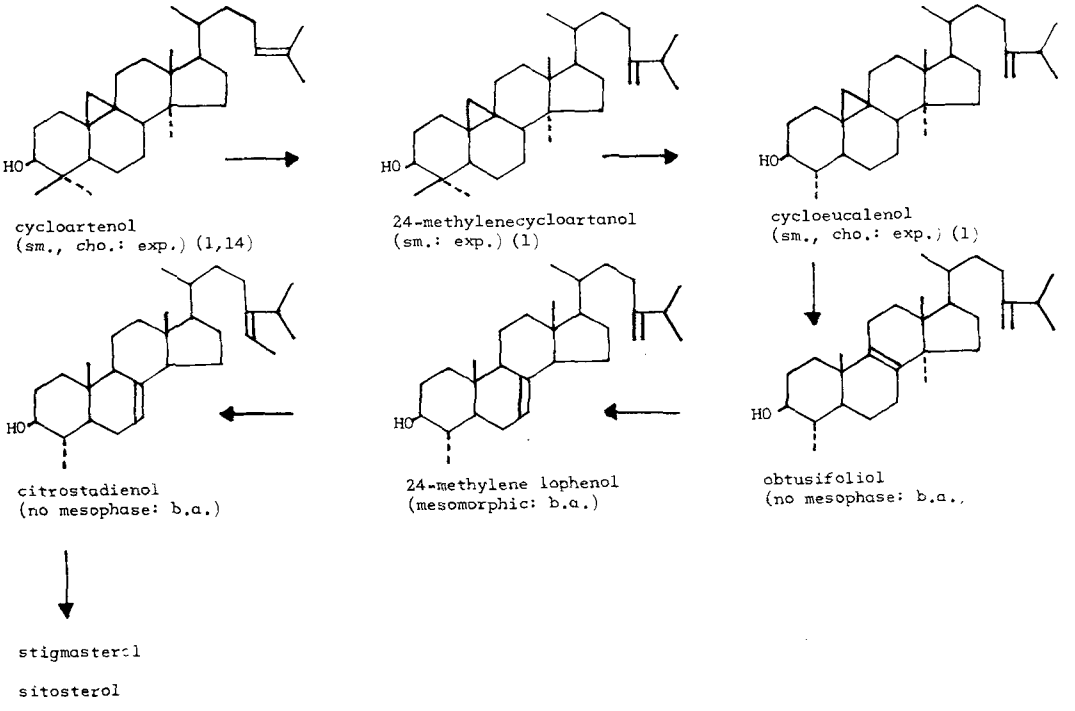

FIG. 4. Possible biosynthetic conversion of cycloartenol into phytosterol, involving side chain methylation (24). sm = Smectic, cho = cholesteric, exp = experimental, and b.a. = by analogy.

that many of the key intermediates form no mesophase at all. Whether or not these selective mesophase transitions, or lack of them, in these biosynthetic sequences are merely coincidental or have a specific function based upon their fluidity or viscosity at the mesophase transition has been one objective of our proposed hypothesis. For example, such changes in viscosity at critical steps in the biosynthetic pathways may influence changes in membrane permeability with consequent effects upon the chain of metabolic events. This comment is based upon the assumption that steryl esters are present in the membranes as an integral structural unit, something that has not been established yet.

METHODS AND RESULTS

This article attempts to discuss in a partial summary manner what is known about the role of steryl esters in living tissues, especially plant tissues, and to present a hypothesis that their liquid crystalline properties may, in some way, play an active role in their function. The formation of mesophases by substances capable of exhibiting such a state is influenced by heat, electric currents, trace solvents, pressure, and many other physical factors (16,17). In approaching the overall hypothesis, it was assumed that the liquid crystalline properties of plant steryl esters manifest themselves at the

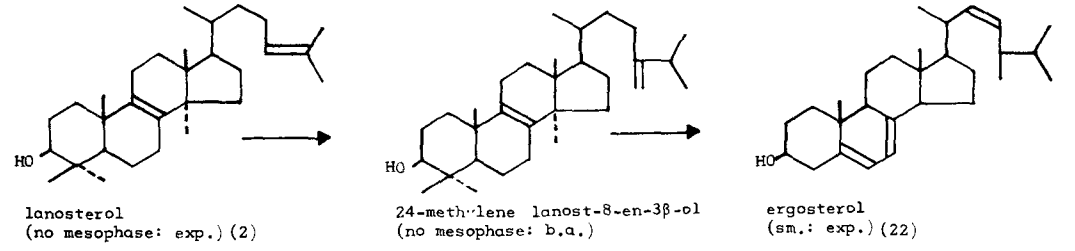

FIG. 5. Biosynthetic conversion of lanosterol into ergosterol (24). sm = Smectic, exp = experimental, and b.a. = by analogy.

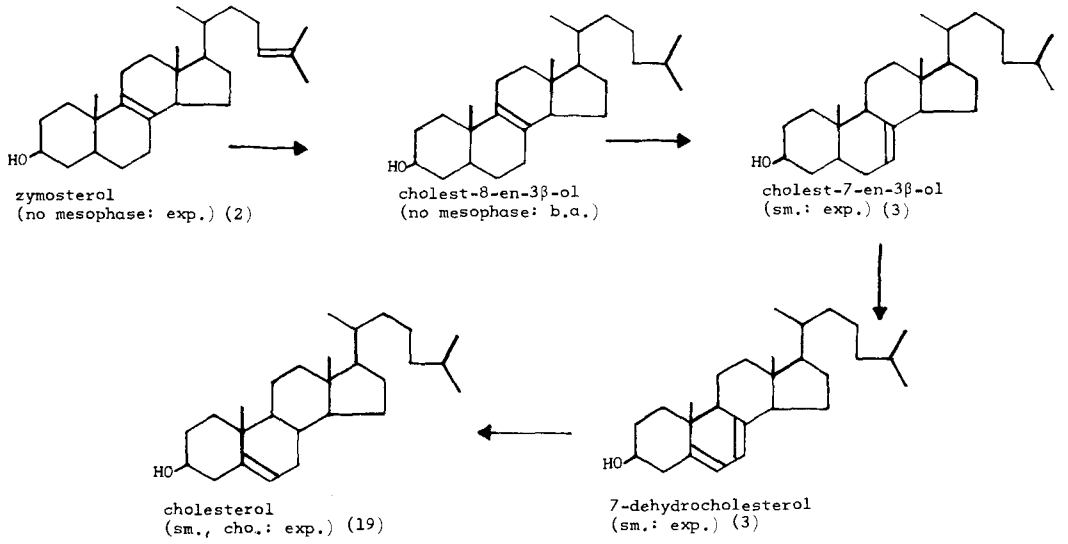

FIG. 6. Possible biosynthetic sequence in the formation of cholesterol from zymosterol (24). sm = Smectic, cho = cholesteric, exp = experimental, and b.a. = by analogy.

subcellular level. Attempts to show this with subcellular organelles from living plant tissues is a difficult problem. A large number of experiments have been performed in our laboratory with this objective in mind, using heat as the initial parameter for such studies. In retrospect, these studies, as performed, were an oversimplified approach to this most complex problem. Accordingly, only a general summary of our preliminary studies follows.

Using etiolated bean seedlings (*Phaseolus vulgaris*, L., var. Bush Bean-Green Podded Top Crop, Hummert Seed Co., St. Louis, Mo.), cell-free extracts were prepared according to the general method of Brandt and Benveniste (25). These cell-free extracts were incubated in the dark with cholesteryl-1,2-^{3}H palmitate, β-sitosteryl-22,23-^{3}H(N)-palmitate, and the respective free ^{3}H-labeled sterols, each for 2 hr at 20, 30, and 40 C. Following the incubation period, purified etioplasts were prepared via the discontinuous sucrose density gradient method of Kemp and Mercer (26), and mitochondrial and microsomal fractions were prepared by the same method with slight modification.

Total lipids subsequently were extracted from each subcellular fraction by successive extraction with hot acetone and chloroform-methanol (2:1). The total ^{14}C incorporation into the respective organelle lipid extracts is shown in Table III. Both free cholesterol and sitosterol were incorporated into mitochondria to a higher degree than the respective palmitates of these sterols. Both free sterols and their respective palmitates were incorporated in ca. equal amounts into microsomes. There was no marked effect of incubation temperature upon the degree of incorporation into either mitochondria or microsomes, unless one considers the value of 11% for microsomal sitosterol at 40 C, as opposed to 7 and 5% at 20 C and 30 C, respectively. These data must be considered equivocal at this time. There was no significant incorporation into etioplasts in any case.

It was concluded that there is some degree of selective uptake of the sterols and their palmitates by the subcellular organelles under the given experimental conditions.

Thin layer chromatography (TLC) analysis of lipid extracts obtained (above) showed that both mitochondria and microsomes exhibited the capacity to hydrolyze added ester (maximum 15% with either steryl ester) and esterify added free sterol (maximum 10% with either free sterol) (Table IV). Again, temperature effects were too inconsistent to validate any fixed conclusions. Attention should be called, however, to the marked variations seen in some cases, for example the variations with temperature indicated for free cholesterol and sitosterol in mitochondria following incubation with cholesteryl palmitate and sitosteryl palmitate, respectively, and the variations with temperature of steryl ester formation in microsomes following incubation of cell-free extracts with free sitosterol.

It was concluded that *P. vulgaris* mitochondria and microsomes are both capable of synthesizing and hydrolyzing added free cholesterol and sitosterol and their respective pal-

TABLE III

Incorporation of Cholesterol-1,2-^{3}H, Sitosterol-22,23-^{3}H(N), and Their Palmitates into Subcellular Organelles of *Phaseolus vulgaris* Following 2 Hr Incubation with Cell-Free Preparations[a]

Temperature, C	Percentage of ^{3}H administered			
	Cholesteryl palmitate	Cholesterol	Sitosteryl palmitate	Sitosterol
		Mitochondria		
20	1	6	1	5
30	2	8	<1	8
40	3	8	2	8
		Microsomes		
20	6	6	6	7
30	4	8	6	5
40	4	7	2	11
		Etioplasts		
20	<1	<1	<1	<1
30	<1	<1	<1	<1
40	<1	<1	<1	<1

[a]Total ^{3}H content calculated from a 10% aliquot of the total lipid extracts (2:1 $CHCl_3$-MeOH) of the organelles indicated, with calculations based upon the ^{3}H originally incubated: cholesteryl palmitate, 458,000 cpm; free cholesterol, 140,000 cpm; sitosteryl palmitate, 451,000 cpm; and free sitosterol, 203,000 cpm.

TABLE IV

Incorporation of Cholesterol-1,2-^{3}H, Sitosterol-22,23-^{3}H(N), and Their Palmitates into Sterol and Steryl Esters of Subcellular Organelles of *Phaseolus vulgaris* Following Incubation with Cell-Free Preparations[a]

	^{3}H, Percentage incorporation (from TLC)			
	Mitochondria		Microsomes	
	Free sterol	Ester	Free sterol	Ester
Cholesteryl palmitate				
20	15	76	12	80
30	11	80	10	82
40	11	75	6	91
Free cholesterol				
20	50	9	87	8
30	82	3	87	4
40	28	4	79	7
Sitosteryl palmitate				
20	10	85	8	96
30	7	33	3	97
40	3	83	6	90
Free sitosterol				
20	74	10	92	8
30	37	6	80	10
40	46	3	100	0

[a]The total lipid extracts (90% original extracts) indicated in Table III were subjected to thin layer chromatography (TLC). The above values indicate figures obtained by scraping the free sterol and ester regions, extracting the labeled material from the silica gel and determining the ^{3}H content. ^{3}H Values for other regions of the plates are not shown.

[b]Temperatures given in C.

TABLE V

Distribution of ^{14}C into Subcellular Organelles of *Phaseolus vulgaris* Etiolated Seedlings Incubated with 2-^{14}C-MVA for 24 Hr at Different Temperatures[a]

Organelle	Temperature, C	Total cpm
Microsomes	20	189,000
Microsomes	30	120,000
Microsomes	40	198,000
Swollen mitochondria	20	200
Intact mitochondria	20	300
Swollen mitochondria	30	200
Intact mitochondria	30	200
Swollen mitochondria	40	300
Intact mitochondria	40	300
Etioplasts	20	1,300
Etioplasts	30	1,100
Etioplasts	40	1,400

[a]Values represent total ^{14}C-incorporation into $CHCl_3$-CH_3OH (2:1) extracts of the organelles indicated. The etioplasted cut seedling sections each had been allowed to absorb 10 μCi 2-^{14}C-MVA at the temperature indicated, following which each section was kept in distilled H_2O for 24 hr also at the temperature indicated.

mitate esters. The effect of incubation temperature on these phenomena was inconsistent.

The experiments reported above were conducted with cell-free preparations which exhibited only a weak capacity to synthesize total nonsaponifiable material from 2-^{14}C-mevalonic acid (MVA-2-^{14}C) (unpublished experiments). This may represent a limiting factor in metabolic studies, since it generally is recognized that obtaining a cell-free extract of a higher plant that can synthesize isoprenoids is difficult at our present stage of knowledge. Accordingly, we approached the problem in a manner previously explored in this laboratory (27). MVA-2-^{14}C was administered to etiolated *P. vulgaris* seedlings held at temperatures of 20, 30, and 40 C (respectively) for 24 hr, followed by fractionation of the seedlings into subcellular fractions according to the method of Kemp and Mercer (26) and Brandt and Benveniste (25). For all practical purposes, there was no incorporation of ^{14}C into either swollen or intact mitochondria (25) or etioplasts (Table V). This is consistent with our previous observations (27) that the microsomal plus cytosol fractions are responsible for the synthesis of sterol from MVA-2-^{14}C in higher plants. There was no consistent effect of temperature of incubation upon total ^{14}C-incorporation into microsomes. When the total ^{14}C-labeled microsomal lipids were subjected to TLC, the percentage of ^{14}C-ester in the microsomal fractions was (2-3%) (Table VI). No effect of incubation temperature upon ester formation in these fractions was apparent.

DISCUSSION

The purpose of this article has been twofold. First, we have suggested a hypothesis that one of the functions of steryl esters (we include the methylated sterols commonly referred to as tetracyclic triterpenes in this term, e.g. lophenol, lanosterol, etc.) in plants may be related to their liquid crystalline properties, possibly in the sterol biosynthetic sequence, since mesophase formation (Figs. 2-6) seems selective in this complex sequence. Successful proof of this hypothesis depends upon experimental demonstration that these compounds can, or do, assume the liquid crystalline state (mesophase) at the temperature of the living organism, whether it be plant or animal. Since most of the mesophases determined on a number of pure compounds (Table I) are well above this temperature, it would appear that mesophase formation at living temperature is unlikely. One argument in disputing this has been presented by the observations of Scanu and Tardieu (28) and Frank and Byrd (3), both of which indicate that mixtures of cholesteryl esters, especially those containing unsaturated fatty acids, are probably fluid (mesomorphic) at body temperature. Frank and Byrd state, for example: "all mixtures of cholesteryl oleate and cholesteryl linoleate are mesomorphic at

TABLE VI

Incorporation of ^{14}C into Microsomes of *Phaseolus vulgaris* Etiolated Seedlings Incubated with 2-^{14}C-MVA for 24 Hr at Different Temperatures[a]

		Percent ^{14}C incorporated	
Organelle	Temperature, C	Sterols	Esters
Microsomes	20	46	2
Microsomes	30	50	2
Microsomes	40	53	3

[a]The total lipid extracts 2:1 $CHCl_3$-CH_3OH (90% original amount obtained) shown in Table V were subjected to thin layer chromatography as indicated in "Methods and Results." The ester and free sterol regions were scraped, extracted, and the ^{14}C content of the regions determined. Values for other regions of the plate are not shown.

body temperature." This is consistent with the work of Small (29). The methylated sterol esters synthesized in our laboratories have not as yet been examined for their mesomorphic behavior in mixtures, although this work is in progress. It seems most probable, however, that they occur in all tissues as complex mixtures containing fatty acids, both saturated and unsaturated and of varying chain length, e.g. pollen (13), and are accordingly capable of forming mesophases at body temperature. Our approach to demonstrate this has been rather crude for such a potentially complex property. It may be that we chose the incorrect steryl esters for this preliminary work. Our objective was to investigate one ester known to exhibit a mesophase, cholesteryl palmitate (16), and one which does not, sitosteryl palmitate (18). The role of cholesterol in plant sterol metabolism is not established firmly yet (30). Hence, further studies upon established intermediates like 31-norcycloartanol, lophenol, and pollinastanol may provide more meaningful data.

Although not specifically delineating the role of steryl esters in plant sterol metabolism, it seems of particular interest that microsomes and mitochondria (and possibly chloroplasts on more extensive investigation) possess the capacity to both esterify sterol (cholesterol and sitosterol) added to cell-free preparations in vitro and also hydrolyze the esters of these same sterols. The significance of this is not clear but certainly points to the likelihood that steryl esters are not formed without some metabolic purpose.

The experiments presented in this article resulting from administration of MVA-2-^{14}C to etiolated seedlings again support our previous observations (Knapp, et al. [27]) that the endoplasmic reticulum (microsomes in particular) is the site of sterol biosynthesis. Several questions naturally arise: (A) How do the mitochondria receive their transported sterols? and (B) How and in what order do specific sterols form the major portion of these subcellular organelles? Brandt and Benveniste (25) appear to have solved the problem of the specific nature of the sterols of subcellular organelles of *P. vulgaris* and have suggested the possible importance of steryl esters in the organelles.

Cholesterol is an intermediate in plant sterol biosynthesis; and sitosterol, except where converted to minor sterols of questionable physiological significance, is an end product. Accordingly, we were hoping that the palmitate of cholesterol (a liquid crystal) as indicated in our in vitro brain experiments (8) would be converted to other compounds, whereas the free sterol would not, or would, indicate a lesser degree of conversion. This hope was not realized in the present experiments, since the free sterol obtained from the incubation mixtures following addition of cholesteryl palmitate appeared to be cholesterol, just as in the case where free cholesterol was incubated. However, this approach was difficult to evaluate on a quantitative basis using the radioactive monitoring system (unpublished observations) for evaluation, and more intensive study may indicate that the ester does, indeed, penetrate the endoplasmic reticulum of plant tissues more readily than the free sterol for further metabolism. This has been our interpretation with the brain experiments (8). Whether these observations can be related to cholesteryl palmitate as a liquid crystal will require further investigation.

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Improved Synthesis of 7-Dehydrositosterol[1,2]

HENRY W. KIRCHER, Department of Agricultural Biochemistry,
The University of Arizona, Tucson, Arizona 85721

ABSTRACT

Sitosteryl acetate was brominated in petroleum ether by the gradual addition of N-bromosuccinimide to a refluxing, illuminated solution and dehydrobrominated by the dropwise addition of the bromo derivative to a refluxing solution of collidine in mesitylene to give a 38% yield of 7-dehydrositosteryl acetate.

INTRODUCTION

7-Dehydrositosterol (trivial name for 5,7-stigmastadien-3β-ol) was first prepared in Windaus' laboratory in 1936, in 2% yield from sitosteryl acetate to test the antirachitic properties of its irradiated products (1). 7-Dehydrositosteryl benzoate has been prepared in unspecified yield by the photo bromination and dehydrobromination of sitosteryl benzoate (2); the purity of this material, however, is in doubt. A mp of 153-4 C was reported (lit [1] 149 C), and the authors mention the presence of tachysteryl derivatives in the product. Our recent isolation of 99.5% pure sitosteryl acetate (3), together with an improved method for the preparation of 5,7-dienes from Δ^5-steryl acetates (4), enabled us to prepare chromatographically pure 7-dehydrositosterol in good yield.

EXPERIMENTAL PROCEDURES

Petroleum ether (Skellysolve B) was stirred a week with 1% $KMnO_4$ in water, several days with conc. H_2SO_4, shaken with 0.1% $KMnO_4$ to remove SO_2, dried ($MgSO_4$), and distilled, bp 65-67 C. Collidine and mesitylene were distilled under N_2 and dried over KOH. Reagent grade acetone, absolute ethanol, and N-bromosuccinimide (NBS) were used as obtained. The sitosteryl acetate used, mp 120.8-121.5 C, was a portion of fraction 42 mentioned in a previous communication (3). Mp's were taken in vacuo and are corrected. All crystallizations and reactions of the $\Delta^{5,7}$ derivatives were performed in solvents flushed with nitrogen and in a nitrogen atmosphere.

7-Dehydrositosteryl acetate: A 1 cm bore stopcock topped with a powder funnel was cemented (GE Silicone RTV) to a 24/40 glass joint and placed in the center opening of a 1 liter 3-necked flask equipped with a reflux condenser and nitrogen inlet. The lower stem of the stopcock protruded ca. one fourth of the way into the flask. The flask was clamped over a magnetic stirrer and was heated and illuminated by 2 GE DSB Photospot bulbs 5 cm away. Sitosteryl acetate (65 g, 0.142 mole) was dissolved and brought to a reflux in 350 ml Skellysolve B under N_2 with rapid stirring. NBS (32 g, 0.187 mole, 30% excess) was added in small portions during 30 min through the stopcock with the aid of 5-10 ml solvent for each addition. The lights were removed 5 min later and replaced with an ice bath. The cooled mixture was filtered into a 2 liter suction flask that contained 35 ml mesitylene and a magnetic stirring bar and the contents of the flask evaporated with an oil pump at 30-40 C until a thick, honey-colored syrup remained. Too high a temperature during this step results in low yields of product.

The bromosteryl acetate was transferred to a dropping funnel with 130 ml mesitylene and added during 30 min to a well stirred, refluxing solution of 50 ml collidine in 300 ml mesitylene. Five min later the mixture was cooled in an ice bath, low-boiling petroleum ether was added (400 ml) and the mixture filtered and evaporated at 50-60 C in vacuo. The resulting brown semisolid was brought to boiling under N_2 with 250 ml acetone. After cooling to room temperature, 31.0 g 7-dehydrositosteryl acetate that contained small amounts of sitosteryl and 4,6-stigmastadienyl acetate was filtered off (thin layer chromatography: 10% $AgNO_3$-SiO_2 plates, 1:1 $CHCl_3/CCl_4$, R_f: $\Delta^5 > \Delta^{4,6} > \Delta^{5,7}$ acetates). An additional 9.4 g less pure product precipitated from the acetone filtrate in the refrigerator; this was recrystallized from 150 ml acetone to give 3.8 g purer material. The combined products (34.8 g) were recrystallized again from acetone (900 ml) and then from Skellysolve B (200 ml) to yield 17.0 g 7-dehydrositosteryl acetate, mp 146.5-147.2 C (lit. [1] 151-2 C). An additional 7.7 g material, mp 146-7 C precipitated from the Skellysolve B filtrate in the refrigerator.

[1]One of eight papers presented in the symposium "Phytosterols," AOCS Spring Meeting, New Orleans, April 1973.

[2]Contribution 2015, Arizona Agricultural Experiment Station.

7-Dehydrositosterol: A solution of sodium ethoxide (1.5 g Na plus 300 ml absolute ethanol) was added to a suspension of 25 g 7-dehydrositosteryl acetate in 1 liter ethanol, and the mixture was stirred at room temperature 20 hr under N_2. Acetic acid (7 ml) was added and the mixture cooled several hours in a refrigerator. The precipitated 7-dehydrositosterol was washed thoroughly with methanol and dried in vacuo; wt 17.5 g, mp 145-145.5 C (lit. [1] 144-5 C), single spot on thin layer chromatography (10% $AgNO_3$-SiO_2 plate, 95:5 $CHCl_3$/acetone), single peak on gas liquid chromatography (5% OV-101, 260 C). A benzoate was prepared, mp 149-149.2 C (lit [1] 149 C).

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A Facile Synthesis of Ergostanol[1,2]

HENRY W. KIRCHER, Department of Agricultural Biochemistry, The University of Arizona, Tucson, Arizona 85721

ABSTRACT

Ergosteryl acetate is hydrogenated in one step to ergostanyl acetate over 10% Pd/C in ethyl acetate-acetic acid at 150 C.

INTRODUCTION

The classical preparation of ergostanol (trivial name for ergostan-3β-ol) (1-3) is time consuming. An ergosteryl ester (acetate or benzoate) is hydrogenated in the presence of acetic acid to the $\Delta^{8(14)}$ derivative; the latter is rearranged in chloroform with dry HCl to the Δ^{14} derivative which then is reduced to the appropriate ergostanyl ester. This procedure took 2.5 months in our laboratory. The rearrangement of $\Delta^{8(14)}$ to Δ^{14} steryl esters is an equilibrium reaction; and, although the Δ^{14} isomers crystallize preferentially from solution in the ergostane series, complete separation of 14-ergostenyl acetate from small amounts of 8(14)-ergostenyl acetate only could be accomplished by chromatography on 20% $AgNO_3$/SiO_2 columns.

A rapid preparation of ergostanyl acetate was developed. Ergosteryl acetate was hydrogenated over palladium in a mixture of ethyl acetate and acetic acid at 150 C. Traces of Lieberman-Burchard positive material were removed with CrO_3, and pure ergostanyl acetate was isolated in 55% yield. The hydrogenation undoubtedly goes through the $\Delta^{5,7,22} \rightarrow \Delta^{7,22} \rightarrow \Delta^{7} \rightarrow \Delta^{8(14)} \leftrightharpoons \Delta^{14} \rightarrow \Delta^{0}$ route; the next to the last step occurs in the hot acetic acid solution. At room temperature, the reaction stops at the $\Delta^{8(14)}$-ergostenyl acetate stage.

EXPERIMENTAL PROCEDURES

Ergosteryl acetate, mp 177-8 C (25 g), 10% Pd/C (2 g), ethyl acetate (250 ml), and glacial acetic acid (100 ml) were placed into a 1 liter Parr stirred autoclave and flushed 3 times with hydrogen. The pressure was adjusted to 13.5 atm and the temperature raised to 150 C over 1.5 hr. The pressure then was raised to 34 atm and stirring continued at 150 C for 1 hr, after which the heater was shut off. When the autoclave had cooled to 60 C (1.5 hr) it was opened, the catalyst filtered off, washed with benzene, and the filtrate (500 ml) cooled in an ice bath. A solution of 5 g CrO_3 in 10 ml water and 30 ml acetic acid was added dropwise to the cold (10 C) solution during 30 min and the mixture allowed to warm to room temperature. Methanol (100 ml) was added to decompose the excess CrO_3, 1 liter water was added and the mixture extracted with ether. Evaporation of the ether and crystallization of the residue (22.8 g) from 500 ml methanol-benzene gave 13.9 g chromatographically pure (gas chromatography, thin layer chromatography) ergostanyl acetate, mp 145-146 C (in air, corrected), negative Lieberman-Burchard test on 10 mg sample (lit mp 144-5 C [1-3]). A sample was hydrolyzed to ergostanol, mp 145.5-6 C after crystallization from ethanol (lit mps 144-5 C [1], 143-4 C [2], 141.5 C [3]).

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Recent Progress in the Biochemistry of Plant Steroids Other than Sterols (Saponins, Glycoalkaloids, Pregnane Derivatives, Cardiac Glycosides, and Sex Hormones)[1]

ERICH HEFTMANN, Western Regional Research Laboratory,[2] Berkeley, California 94710

ABSTRACT

Recent studies concerning the biosynthesis, metabolism, and possible functions of steroids other than sterols in plants are reviewed and discussed. These studies embrace the saponins, glycoalkaloids, pregnane derivatives, cardiac glycosides, as well as their aglycones, and the sex hormones.

INTRODUCTION

Previously, it has been pointed out (1) that one of the important functions of sterols in plants is to serve as precursors for the synthesis of other steroids. This article discusses the metabolism of steroids in plants in greater detail and makes suggestions for further studies aimed at bringing about a closer understanding of the physiological significance of plant steroids. To shorten the reference list, I shall refer the reader as much as possible to recent reviews and the literature cited therein, rather than to the original papers.

Recent advances in the biochemistry of plant steroids have been facilitated greatly by the use of modern physical methods of analysis (2), notably chromatography (3) and tracer methodology, and by previous knowledge about steroid biochemistry in the animal kingdom (4). It is now evident that all living organisms contain steroids of some kind and that all the various classes of animal steroids, with the exception of the C_{24} bile acids (however, Mandava, et al., [*Steroids* 23:357 (1974)] just reported the isolation of 5β-cholanic acid from jequirity beans) and the C_{19} and C_{24} alkaloids, also occur in plants (5). We tentatively may assume that the biosynthesis and metabolism of steroids in these classes will turn out to be similar in all living organisms, but much remains to be done to prove or disprove this hypothesis. We already are aware of minor variations in the way different animal species metabolize steroids, and we should not be surprised to find some variations in the steroid metabolism of different plant species. The distribution of some steroids is, in fact, restricted to a few plant genera.

As far as the distribution of plant steroids in animals is concerned, only the C_{21} and C_{27} alkaloids have not been detected in animals so far. This does not mean that animals can synthesize all the other types of plant steroids, but they incorporate the C_{28} and C_{29} sterols, the sapogenins, and the C_{23} cardenolides in their diet and metabolize them to some extent.

The sterols are the starting materials for the biosynthesis of all the other plant steroids. Others (6-12) already have discussed the biosynthesis of sterols in plants and have pointed out some variations in the biosynthetic pathways and some differences in the nature of the sterols produced by different plant species. These products or intermediates are utilized in various ways by various plants to produce more or less characteristic steroids. We already know that these metabolic processes do not go on in all parts of the plant at all times, but we need to learn much more about the time and place at which various reactions occur before we can say much about their possible significance.

C_{27} SAPOGENINS

One of the most tantalizing examples of the appearance and disappearance of steroids in plants is that of the C_{27} sapogenins and alkaloids (13-15). The ability to synthesize steroidal sapogenins is rather widely shared by plants belonging to the monocots and dicots. Because some of them accumulate considerable quantities of diosgenin (Fig. 1) and other valuable raw materials for the manufacture of progesterone, there has been considerable interest in the biosynthesis of sapogenins. In a series of studies that began in 1961, we have established that cholesterol (Fig. 1), which is rather ubiquitous but generally present in low concentrations in plants (5,13,16) is the starting material for the biosynthesis of diosgenin.

Either free cholesterol or some conjugated form, such as a cholesterol glycoside, is believed to be oxidized at C-16 and C-22 and at one of the two terminal carbon atoms of the side chain. The sequence in which these oxidations

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[1]One of eight papers presented in the symposium "Phytosterols," AOCS Spring Meeting, New Orleans, April 1973.

[2]ARS, USDA.

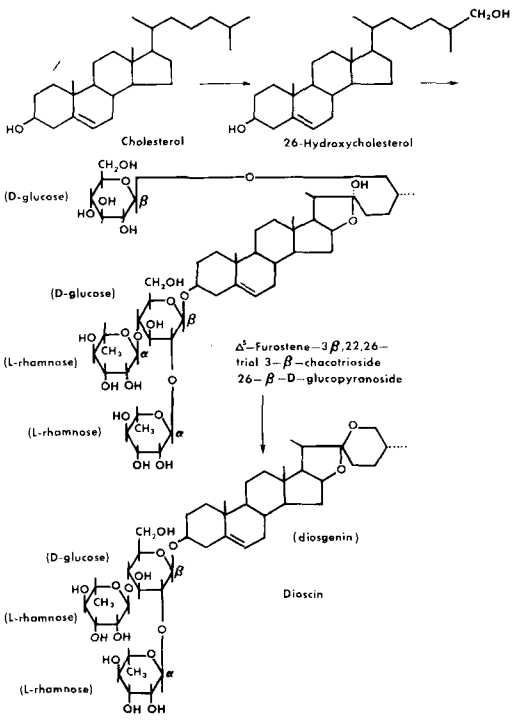

FIG. 1.

occur is not known completely. Tschesche, et al., (17) reported that neither 22-hydroxy- nor 22-ketocholesterol-22-^{14}C was converted to sapogenins by *Digitalis lanata* plants. We have prepared 26-hydroxycholesterol-26-^{14}C by reduction of kryptogenin-26-^{14}C (18) and have applied both compounds to the leaves of *Dioscorea floribunda* plants over a period of 5 weeks. Whereas kryptogenin was not converted to known sapogenins, we were able to trace the conversion of 26-hydroxycholesterol (Fig. 1) to diosgenin (19). This suggests that oxygenation at C-26 may be the first step in the biosynthesis of diosgenin from cholesterol. More recently, Tschesche and Fritz (20) have extended this observation by demonstrating the conversion of 16β,26-dihydroxycholestanol to tigogenin and gitogenin by *Digitalis lanata* (Fig. 2).

In another experiment, we were able to isolate the furostanol glycoside shown in Figure 1 in radioactive form after the administration of cholesterol-4-^{14}C to the leaves of *Dioscorea floribunda* (21). This bisdesmosidic saponin lacking ring F had been isolated earlier from *Dioscorea gracillima* by Kiyosawa, et al. (22). When it was incubated with a homogenate of a *Dioscorea floribunda* leaf, it was rapidly and almost completely converted to dioscin (Fig. 1) (23). We have used a cell-free preparation from the plant that synthesizes dioscin, but any glucosidase or even dilute acids (22) can hydrolyze the glucosidic linkage. Canonica, et al., (24) isolated labeled tigogenin from the leaves of a *Digitalis* plant to which tritiated 5α-furostan-3β,26-diol had been applied. After hydroxylation at C-22, the hemiketal spontaneously forms the cyclic ketal (spirostanol). This, incidentally, makes it unlikely that hydroxylation at C-26 is the last step in the sequence, because then ring F would close before the glucose can be attached.

Our observation that the label in 26-hydroxycholesterol-26-^{14}C is incorporated into diosgenin but not its 25-epimer, yamogenin, by *Dioscorea floribunda* indicates that—at least in this plant—the stereochemistry at C-25 of the sapogenin is fixed when cholesterol is oxygenated at one of the two terminal methyl groups and no interconversion of epimers takes place (19). It has been observed earlier that in the biosynthesis of tigogenin from mevalonic acid-2-^{14}C in *Digitalis lanata* C-26 was labeled, but C-27 was not (16). The selective oxygenation of either C-26 or C-27 originally was attributed to the presence of a double bond at C-24, and it was suggested that cholesterol may be dehydrogenated reversibly to Δ^{24}-dehydrocholesterol by plants. To test this theory, two groups of investigators have administered cholesterol-4-^{14}C-25-^{3}H to plants and determined the 3H/^{14}C ratio in the sapogenins formed. They found simultaneously that the isotope ratio in tigogenin isolated from *Digitalis* (25) and in diosgenin isolated from *Dioscorea* (26) was the same as in the administered cholesterol. This proved that cholesterol was not dehydrogenated in the process and that the stereospecific oxygenation of cholesterol is due to the presence of a prochiral center at C-25.

Another dehydrogenation has been postulated to intervene in the conversion of cholesterol to saturated sapogenins. It has been previously established (16) that the reduction of cholesterol to cholestanol (Fig. 2) in potato leaves passes through Δ^4-cholestenone (Fig. 2) as an intermediate, and Tschesche, et al., (27) demonstrated the other sterol interconversions shown in Figure 2 in intact *Digitalis lanata* plants. Each of the sterols shown was transformed into tigogenin and gitogenin, except cholesterol-3-^{3}H, because tritium was lost in the oxidation to cholestenone (17). Conversion of cholesterol-4-^{14}C to neotigogenin (Fig. 2) in *Lycopersicon pimpinellifolium* was observed in our laboratory (16).

Sapogenins undergo a series of metabolic reactions similar to those outlined for sterols in Figure 2. In these transformations, which are shown by partial structures in Figure 3, smila-

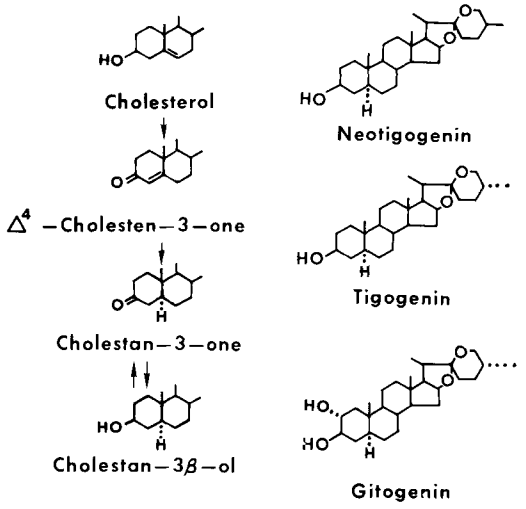

FIG. 2.

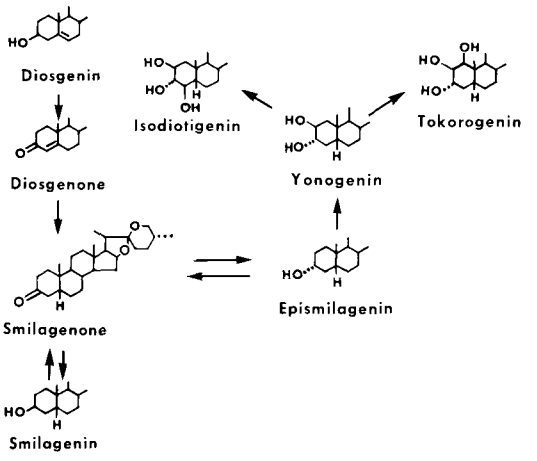

FIG. 3.

genone takes the place of cholestanone in Figure 2 (28,29). The 3α-hydroxylated sapogenins in *Dioscorea tokoro*, which apparently are formed through such pathways, do not have any sugars attached to the 3 position. Although the work of Takeda, et al., (9,29,30) and microbiological experiments (16,31) indicate that sapogenins can be enzymatically hydroxylated, Tschesche (15) has raised some doubt about the less oxygenated sapogenins being precursors of the more highly oxygenated ones.

Aside from occasional observations that sapogenins are extensively metabolized by higher plants and are converted partly to saponins (19), not much is known about their metabolic fate. We recently have discovered that tomatine is enzymatically degraded to allopregnenolone (3β-hydroxy-5α-pregn-16-en-20-one) (Fig. 6) (32), identical with the product of the Marker degradation on which the partial synthesis of progesterone from sapogenins is based (5,33). On the basis of the close structural analogy between steroidal alkaloids and sapogenins, we may assume that sapogenins are degraded to analogous products. This assumption is supported by the simultaneous occurrence of diosgenin and 3β-hydroxy-5,16-pregnadien-20-one in the same plant source, the fruits of *Solanum vespertilio* (34-36). *Mycobacterium phlei* degrades diosgenin to Δ^4-androstene-3,17-dione and $\Delta^{1,4}$-androstadien-3,17-dione (Fig. 13) (37). Perhaps this also can happen in higher plants.

Analytical data about the amount and kind of sapogenins present in various parts of plants at certain times give only a limited amount of information about the biochemical changes that go on, because they represent the balance of anabolic, metabolic, catabolic, and translocation processes during plant life. However, I shall review this information, because it is all we have at present and it may point to future discoveries concerning the role of saponins in plant physiology. Earlier observations already have been summarized (13,38).

Biosynthesis of saponins starts when the seeds germinate (39-41) and reaches a peak with the development of cotyledons and another peak when the first true leaves develop (42). As the plants mature, their sapogenin composition changes (43-55). The growing root tip and developing inflorescence are rich in saponins, as are the ripening fruits (46,47) and seeds (45). There are marked differences in amount and composition of the saponin fraction between female flowers and seeds (48), and rhythmic changes occur in stored seeds (49). The photoperiod affects the sapogenins in the epigeous portions of *Dioscorea* plants (50,51) and seasonal variations in sapogenin yields from the tubers have been noted (52). The distribution of steroidal sapogenins in the genus *Dioscorea* has been reviewed (53,54). The highest sapogenin content of tubers, on a dry wt basis, was 15% for *D. spiculiflora*, 13% for *D. composita*, and 10% for *D. floribunda* (53). The saponin content of tubers increases with age and is highest when they are dormant (55).

In vitro experiments on sapogenins have a great potential for revealing details of enzymatic reactions, but they must be interpreted with caution. Whereas cultures of differentiated *Dioscorea* tissues produce negligible amounts of sapogenins, undifferentiated tissues grown in

the presence ot 2,4-dichlorophenoxyacetic acid are capable of producing diosgenin (56,57). Other auxins enhance diosgenin production in *Solanum xanthocarpum* tissue cultures (58). The biosynthesis of sapogenins in tissue cultures of *D. deltoidea* (59-61), *D. tokoro* (62), *S. laciniatum* (63), and *Digitalis purpurea* (64) has been studied under various conditions. The metabolism of sapogenins also has been traced in vitro. When homogenates of *Balanites* fruits (47,65,66) or of young *Dioscorea* shoots (44) are incubated, the sapogenin yield increases. This can be explained on the basis of the previously discussed conversion of open chain precursors to saponins.

C_{27} ALKALOIDS

The organic (67-71) and biological (13,16,72,73) chemistry of the C_{27} alkaloids recently has been reviewed. We already know that cholesterol (74,75) and its precursors, cycloartenol and lanosterol (76) are converted into tomatidine (Fig. 4) in tomato plants, but we are still in the dark about the steps intervening between cholesterol and tomatidine, e.g. at what stage the nitrogen atom is introduced and where it comes from. When radioactive solasodine (Fig. 4) was administered to excised stems of *S. laciniatum*, the glycoalkaloids solamargine and solasonine (Fig. 4) were obtained in labeled form (77). Incubation of solasodine with uridine diphosphate-glucose and a crude enzyme preparation made from the leaves of this plant produced the 3-glucoside of solasodine.

Solanidine (Fig. 4) also is known to be synthesized from cycloartenol and lanosterol (76) via cholesterol (78,79). Kaneko, et al., (80) isolated an alkaloid lacking ring E, etioline (Fig. 4), from budding *Veratrum grandiflorum* plants. Because the etioline content of the leaves decreases during etiolation, while their solanidine content increases, they have postulated that etioline is a precursor of solanidine. Another C_{27} alkaloid with condensed ring system, solanocapsine (Fig. 4), now has been shown to be biosynthesized from cycloartenol and lanosterol (76).

The hypothesis that the C-nor-D-homosteroids in certain Liliaceae, e.g. *Veratrum*, also are derived biogenetically from cholesterol involves a Wagner-Meerwein rearrangement (13). That this reaction actually takes place in plants recently was demonstrated by Kaneko, et al., (81), who administered radioacetate or cholesterol labeled at either C-4 or C-26 to *V. grandiflorum* and isolated labeled solanidanine, jerveratrum, and ceveratrum alkaloids. The solanidanine alkaloid rubijervine (Fig. 4), which was labeled, or, more likely, 12-epirubijervine, which was not found, presumably undergoes rearrangement to the jerveratrum alkaloid 11-deoxojervine (Fig. 5), which also was labeled. In another experiment, the same group showed that growing *Veratrum* plants convert administered 11-deoxojervine-^{14}C to jervine but not to veratramine (Fig. 5) (82). More recently, Kaneko, et al., (83) observed that, when *V. grandiflorum* was kept in the dark, administered radioacetate produced an accumulation of radioactivity in solanidine; but, when the plant

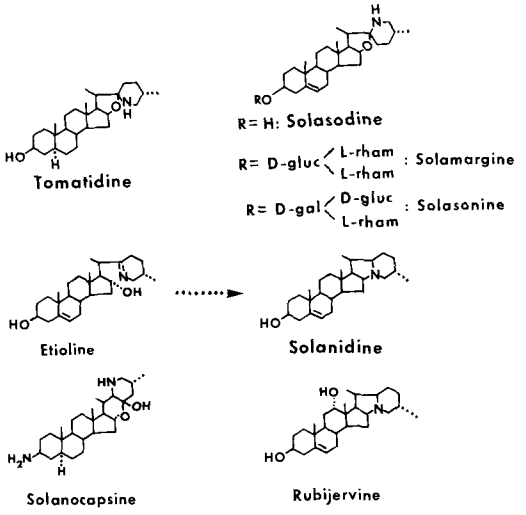

FIG. 4.

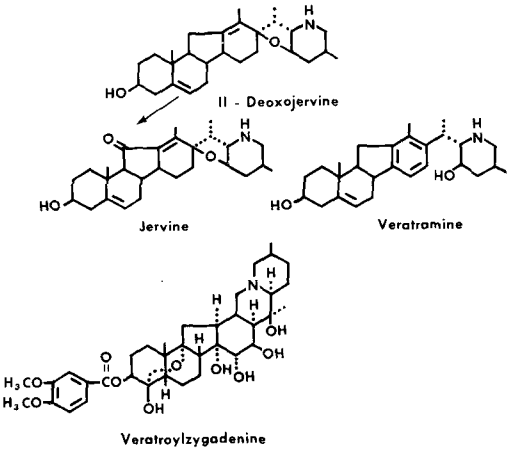

FIG. 5.

was illuminated, the radioactivity disappeared from the solanidine and was rapidly incorporated into the jerveratrum alkaloids jervine and veratramine. Theoretically, the ceveratrum alkaloids also could be products of solanidine metabolism. So far, only the incorporation of acetate-1-^{14}C into veratroylzygadenine (Fig. 5) actually has been demonstrated (81). A number of alkaloids lacking ring E has been isolated from *Veratrum* (5,13). Some of them probably will turn out to be intermediates in the biosynthesis of the Liliaceae alkaloids.

There has been a good deal of interest in the physiological variations of the alkaloid content of plants that are promising sources of raw material for the partial synthesis of steroid hormones. *S. laciniatum* seeds contain only traces of solasodine (84). As the plant matures, the solasodine content of the leaves increases (85), reaching a maximum when they wilt (86). The alkaloid concentration is subject to a diurnal rhythm, being highest in the daytime and lowest at night (85,87-89). The highest yield is obtained during the flowering period (84,87,88). Of the various plant organs, unripe berries have the highest solasodine concentration (87,88,90). The steroidal alkaloids in two chemotypes of *S. dulcamara* (13) have been studied further. Both the West European tomatidenol variety and the East European soladulcidine variety show increases in the alkaloid content of their fruits as they develop (46). No translocation of alkaloids from the shoots to the fruits was observed (91). In full grown green fruits of both varieties, solasodine becomes the main alkaloid (92,93). As the fruits mature, solasodine disappears (46).

Keeler (94) has identified one of the teratogenic compounds in *V. californicum* as 11-deoxojervine (Fig. 5) and named it cyclopamine. It occurs in the plant as the 3-glycoside, which he named cycloposine (95). The substance, which produces cyclopian malformations in lambs, first appears in the leaves of *V. californicum*. Later in the season, it is found in the stems and still later in the root system (96). Recent work on the production of solanidine glycosides by potato tubers has confirmed that they are formed in young shoots (97,98). Fungus infections apparently increase the alkaloid production (99,100). Whereas tissue cultures of *S. laciniatum* have not yielded any alkaloids (63,101), solasonine has been obtained from tissue cultures of *S. xanthocarpum* (102) and tomatine from cultured tomato roots (103,104).

Nothing is known about the degradation of C_{27} alkaloids by higher plants, except for our observation that ripe tomatoes convert administered radioactive tomatine to labeled allopregnenolone (Fig. 6) (32). In addition to the microbiological hydroxylations reviewed earlier (13), a dehydrogenation now has been reported. *Nocardia restrictus* converts tomatidine to its 3-keto, Δ^1-3-keto, and $\Delta^{1,4}$-3-keto analogues (105). New data confirm that glycoalkaloids are fungitoxic (106-110) and that some of them also have growth inhibiting activity in higher plants (111,112). However, the previously reported auxin activity of tomatine (13) could not be confirmed (113).

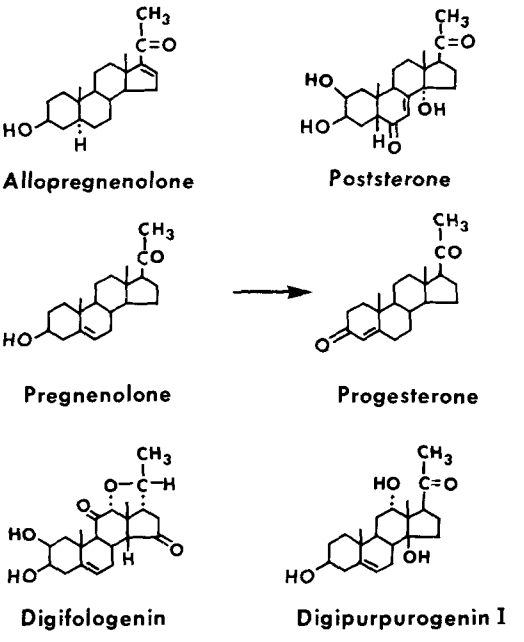

FIG. 6.

PREGNANE DERIVATIVES

As explained earlier (1,4,15,16), pregnane derivatives are synthesized in plants by degradation of steroids with a greater number of carbon atoms. Examples are: the degradation of cholesterol to pregnenolone (Fig. 6) by *Haplopappus heterophyllus* (114), *Digitalis purpurea* (115,116), and *Punica granatum* (117), the conversion of sitosterol to progesterone (Fig. 6) by *D. lanata* (118), and the degradation of tomatine to allopregnenolone (Fig. 6) by tomato fruits (32). The postulated (119) degradation product of ecdysterone has now been isolated from *Cyathula capitata* and named poststerone (Fig. 6) (120). Direct evidence for this degradation is still lacking, but it recently has been observed in silkworm larvae (121). The work of Stohs and El-Olemy (122) indicates that 20α-hydroxycholesterol may be an intermedi-

ate in the transformation of cholesterol to pregnenolone. Leaf homogenates of *Cheiranthus cheiri* and of *Nerium oleander*, but not *D. purpurea* or *Strophanthus kombé*, were capable of performing this degradation. Tissue cultures of *D. purpurea* also have been reported to be incapable of cleaving the side chain of cholesterol (116).

Many genera, especially in the Scrophulariaceae and Asclepiadaceae families contain digitanol glycosides (4,5). The digitanols are C_{21} steroids, some of which have rings C and D *trans*-fused, like the cardiac genins, and are combined with some of the rare hexoses otherwise only found in cardiac glycosides. Two of the Δ^5-3β-hydroxypregnane derivatives in *Digitalis*, digifologenin and digipurpurogenin I (Fig. 6), have been shown to be metabolites of pregnenolone (16). Many genera in the Apocynaceae family contain C_{21} alkaloids (123,124) that are likewise synthesized from pregnenolone (16). Figure 7 shows the structures of three alkaloids for which this has been established experimentally (16): holaphyllamine, holaphylline, and conessine. The conversion of pregnenolone to holaphyllamine is reversible.

Although progesterone has so far been isolated only from *Holarrhena floribunda* leaves and from apple seeds (125), minute amounts of this hormone must exist in many plants, because the ability to synthesize progesterone from pregnenolone is rather widespread. It has been observed in vivo not only in *H. floribunda* (126), but also in *D. lanata* (127,128), and in vitro in tissue cultures of *D. purpurea*, *D. lutea*, and *Nicotiana tabacum* (129), in leaf homogenates of *D. purpurea* and *C. cheiri* (130), and in pineapple slices (131). The reverse reaction, conversion of progesterone to pregnenolone, has been observed in *D. lanata* (132).

According to Caspi and Hornby (133), the oxidation of pregnenolone to progesterone is a preliminary step in the reduction to saturated pregnane (5β) and allopregnane (5α) derivatives. The reaction sequence is analogous to the previously discussed oxidation and reduction of cholesterol (Fig. 2) and of diosgenin (Fig. 3). When either pregnenolone (127) or progesterone (132) was fed to *D. lanata*, the following reduction products were isolated (Fig. 8): 5β-pregnane-3,20-dione, 5β-pregnane-3β,20β-diol, 5α-pregnane-3,20-dione, and 5α-pregnan-3β-ol-20-one. After the administration of radioactive progesterone to *S. kombé*, we also isolated labeled 5β-pregnan-3β-ol-20-one and pregnane-3β,5β-diol-20-one (Fig. 8) (134). The hypothetical pathways leading to these products are indicated in Figure 8 by dotted arrows. The 5α-pregnane derivatives, which had a higher radioactivity than the 5β-pregnane derivatives, were found in *S. kombé* for the first time. Work with tissue cultures (129,135,136) and leaf homogenates (130) also has shown that many plants reduce pregnenolone and progesterone preferentially to the 5α-pregnane derivatives. Microsomes from *C. cheiri* and *Dioscorea deltoidea* converted progesterone to 5α-pregnane-3,20-dione (Fig. 8) in the presence of nicotinamide

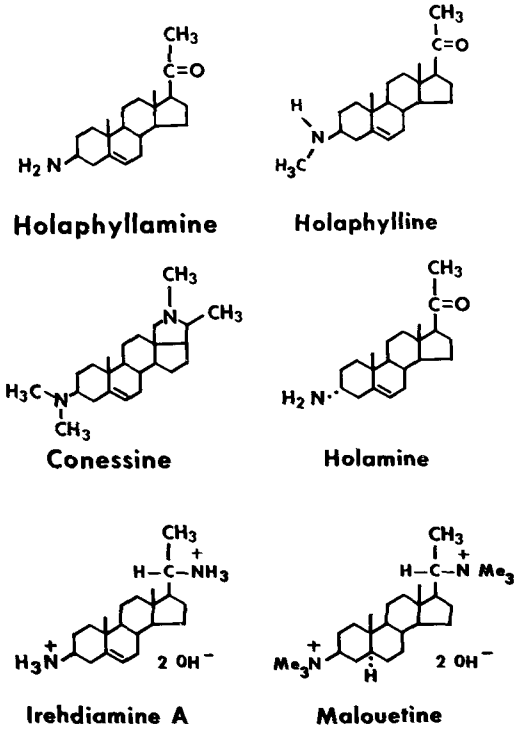

FIG. 7.

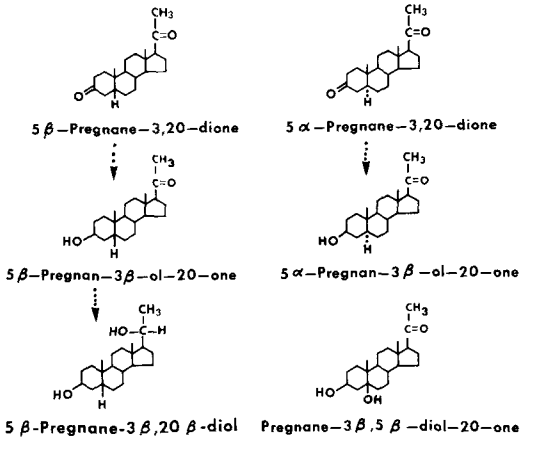

FIG. 8.

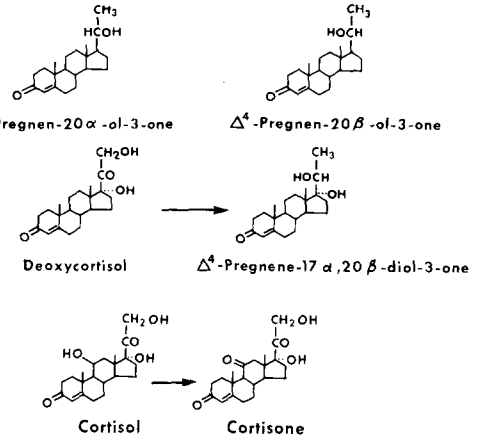

FIG. 9.

adenine dinucleotide phosphate reduced form (NADPH) (137).

Parthenocissus and *Rosa* tissues reduced progesterone to Δ^4-pregnen-20α-ol-3-one and Δ^4-pregnen-20β-ol-3-one (Fig. 9) (129). The 20-acetate of the latter compound also has been found in the bark of *Khaya grandifoliola* (138). Reduction at C-20 also was observed in fruit bodies of mushrooms supplied with deoxycortisol (Fig. 9) (139). When we administered cortisol-4-^{14}C to a *Mallotus paniculatus* plant, it was oxidized to radioactive cortisone (Fig. 9) (140).

Caspi, et al., (141) demonstrated the 21-hydroxylation of progesterone to the adrenocortical hormone, deoxycorticosterone (Fig. 10), in a *Digitalis lanata* plant. Hydroxyl groups at C-5 were introduced by *S. kombé* when progesterone was converted to pregnane-3β,5β-diol-20-one (Fig. 8) (134) and by *Helleborus atrorubens*, when Δ^5-pregnen-3β-ol-20-one was converted to hellebrigenin (Fig. 12) (142). Contrary to Caspi and Hornby (133), who postulated a 3-ketosteroid as an obligatory intermediate in the reduction of the Δ^5-double bond, Tschesche, et al., (142) found in a double labeling experiment that the 5-hydroxyl group is introduced directly by hydration of that bond.

The transformations of pregnane derivatives carried out by microorganisms are too numerous to be included in this review, but some recent work will be mentioned in the section on sex hormones. For references to the older literature, the reader is referred to other reviews (16,143) and books (4,5,144).

There is practically no information on the concentration changes of C_{21} steroids in plants in relation to their physiological condition, except for the C_{21} alkamines. Leboeuf, et al., (145) observed that holaphyllamine (Fig. 7) gradually disappears from the leaves of *Holarrhena floribunda* during the growing season, while their holamine (Fig. 7) content increases from 0 to 75% of the total alkaloid fraction. Only holaphylline (Fig. 7) is always present. Also, there is practically no information on the functions in or effects upon plants of the C_{21} steroids, which include several compounds with hormonal activity in animals. Some of them are precursors of other plant steroids, but only in the case of the C_{21} alkamines can we obtain a glimpse of their actions.

Only the *Funtumia* alkaloid irehdiamine A (Fig. 7) and the bis-trimethylammonium base malouetine (Fig. 7) in *Malouctia bequaertiana* have been studied in detail. Both compounds inhibit the bacteriophage-directed deoxyribonucleic acid (DNA) synthesis in infected *Escherichia coli* (146). Whereas Silver, et al., (147) maintains that steroidal diamines attack mainly the bacterial cell membrane, causing leakage of small molecules, Waring (148) has obtained evidence for binding of the steroids to the closed circular φX-174 replicative form of DNA. Although they do not intercalate like other drugs and antibiotics, these diamines apparently uncoil and reverse the DNA supercoils (149).

CARDIAC GLYCOSIDES

The biosynthesis of cardiac glycosides has been reviewed so recently and thoroughly (15,16,150-152) that we can restrict ourselves to a discussion of the salient facts in outline form. Information on the chemistry of cardiac glycosides (5,153), particularly the cardenolides (154) and bufadienolides (155), also has been summarized recently.

Cardenolides and bufadienolides are formed by condensation of as yet unidentified C_2 and C_3 fragments, respectively, with a C_{21} steroid. Genins with 23 and 24 carbons have not been found in the same plant so far. Cholesterol (156), sitosterol (157), and presumably other sterols can act as precursors of the C_{21} steroid. Although the involvement of a C_{21} intermediate can be taken for granted, its nature is still far from clear (158). Caspi, et al., (159) who had originally implicated progesterone (Fig. 6) (129) or at least a 3-ketosteroid (133) as the obligatory intermediate, later concluded from double labeling experiments that neither pregnenolone (Fig. 6), progesterone (156), nor deoxycorticosterone (Fig. 10) (141) could be intermediates of major importance.

Be this as it may, the following C_{21} steroids—in addition to the aforementioned precursors—have been shown to be converted to

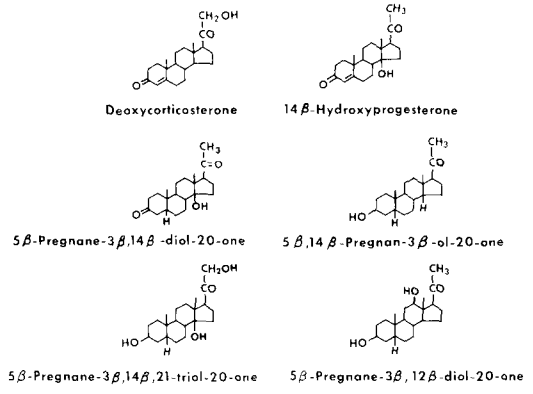

FIG. 10.

cardiac genins by (unless otherwise stated) *D. lanata* plants: 14β-hydroxyprogesterone (Fig. 10) to digitoxigenin and gitoxigenin (Fig. 11) (160); 5β-pregnane-3,20-dione and 5β-pregnan-3β-ol-20-one (Fig. 8) to digitoxigenin, digoxigenin, and gitoxigenin (Fig. 11) (161); 5α-pregnan-3β-ol-20-one (Fig. 8) to uzarigenin (Fig. 11) by *S. kombé* (162); 5β-pregnane-3β,14β-diol-20-one (Fig. 10) (163) and 5β,14β-pregnan-3β-ol-20-one (Fig. 10) (161) to digotoxigenin; 5β-pregnane-3β,14β,21-triol-20-one (Fig. 10) to digitoxigenin, digoxigenin, and gitoxigenin (164); and 5β-pregnane-3β,12β-diol-20-one (Fig. 10) to digoxigenin (161).

Confirmatory evidence for the conversion of progesterone to gitoxigenin by *D. purpurea* also should be mentioned (165). Negative evidence should be treated with caution, because the incorporation of precursors depends upon many factors, such as the mode and place of administration, age and condition of the plant, season, and other environmental conditions. The failure of Δ^5-14-deoxy-14α-digitoxigenin (163), 14-anhydrodigitoxigenin (163), 14α-hydroxyprogesterone (166), and Δ^{14}-pregnen-3β-ol-20-one (161) to be converted to cardiac genins indicates but does not prove that the 14β-hydroxyl group is introduced into the C_{21} precursor before the lactone ring is formed. No label was removed from precursors tritiated in the 15-position (167).

The hydroxylation of digitoxigenin (Fig. 11) at C-12 to form digoxigenin has been observed in vivo (161,164,168), and the 16-hydroxylation of digitoxigenin to gitoxigenin occurred in *Digitalis* tissue cultures (169). The 16-hydroxylation involves direct replacement of the 16β-hydrogen (170). As previously mentioned, a 5β-hydroxyl group may be formed by the addition of water to the Δ^5-double bond of pregnenolone (142).

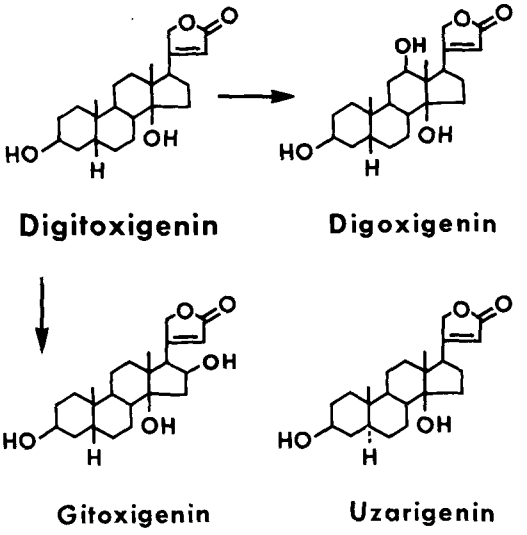

FIG. 11.

The angular methyl group at C-10 in pregnenolone or progesterone apparently undergoes stepwise oxidation, because Tschesche (142,171) has observed that *Helleborus atrorubens* converts pregnenolone to hellebrigenin (Fig. 12), and we have demonstrated the biosynthesis of periplogenin, strophanthidol, and strophanthidin (Fig. 12) in *S. kombé* (172). Many plants contain cardiac glycosides in which C-19 appears at different stages of oxidation (5). Porto and Gros (173) also have observed the conversion of pregnenolone to hellebrigenin in *Scilla maritima*, but not in the toad, *Bufo paracnemis*. Although cholesterol is known to be a precursor of bufadienolides in toads (174,175), no significant incorporation of labeled pregnenolone has so far been obtained (176,177).

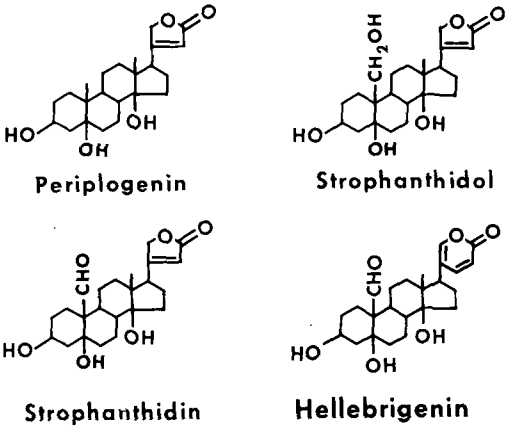

FIG. 12.

Since the first experiments on the conversion of labeled glucose to digitoxose by *Digitalis* plants (178,179), uridine diphosphate gitoxose has been isolated from this plant (180) and the glycosylation of digitoxin by uridine 5'-diphosphate-glucose in the presence of an enzyme preparation from *Digitalis* leaves has been demonstrated (181). Otherwise, the biosynthesis of the unusual sugars and their glycosides still is unexplained.

Evans and Cowley (42,45) have studied the variations in the relative proportions and amounts of cardenolides during the development of *D. purpurea* plants. Germination was accompanied by a decrease in cardiac glycoside content, which was followed by an increase at the time of the first leaf development. The digitoxigenin fraction decreased as the gitoxigenin fraction increased. The cardenolide concentration reached a maximum 6-7 months after germination. Young leaves and the developing inflorescence and fruits were rich in cardiac glycosides. Digitoxigenin was the major component of the cardiac glycoside fraction, with the exception of the inflorescence and roots, which contained principally gitoxigenin. The role of external factors in the accumulation of cardiac glycosides in *Digitalis* plants has been reviewed recently (182).

Information concerning the possible functions of cardiac glycosides in plants is very meager indeed. There has been much talk about the raison d'être of toxic plant steroids as protection from insects (183). When it was discovered that many insects can ingest cardiac glycosides with impunity, the talk shifted to the protection of insects from predators by ingested cardiac glycosides (184). It now seems that some butterflies containing cardiac glycosides taste good to some birds (185) and that mice, lizards, and ants do not mind feeding on these butterflies (186). Undoubtedly, cardiac glycosides are capable of regulating ion transport through membranes. Cram (187) has shown that ouabain inhibits sodium efflux in excised root tissue of carrot, but the effects of cardiac glycosides upon cation transport in myocardial tissue are much better documented (188,189). Kupchan, et al., characterized a number of cardiac genins as cytotoxic agents in the extracts of *Apocynum cannabinum* (190), *Asclepias curassavica* (191), and *Bersama abyssinica* (192-194). This raises more questions about the mechanism of the cytotoxic action and the way cardiac glycosides may affect the plants that contain them.

SEX HORMONES

More biochemical information about sex hormones in plants has accumulated since this topic was last reviewed (195,196). Evidently, a great number of microorganisms degrade sterols to sex hormones and to various secosteroids (197-199). Such degradation products as androstenedione (Fig. 13) lately have been identified in cultures of *Arthrobacter* (200), *Bacillus* (201), *Brevibacterium* (201), *Corynebacterium* (202) *Microbacterium* (201), *Mycobacterium* (203-206), *Nocardia* (202), *Protaminobacter* (201), *Serratia* (201), and *Streptomyces* (207). Degradation of the side chain of progesterone (Fig. 6) to androstenedione and testosterone (Fig. 13) was observed in *Penicillium* (208), aromatization of androstenedione to estradiol (Fig. 15) in *Escherichia* (209), and degradation, as well as aromatization of sterols to estrone (Fig. 15), in *Mycobacterium* and *Proactinomyces* (210).

Gliocladium virens produces an antifungal metabolite, viridin (Fig. 13), apparently by some analogous mechanisms (211). Rubrosterone (Fig. 13), a C_{19} steroid in *Achyranthes rubrofusca* (212), and 5α-androstane-3β,16α,17α-triol (Fig. 13), a C_{19} steroid in *Happlopappus heterophyllus* (213), are almost certainly degradation products of sterols, but we have been unable to demonstrate this so far. Evidence for the occurrence of testosterone (Fig. 13), epitestosterone, and androstenedione (Fig. 13) in the pollen of *Pinus silvestris* has been reported recently (214).

Higher plants undoubtedly have the ability to metabolize C_{19} steroids. When androstenedione (Fig. 13) was incubated with a suspension culture of *Dioscorea deltoidea*, it was converted to 5α-androstan-3β-ol-17-one and 5α-androstane-3β,17β-diol (Fig. 14) (215). Dehydroepiandrosterone (Fig. 14) and other C_{19} ster-

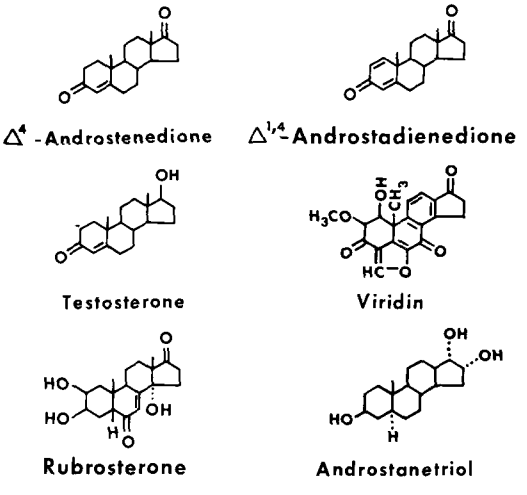

FIG. 13.

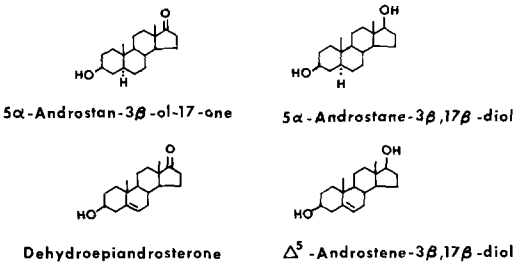

FIG. 14.

oids, incubated with potato tuber slices, were conjugated with glucose (131,216) and hydroxylated (217). Slices of green beans reduced dehydroepiandrosterone to Δ^5-androstene-3β,17β-diol (Fig. 14), and pineapple slices oxidized dehydroepiandrosterone to androstenedione (Fig. 13) and oxidized androstenediol (Fig. 14) to testosterone (Fig. 14) (131).

The occurrence of steroidal estrogens in higher plants has been doubted as late as 1965 (218) but may now be considered a fact (219). We have been unable to confirm an earlier report of the isolation of estriol from female willow flowers (220), but the occurrence of estrone (Fig. 15) in palm kernels (221) and pollen (222,223) now has been established definitely (224-226). Estrone also was identified in pomegranate (227,228) and apple (229) seeds, in moghat root (*Clossostemon bruguieri*) (226) and in a mixed pollen sample (230). There is also some evidence for the occurrence of estrone in developing bean plants (231-233) and in flowering *Perilla* (234) and *Hyoscyamus* (235), but the source of estrogenic activity in other plants has not yet been adequately characterized (236,237). The nature of certain fungal sex hormones is also still unknown (238-241). There is some evidence that the yeast sex hormones may be steroids, because testosterone and estradiol have hormonal activity (242-244).

In spite of considerable effort, we can no more than guess what functions sex hormones may perform in plants. In principle, there is not so much difference between plant and animal cells that we cannot conceive the same basic

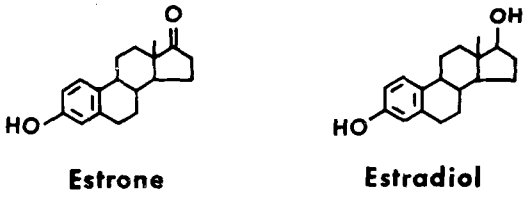

FIG. 15.

interactions between sex hormones and cellular components in plants and animals (245-254). Thus, male sex hormones, such as testosterone and rubrosterone, which stimulates spermatogenesis in the insect (255) and protein synthesis in the mouse liver (256), may have anabolic effects in the plant. However, it will be difficult to test such a hypothesis. Results of the direct application of hormones to plants are notoriously difficult to reproduce. Nevertheless, it will be profitable to review such results of recent experiments. Older work has been reviewed elsewhere (38,257,258).

The growth of microorganisms can be influenced by the addition of steroid hormones to the medium. Thus, androgens and progestational hormones promote the growth of *Staphylococcus aureus* and *Corynebacterium pyogenes* (259); cortisol stimulates the growth of mycobacteria (260); but corticosteroids generally inhibit the growth of *Mycoplasma gallisepticum* (261). Deoxycorticosterone and progesterone are strong inhibitors of cell growth and division for *Tetrahymena* (262), and estradiol administration causes unequal cleavage and growth delays in the embryos of *Fucus distichus* (263). Progesterone promotes the oospore production in *Pythium artotrogus* (264), but estradiol abolishes the sterol-induced reproduction in *Pythium periplocum* (265). The respiration of yeast is stimulated by steroid hormones at low concentrations but inhibited at high concentrations (266,267). Hendrix (268) has reviewed some of the earlier work on the effects of steroids upon growth and reproduction of fungi.

The administration of steroidal estrogens to higher plants increases their gibberellin (269,270) and auxin (271,272) content and stimulates growth (270,273) and seed germination (274). Estrogens promote flowering in *Cichorium intybus*, a long-day plant, grown under noninductive conditions (275). Estrone, as well as androsterone, stimulates floral development and rooting of broccoli curd cuttings (276). Treatment of monoecious cucumber plants with either estradiol or testosterone induced femaleness in the flowers (277), but experiments with *Ecballium elaterium*, another member of the Curcubitaceae, showed opposite effects on sex expression (278). Estrogens increased the number of flowers and the ratio of female to male flowers; androgens decreased that ratio but had no effect upon the number of flowers, whereas cortisone increased that number without affecting sex expression (278). When femaleness was induced in *Curcubita pepo* by treatment with 2-chloroethylphosphonic acid, endogenous production of estrogens ap-

peared to increase (279).

The ability to synthesize sex hormones and their distribution in the plant kingdom are apparently widespread. The reported effects of sex hormones upon microorganisms and higher plants and our present concepts of the mechanisms of hormone action at the molecular level make it seem likely that certain steroids have regulatory functions in plants that are analogous to those in animals. To verify this hypothesis, further work, mainly on whole plants, will be required.

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SHORT COMMUNICATIONS

Saussurea candicans Seed Oil—Alternative Source of Crepenynic Acid

ABSTRACT

Crepenynic acid represents 33% of the constituent fatty acids of *Saussurea candicans* seed oil. This important acetylenic acid, after isolation by countercurrent distribution, was characterized unambiguously by derivatives and by degradative procedures.

INTRODUCTION

In their recent book, (1,2), Bohlmann, et al., remarked that crepenynic acid is "perhaps the most important acetylenic fatty acid." Crepenynic (*cis*-9-octadec-12-ynoic) acid originally was isolated from *Crepis foetida* seed oil (3), in which it occurs to the extent of 60% of the total fatty acids. Widespread interest in this acetylenic analogue of linoleic acid has been evidenced in several ways. The biogenesis of crepenynic acid has been examined (4,5), and there have been experimental studies, as well as speculations, regarding its role in the biosynthesis of more highly unsaturated acetylenes (5-8). Its metabolic behavior has been studied (9-11). Crepenynic acid has been synthesized chemically (12,13), and additional natural sources have been revealed, including seed oils of various *Crepis* sp. (14), of *Helichrysum bracteatum* (15), and of *Afzelia* species (16).

In this note, we wish to draw attention to an alternative natural source of this acid. We have unambiguously identified crepenynic acid as a constituent of *Saussurea candicans* seed oil representing 33% of the derived methyl esters. *Saussurea*, a genus in the plant family Compositae, constitutes a group of herbaceous plants which vary in habit and sometimes are used as ornamentals (17). Our sample of *S. candicans* seeds originated in Pakistan and yielded 30% oil. Seed oils of a few other *Saussurea* species have been examined by gas liquid chromatography (GLC), but their apparent content of crepenynic acid did not approach that of *S. candicans* (unpublished results from this laboratory).

METHODS

Coarsely ground seeds (33.2 g) of *S. candicans*, when extracted with petroleum ether (bp 30-60 C) by the Soxhlet procedure, provided 10.0 g oil. Mixed methyl esters were prepared by acid-catalyzed transesterification with 1% sulfuric acid-methanol; GLC analyses indicated that these esters contained 32.7% crepenynate, along with esters of common acids in the following amounts (expressed as area percent): 16:0, 6.2; 18:0, 3.1; 18:1, 19.4; 18:2, 36.3; and 18:3, 0.5. A 7.9 g portion of the mixed methyl esters was subjected to countercurrent distribution (CCD) in an acetonitrile/hexane system essentially as described previously for *Crepis foetida* esters (3), except that 10 ml upper phase was used throughout. As in the previous instance (3), methyl crepenynate was well resolved from other esters; its maximum in the wt distribution was observed between transfers 580-600. The apparent content of methyl crepenynate derived from a CCD wt curve (23%) was significantly lower than the GLC value (R-446 column) of 32.7%. This discrepancy may have resulted from the presence of lipids in the fatty acid methyl ester mixture which are recovered in he CCD but not in the GLC analysis.

Methyl crepenynate thus purified by CCD was characterized structurally, for the most part, by the same chemical and degradative procedures described in our previous publication (3). However, m-perchlorobenzoic acid (18) was used instead of peracetic acid to epoxidize crepenynic acid. A solution of the peracid (0.67 molar excess) in chloroform was added dropwise to a continuously stirred solution of crepenynic acid in the same solvent at ambient temperature. The reaction was terminated after 1 hr by addition of 10% sodium sulfite solution, and the product was isolated by conventional extraction procedures. Without purification, this epoxyacetylene was subjected to acetolysis followed by saponification. Recrystallization of the resulting product from hexane-ethyl acetate provided (±)-threo-9,10-dihydroxyoctadec-12-ynoic acid, mp 71-72.5 C, undepressed upon admixture with an authentic specimen. Hydrogenation of this dihydroxy acetylene (Adams catalyst, methanol solution) gave (±)-threo-9,10-dihydroxyoctadecanoic acid, mp 92-93.5 C after recrystallization from

aqueous methanol, undepressed upon admixture with an authentic specimen of the compound. Positions of unsaturated linkages in the various derivatives were confirmed by periodate-permanganate oxidation followed by GLC. (The IR spectrum of methyl crepenynate is nondescript, and its NMR spectrum is rather similar to that of methyl linoleate.)

C.R. SMITH, JR.
Northern Regional Research Laboratory
ARS, USDA
Peoria, Illinois 61604

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Free and Protein-Bound Gangliosides in Triton X-100 Extracts from Rat Brain

ABSTRACT

Gangliosides that are extracted from brain tissue samples with Triton X-100 exist in a free form and as a ganglioside-protein complex. The two forms can be separated by diethylaminoethyl-cellulose chromatography. Whole brain tissue yields only ganglioside-protein complexes. Crude mitochondrial and microsomal fractions and synaptosome-enriched fractions yield both free and protein-bound gangliosides. The myelin and mitochondria-enriched fractions yield only free gangliosides. The isolation of ganglioside-protein complexes and free gangliosides appears to be dependent upon the nature of the starting material.

INTRODUCTION

The association of protein or peptide material with gangliosides that had been extracted from rat brain has been reported by many workers (1-7). The presence of peptides in gangliosides prepared by chloroform-methanol extraction is largely dependent upon conditions of extraction. They can be removed from the lipids by thin layer chromatograhic (TLC) procedures (4,8). Wolfe (9) subjected brain homogenates to ultrasonic radiation and found that this caused a decrease in the amount of gangliosides that could be extracted with chloroform-methanol. It was thought that the treatment liberated basic protein from the nucleus which then combined with the gangliosides, thereby preventing complete extraction with chloroform-methanol. In the present article it is shown that protein-bound gangliosides can be obtained by extracting tissue with the detergent, Triton X-100, and that protein-bound gangliosides can be separated from protein-free gangliosides by means of diethylaminoethyl (DEAE)-cellulose chromatography. The association between ganglioside and protein appears to

aqueous methanol, undepressed upon admixture with an authentic specimen of the compound. Positions of unsaturated linkages in the various derivatives were confirmed by periodate-permanganate oxidation followed by GLC. (The IR spectrum of methyl crepenynate is nondescript, and its NMR spectrum is rather similar to that of methyl linoleate.)

C.R. SMITH, JR.
Northern Regional Research Laboratory
ARS, USDA
Peoria, Illinois 61604

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be dependent upon the nature of the starting material.

EXPERIMENTAL PROCEDURES

Rat brain tissue or subcellular fractions prepared therefrom (10) were extracted with Triton X-100 (0.5% in 0.005 M potassium phosphate, pH 6.8) (11) and centrifuged at 100,000 g for 90 min to remove insoluble material. The detergent solubilized 40% of the gangliosides and 50% of the proteins. Most of the protein content of the extract was removed by adsorption on calcium hydroxylapatite (11). The unadsorbed gangliosides were subjected to anion exchange chromatography on DEAE-cellulose (1.9 x 45 cm column) that had been packed under a pressure of 2.5 psi. The column had been equilibrated overnight with 0.005 M potassium phosphate, pH 8.0 in 0.2% Triton X-100. The columns were charged with 4 mg protein (or less) in the same buffer/ml bed volumn. Gangliosides and proteins that were adsorbed were eluted with a linear gradient of NaCl in 0.005 M potassium phosphate, pH 8.0, and 0.2% Triton X-100. All of the gangliosidic N-acetylneuraminic acid (NANA) in the original Triton extract was recovered. The gangliosides and ganglioside-protein complexes were analyzed for fatty acids (3,12), sphingosine (13), N-acetylneuraminic acid (14), hexosamine (15), galactose and glucose (16,17), amino acids (18), and protein (19).

RESULTS AND DISCUSSION

Gangliosides that had been obtained by extraction of the tissue with chloroform-methanol and partitioned into an aqueous upper phase by the method of Suzuki (20) normally are bound to DEAE-cellulose. Subsequent elution, using a gradient of increasing concentration of monovalent anions, has permitted the separation of the ganglioside mixture to provide

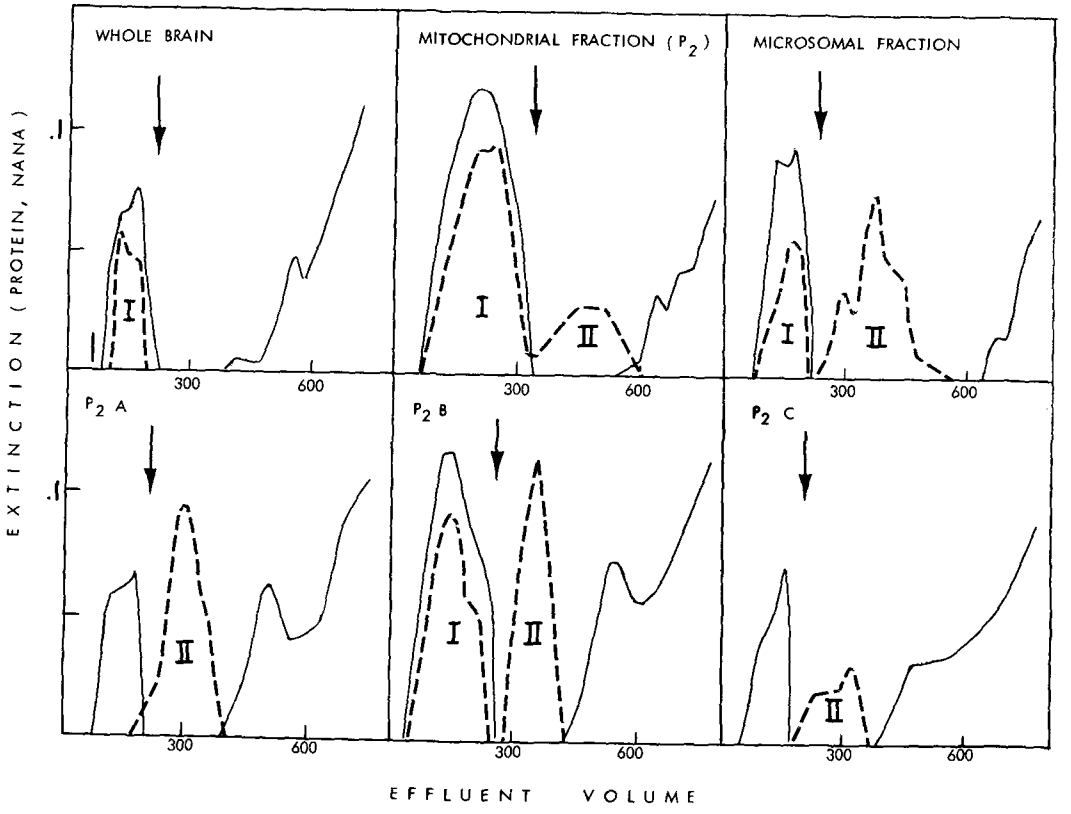

FIG. 1. Triton X-100 extracts from which the bulk of the proteins had been removed by adsorption to calcium hydroxylapatite were fractionated on diethylaminoethyl-cellulose columns (1.5 x 45 cm). A linear gradient of NaCl in 0.005M potassium phosphate buffer, pH 8.0, containing 0.2% Triton X-100, was applied as indicated (arrow). Gangliosides of peak II were eluted at concentrations of NaCl between 0.02 M. P_2A, P_2B, and P_2C are myelin-, synaptosomal-, and mitochondria-enriched fractions that had been prepared from P_2 (crude mitochondrial fraction) by sucrose-density gradient centrifugation (10). ——— = protein and – – – = nana.

mono-, di-, and trisialogangliosides (21). In the present experiment, a major part of the gangliosides solubilized by Triton X-100 is not retained by the anion exchanger (Fig. 1). A ganglioside that is free of associated proteins (peak II) is adsorbed and subsequently eluted between 0-0.02 M sodium chloride. The ganglioside-protein complex recovered from mitochondrial and microsomal fractions (peak I), which is not adsorbed to DEAE-cellulose, also failed to be adsorbed to or dissociated by a cation exchanger. The material was applied to carboxymethyl (CM)-cellulose in the presence of 0.005 M potassium phosphate, pH 6.8, containing 0.2% Triton X-100. The protein associated with the gangliosides of peak I contained (percent of total amino acids) leucine, 16; arginine, 15; lysine, 14; glutamic acid, 11; glycine, 10; alanine, 6; phenylalanine, 4; serine + threonine, 10; α-aminobutyric acid (GABA), 4; tyrosine, 3; cysteine, 2; histidine, 2; aspartic acid, 1; and trace amounts of valine and isoleucine. The gangliosides were shown to contain sphingosine, fatty acid (mainly stearic acid), galactosamine, galactose, glucose, and NANA in a molar ratio of 1:1:1:2:1:1.5. The absence of mannose and fucose assured that glycoproteins were absent.

The protein-linked ganglioside migrated with a single sharp band when subjected to electrophoresis (22) in a 5% acrylamide gel at pH 9.2 (0.1 M Tris-ethylenediaminetetraacetic acid-borate buffer, 24 V/cm). The results were similar for ganglioside-protein complexes obtained from mitochondrial, microsomal, and whole brain preparations.

The relative proportion of protein-bound (peak I) and free gangliosides (peak II) in Triton extracts was dependent upon the nature of the material from which they had been extracted (Fig. 1). Triton X-100 preparations from whole rat brain yielded only protein-bound gangliosides. The crude mitochondrial (P_2), the microsomal (P_3), and the synaptosome-enriched (P_{2B}) fractions yielded both protein-bound and free gangliosides. The mitochondria-enriched (P_{2C}) and myelin-enriched (P_{2A}) fractions yielded only free gangliosides; basic protein in these fractions failed to bind to the gangliosides. Appearance of free gangliosides in the subcellular fractions may be due to the prior removal of the nuclear fraction, a probable source of some of the basic proteins. Formation of protein-ganglioside complexes after extraction of subcellular fractions with Triton X-100 is dependent upon the presence of both gangliosides and basic proteins in the treated sample. It is also possible that such complexes may exist in situ.

ERIC G. BRUNNGRABER
VINCENT A. ZIBOH
Research Department
Illinois State Psychiatric Institute
Chicago, Illinois 60612

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ERRATUM

An error occured in Figure 1 of "Acyl Specificity in Glyceride Synthesis by Lactating Rat Mammary Gland," by Hiroaki Tanioka, Chu Yuan Lin, Stuart Smith, and S. Abraham (*Lipids*, 9:229 [1974]).

The units of protein concentration in Figure 1 should read: 0.05, 0.10, 0.15. 0.20. and 0.25.

Model of Interaction of Polar Lipids, Cholesterol, and Proteins in Biological Membranes

H. BROCKERHOFF, New York State Institute for Basic Research in Mental Retardation, Staten Island, New York 10314

ABSTRACT

Membranes are proposed to consist of a hydrophobic core, two hydrogen belts, and two polar zones. The hydrogen belts consist of hydrogen bond acceptors, i.e. the carbonyl groups of phospholipids and sphingolipids, and hydrogen bond donors, i.e. the labile hydrogens of cholesterol, sphingosine, proteins, and water. The density of anhydrous hydrogen bonding and the impermeability of the membrane increase with increasing concentrations of cholesterol, sphingolipids, α-hydroxy acyl residues, plasmalogens, and ether phospholipids. Cholesterol owes its membrane-closing properties to its rigid longitudinal orientation in the membrane combined with the latitudinal orientation of the O-H bond. It is suggested that the intrinsic proteins of membranes are held in position by hydrogen bonding, as well as by hydrophobic and electrostatic forces, and that hydrogen bonding also mediates the penetration of membranes by proteins.

INTRODUCTION

Cholesterol is a major component of many biological membranes and obviously fulfills an important function in them, but it is not known what this function is. The sterol appears to form a complex with phospholipids, as shown by calorimetric scanning (1,2) and by the contraction of mixed phospholipid-cholesterol layers (3-5). Cholesterol suppresses the permeability of phospholipid membranes for water (6), cations, glycerol, and glucose (7,8) and the penetrability of monolayers by proteins (9). The effect upon permeability is shown only by cholesterol and related sterols having a β-OH group, a planar ring system, and an aliphatic side chain (8). Spin-label (10) and NMR (2) studies show that cholesterol reduces the flexibility of the carboxyl half of the aliphatic phospholipid chains in liquid-crystalline films. The spatial fit between the rigid ring structure of cholesterol and the paraffinic part of the common unsaturated fatty acids has been noted (11). It seems to be the present consensus of opinion that cholesterol condenses and rigidifies membranes without solidifying (gelling) them (2,7,12,13).

In the model presented in this article, condensation and rigidification by cholesterol play no role. This does not mean that these effects are negated, but I propose that they are not the only, and probably not the most important, functions of cholesterol in membranes. I suggest that the capacity of cholesterol to donate a hydrogen bond is of greater importance for the functioning of artificial, as well as natural, membranes. This hypothesis will lead to further consequences concerning the function of various phospholipids and sphingolipids and the structure of membrane proteins.

PREMISES

Molecular Models

All following statements concerning distances, bond angles, and other steric properties of the molecules have been obtained from spacefilling Corey-Pauling-Koltun models or in some cases from Dreiding models.

The following assumptions have been made. The conformation of ester groups C-C(=O)-O-C, and amide groups, C-C(=O)-N(H)-C, is *trans*. The conformations between the methylene groups, $-CH_2-CH_2-$, near the polar ends of the chain are also *trans*, and in phosphoglycerides, the first methyglene group of one of the chains must be gauche to the C=O if the chains are to be parallel (14). The further conformations of the chains or of the side chain of cholesterol are immaterial for our discussion. The A-ring of cholesterol has the chair conformation.

The hydrogen bond has a distance between the centers of the oxygen atoms of 2.6-2.8 Å and must be straight, i.e. the bonds in O-H···O form a straight line (15,16). These conditions are built into the commercial molecular models.

Hydrophobic Bonding and Hydration

The transfer of paraffinic chains from aqueous to nonpolar surroundings is accompanied by a loss in free energy of ca. 0.7 kcal/mole for each CH_2 group (17). The maximization of such "hydrophobic bonding" requires the total immersion of fatty acid chains and cholesterol into the nonaqueous phase and the closest possible packing of molecules in this phase.

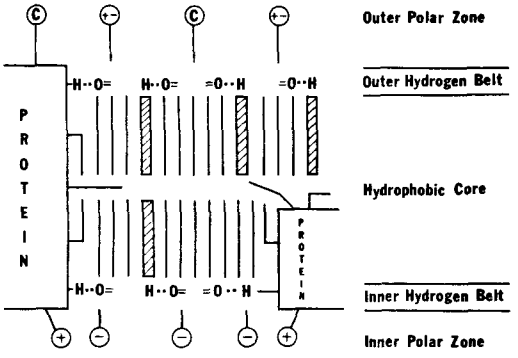

FIG. 1. Schematic model of a plasma membrane. Lines represent hydrophobic lipid chains or amino acids; shaded rectanges represent hydrogen bond donors (cholesterol, sphingolipids); the circles are charged head groups of phospholipids or cationic amino acids residues; C stands for carbohydrate.

Carbohydrate groups, on the other hand, and ionic groups, such as the phosphate, ammonium, and carboxyl groups of phospholipids, have an equally strong affinity for the aqueous environment (18). The head groups of the polar lipids in lipid bilayers must, therefore, be hydrated thoroughly, and any membrane model that implies dehydration of these groups in favor of contact or bonding to nonpolar residues must be discarded. This critique is directed in particular against those models (19-21) which call for phosphate-cholesterol hydrogen bonding with partial extraction of the cholesterol into the polar phase.

MEMBRANE MODEL

The preceding considerations are compatible with the generally accepted membrane model of a lipid bilayer with a hydrophobic core enveloped by two polar zones. The model presented here has, in addition, two interposed hydrogen belts, i.e. well defined planes of lipid-lipid and lipid-protein hydrogen bonding.

In Figure 1, the fluid mosaic membrane model, which envisions the intrinsic proteins floating in a continuous, viscous-liquid lipid bilayers, has been accepted (18,22). Bilayer assymmetry -with acidic lipids on the inner side of the membrane (23,24)–also has been assumed. It should be noted, however, that the concept of the hydrogen belt does not depend upon such conditions. The picture of the membrane (Fig. 1), which might represent a fragment of a plasma or erythrocyte membrane, has been purposely left abstract to suppress any intimations of specific lipid-lipid complexing, steric fit of nonpolar residues, influence of unsaturation, or stoichiometric proportioning of membrane components, all of which are probably of importance but are not essential for the discussion of the basic model.

The =O symbols of Figure 1 represent the carbonyl oxygen atoms of the ester groups of phosphoglycerides and of the amide groups of sphingolipids. These groups accept hydrogen bonds from the OH groups of cholesterol and sphingosine and from labile hydrogens of the amino acids of membrane proteins; if such donors are lacking, hydrogen bonds are accepted from water. The floating proteins are held in position by hydrophobic bonding in the core of the membrane and by electrostatic bonding to the phosphate groups in the polar zone. They are prevented from bouncing and swaying by being buckled into the hydrogen belts.

The permeability of the lipid bilayer in the model is regulated by the density of hydrogen bonding in the hydrogen belts. In layers of phospholipids without cholesterol, the C=O groups bind to water or, as the case may be, to cations or hydrogen bond donors, such as glycerol or glucose, perhaps with the mediation of water. These solutes can, thus, pass into and through the membrane. Hydrogen bonding to cholesterol dehydrates and blocks the C=O groups. Only 50% of them have to be bonded to close the membrane almost completely. A possible explanation for this ratio is given later.

The passage of ions and other molecules through biological membranes generally is believed to be mediated by membrane proteins. Therefore, the C=O groups of the phospholipids in the model do not accept the solutes themselves but the enzymic or carrier proteins that transport the solutes. The surplus of C=O groups is likely to be bonded to cholesterol (or sphingolipid), but there may well be some excess of water-bonded C=O groups. These could cause various degrees of porosity of the lipid matrix in various membranes.

ARGUMENTS

Steric Arguments

The direction vertical to the surfaces of the membrane (Fig. 1) I define as longitudinal. This orientation probably is held, on the average, by the fatty acid chains and by the long axis of cholesterol. The direction parallel to the surfaces and the belts I call latitudinal. A latitudinal angle describes the deviation from this direction; the angle is positive toward the core of the membrane, negative toward the outside. For example, hydrogen bonds lying completely in the plane of a hydrogen belt would have a latitudinal angle of 0°, a bond pointing verti-

cally away into the aqueous phase an angle of -90°.

In the membrane model (Fig. 1), the =O groups function as acceptors of hydrogen bonds from either water or cholesterol; they should, therefore, be approachable from both longitudinal and latitudinal directions. If a phospholipid model is arranged in its most probable conformation, with both aliphatic chains in close contact and the ionic groups longitudinally extended, the C=O bonds can assume an almost perfect latitudinal orientation with an angle around 0°. Such an orientation is obviously ideal for latitudinal lipid-lipid bonding, but it also allows bonding from water molecules. With large negative C=O angles, water bonding will become favored, but such angles are improbable: space filling models show that they would loosen the packing of the aliphatic carboxyl end chains and thus cause the hydration of CH_2 groups; such hydration is energetically unfavorable.

Cholesterol, if arranged longitudinally in the membrane, can have latitudinal O-H angles between ca. +10 and -50°. In the extreme position of +10° (pointing slightly inward toward the membrane core), the hydrogen extends at a right angle from the side of the angular methyl groups, the β-side, of the molecule. Carbonyl···HO hydrogen bonding in this configuration is compatible with tight, parallel packing of fatty acid and cholesterol. The +10° configuration of the cholesterol O-H bond also yields the maximal exposure of the "back" of the oxygen to water, with the possibility of the oxygen accepting one or two hydrogen bonds (Fig. 2). The energetic advantage of such additional bonding is discussed below.

In the alignment shown in Figure 2, the C-3 of cholesterol is situated at a latitude between that of C=O and the first CH_2 group of the fatty acid. Such an alignment is thermodynamically highly probably, because it yields the maximal separation of hydrophilic (polar) and hydrophobic phases. With this alignment, the end methyl group carbons of the cholesterol side chain are ca. equidistant, in fully extended models, with carbon 14-15 of the fatty acid.

Energy of Hydrogen Bond

Hydrogen bond energies usually range from 4-8 kcal/mole (15,16). Dihedral oxygen -O-, forms relatively weak bonds, e.g. the water-water bond energy is ca. 4 kcal/mole. Carbonyl oxygen, C=O, is a stronger hydrogen bond acceptor; to cite an especially relevant example, cholesterol-triglyceride hydrogen bonding is favored strongly over cholesterol-cholesterol bonding (25). It must be assumed that the C=O

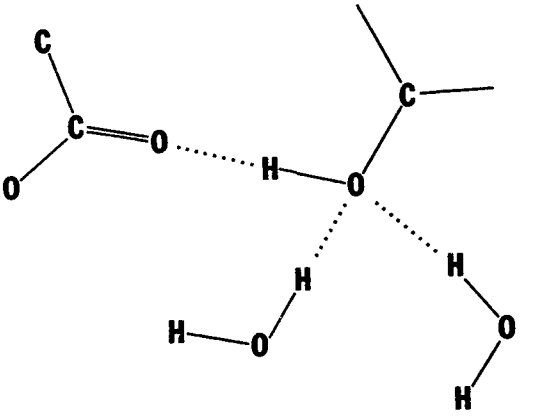

FIG. 2. Hypothetical alignment of phospholipid carbonyl, cholesterol hydroxyl, and water in the hydrogen belt.

group of pure phosphoglyceride bilayers, since they are accessible to water and no other hydrogen donors are available, form hydrogen bonds with water.

The negatively charged phosphate groups of phosphoglycerides increase the electronegativity of the neighboring carboxyl ester groups by induction, and thus make them better hydrogen bond acceptors (15,16). This inductive effect falls off with increasing distance; the C=O of the ester in position 2 of the glycerol can, therefore, be expected to be the better hydrogen bond acceptor. Once established, hydrogen bonding at position 2 will further reduce the acceptor capacity of the carbonyl in position 1 by reverse induction.

It must be understood that the possibility of a strong phosphoglyceride-cholesterol hydrogen bond does not guarantee its existence. The C=O···H-O bond in Figure 2 has to compete with C=O···H_2O hydrogen bonds; such bonds have to be broken, but the overall ΔG must still be negative. The reaction:

$$\text{OH (hydrated)} + \text{OC (hydrated)} \rightarrow \text{OH}\cdots\text{OC} + x\text{H}_2\text{O} \quad [1]$$

has, in fact, been estimated as having a ΔG of +1.4 kcal, regardless of whether the hydrogen bond is formed in polar or apolar environment (26). In our model (Fig. 2), two forces may drive reaction 1. First, because of the close packing of fatty acids and cholesterol and the slightly inward direction of the hydrogen bond, water is expelled from the hydrogen belt and can no longer compete. This means that a part of the hydrophobic bonding energy is expended to balance the positive ΔG of reaction 1. Second, and more important, the cholesterol oxygen, by turning its back towards the aqueous

phase and partly donating its proton to the C=O group, becomes a good acceptor for one or two protons from water. Through this mechanism, the C=O···H-O bond is reinforced heavily. Stated in other words, the positive free energy change reported for equation I (26) refers to the total dehydration of C=O and OH. In the arrangement of Figure 2, however, while there is dehydration of the hydrogen belt, i.e. the C=O, there is no net dehydration of the total system. There is also no reversal of the polarity of the water layer.

EVIDENCE

The preceding arguments lead to the conclusion that the carbonyl groups, as well as the hydroxyl group, must participate in some form of hydrogen bonding and that they are sterically, and also energetically, in a position to bind to each other. Bonding of the cholesterol to the phospholipid phosphate, which has been suggested (20,21), would require the dehydration of the anion, as well as the freezing of it in one position, both energetically improbably. Experiments which appear to show such bonding in anhydrous lecithin-cholesterol mixtures or hydrated multilayers (27,21) or below the liquid-crystalline transition point of the phospholipids (28,29) are irrelevant to the problems of membrane structure. The alternative, then, is a simple one: Are carbonyl and hydroxyl bonded to each other, or is each bonded to water only? We have no direct experimental answer: IR and NMR spectroscopies, which usually detect such bonds, cannot distinguish between the different kinds of hydrogen bonds in the presence of water (1,2). The evidence that can be offered at present for the hypothesis is, of necessity, less direct. It is, mainly, derived from studies originating in the laboratories of van Deenen and his colleagues (7,8,30,31).

Only the β-OH sterols with a flat structure and a side chain reduce the permeability of phospholipid membranes (8). Flat structure and side chain are probably necessary for close packing and hydrophobic bonding; the β-configuration of the hydroxyl is, in the light of our hypothesis, essential for latitudinal hydrogen bonding. Most significantly, epicholesterol, with an α-OH but otherwise identical in structure with cholesterol, does not reduce membrane permeability (8). In this sterol, the possible latitudinal angles of the labile hydrogen vary from $+60^\circ$ to -80°. In the more negative (longitudinal) orientations of the OH groups, both sterols could be expected to be hydrogen bond donors, $OH\cdots OH_2$, to water, and there is no reason to believe that the organization of the water would be much different in both cases, as has been suggested (7,8), but only cholesterol, I postulate, forms a latitudinal hydrogen bond. For epicholesterol to form latitudinal bonds, the hydrogen would have to be directed not vertically out of the β-plane as in cholesterol, but pointing to one of the edges of the molecular plane. This, we must assume, would put C=O and H-O too far apart, under the prevailing conditions of packing, to form a hydrogen bond. Similar steric considerations can explain why cholesterol cannot dose membranes of polyunsaturated phosphoglycerides.

Cholesterol reduces the average molecular area occupied by phospholipids in monolayers (3,4) and abolishes the energy jump at phospholipid phase transitions (2). A large number of sterols and ketosteroids has been tested in an effort to prove that these effects are correlated with the structures of the steroids and with their influence upon membrane permeability (30,8). Such a correlation might indicate that impermeability is the result of closer packing (condensation) of the membrane. The monolayer studies have yielded no support for such a proposal. The planar β-OH sterols all condense the membrane and reduce the permeability, but the (nonplanar, β-OH) coprostanol does not influence the permeability, although it causes considerable condensation. More striking, the keto analog of cholesterol, cholest-5-en-3-one, condenses the membrane as efficiently as cholesterol (30) but leaves it as permeable as before (8); other steroids have similar effects. Cholesterol acetate also condenses membranes (32); I predict that it will not significantly reduce their permeability.

In a recent study (31), it was concluded that there is no specific binding of the sterol-OH to any polar part of the phospholipids. This conclusion was based upon the condensing effect and the liquefying effect (reduction of the ΔE of phase transition) that cholesterol had on some phospholipids that lacked the C=O group in position 2, namely, 1-oleoyl-2-palmitylglycerylphosphorylcholine and 1-oleoyl-2-palmityl-2-deoxyglycerylphosphorylcholine. As pointed out above, membrane condensation is not identical with membrane closure, and the experiments (31) may, therefore, not have been relevant to the problem of membrane function. It is the more surprising that a closer examination of the results (Fig. 1, [31]) shows that the condensing effect of cholesterol upon these lipids amounted to only one-half of the effect that could be achieved upon diacyl phospholipids. This result seems to show that cholesterol does, indeed, establish a bond to car-

bonyls and preferentially to the ester group in position 2 of the glycerol.

SPHINGOLIPIDS, α-HYDROXY FATTY ACIDS, AND PLASMALOGENS

The 3-hydroxy group of sphingosine has no known biochemical function. In conventionally printed structural formulas, this group seems to be buried in the hydrophobic region of sphingolipids, but in a three dimensional model in which the *trans*-configuration of the amide group is taken into account and the chains are arranged parallel, the OH group moves to the same latitude as the amide C=O group. (The N-H group probably is buried between the heads of the chains.) Both C=O and OH now lie in the hydrogen belt. Obviously, the one can act as hydrogen bond acceptor, the other as donor. They cannot link to each other. The range of possible latitudinal sphingosine O-H angles may be much wider than for cholesterol, perhaps from +70° to -70°. The case for latitudinal bonding on the basis of steric arguments is, therefore, not as convincing as it is for cholesterol. Nevertheless, I suggest that sphingolipids are both hydrogen bond acceptors and donors serving as extenders and branches in the hydrogen belts.

The D-α-hydroxy acids found in some galactocerebrosides of the brain introduce an additional hydroxy group into the sphingolipid, and this must be a strong donor because of electronegative induction from the neighboring carbony. These cerebrosides could serve as cross-links in the hydrogen bond network. On the other hand, it is possible for the α-OH to link to the ring oxygen of the galactose while, at the same time, the C=O is linked by the 4-OH of the carbohydrate. This arrangement is possible only with galacto-, not with glucocerebrosides, and not with L but only with D-α-hydroxy acids. A new ring structure would be formed which would be stabilized by concerted electron shifts and which would totally immobilize the galactose in relation to the head groups of the sphingosine and the fatty acid, but would leave hydroxyls 2, 3, and 6 available for $OH\cdots OH_2$ hydrogen bonding. Galactose hydroxyl 3 would be the group farthest extended; it is interesting that this is the group that can carry a sulfate residue. A sphingolipid thus internally complexed would be a hydrogen donor only. It would resemble cholesterol in its largely planar structure (because of the rigidification of the chains by *trans*-methylene configurations), and it labile hydrogen would, at low latitudinal angles, extend out of the plane as in cholesterol. The main physicochemical difference of the sphingolipid would be a greater length and a large, rigid hydrophilic head group.

Plasmalogens, i.e. 1-(alk-1-enyl)-2-acyl phosphoglycerides, and ether lipids, i.e. 1-alkyl-2-acyl phosphoglycerides, have only one C=O group, in position 2. Since there cannot be more than one mole of cholesterol/mole of phosphoglyceride in natural membranes, because the membranes would crystallize, there is always an excess of hydrated CO groups in such membranes, probably mostly the less electronegative CO groups in position 1 of phosphoglycerides. In plasmalogens, even this group is cancelled, and the membrane, according to the hydrogen belt hypothesis, must be of minimal permeability. (Incidentally, removal of the electron-withdrawing CO in position 1 will increase the electron density, and, therefore, the hydrogen bond strength, in position 2). Thus, it becomes clear why plasmalogens abound in plasma membranes, but especially in the myelin membrane. The abundance of sphingolipids in these membranes is similarly explained.

MEMBRANE PROTEINS

The membrane has been treated here as a semipermeable lipid bilayer; biological membranes, however, contain proteins, and the permeation of membranes by solutes is thought to be accomplished mostly by these proteins. This might appear to invalidate all studies on pure lipid monolayers, films, or vesicles. However, lipid bilayers constitute almost certainly the continuous matrix of membranes, and our arguments do apply to this matrix. Furthermore, I believe that the membrane proteins themselves participate in the hydrogen belt. The arguments concerning the hydroxyl hydrogen of cholesterol must also apply to those labile hydrogens that are situated on the border between the hydrophobic and the hydrophilic part of membrane proteins. The required latitudinal orientation of the hydrogen must be a frequent possibility. Membrane proteins, then, can be viewed as being girdled by a hydrogen belt consisting of these protons and the C=O groups of phospholipids. Circumstantial evidence is supplied by the myelin sheath of nervous tissue. This membrane, which has a completely locked lipid-lipid hydrogen belt, also seems to be devoid of any intrinsic protein (33). It also has been shown that cholesterol inhibits the penetration of phospholipid monolayers by proteins (9).

The concept of the hydrogen belt illuminates many aspects of membrane permeability, and it is, as far as I have probed, compatible with all known facts. Further confirmation,

short of direct spectroscopic proof, may be expected from permeability studies with phospholipid analogues lacking C=O groups. More extensive studies on the correlation of membrane condensation and permeability may furnish additional evidence. The concept offers new views on membrane phenomena other than permeability. For example, the action of many hormones might involve the disruption of the hydrogen belt bonding of an acceptor protein and thus initiate the conformational changes that are believed to take place in such proteins on stimulation. Antibiotics, and lysing and fusing agents, such as lysolecithin or polylysine, may function by interrupting and disorganizing the hydrogen belts. Monoglycerides may be absorbed in the gut, because they are hydrogen bond donors. Alcohols, i.e. hydrogen bond donors, are required to solubilize lipids from tissues. The concentrations of cholesterol and protein in inner and outer mitochondrial membranes appear to be inversely related (34); this is understandable if both compete for phospholipid C=O groups. Many more such examples can probably be found.

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Glycosphingolipids of White Cells Lymphoid Tissue and Bone Marrow

G.M. LEVIS and **M. KESSE-ELIAS,** Biochemical Research Laboratory, Department of Clinical Therapeutics, University of Athens, Alexandra Hospital, Athens, Greece

ABSTRACT

Glycosphingolipids of lymphoid tissues, bone marrow cells, mixed blood leucocytes, separated lymphocytes, and granulocytes from large white pigs were analyzed by thin layer and gas liquid chromatographies. The composition of the glycosphingolipids of thymus and blood leucocytes (mixed population) was similar, and trihexosyl ceramide (galactosyl-[1-4]-galactosyl-[1-4]-glucosyl-[1-1]-ceramide) was the major glycosphingolipid component of both tissues. The fatty acid fractions of all glycolipids from the two tissues were analyzed, and gross differences are discussed. Blood lymphocytes had a higher content of glycosphingolipids/mole phospholipid or mg protein than thymus lymphocytes obtained by a gentle washing of sliced tissue. Similar and more pronounced differences were obtained when the glycosphingolipid content of bone marrow cells (>50% polymorphonuclear neutrophils) was compared with that of blood polymorphonuclear neutrophils. In general, most of the blood leucocytes were richer in glycosphingolipids than most of the cells of the lymphoid tissues and bone marrow. These results indicate a marked difference in lipid composition between blood polymorphonuclear neutrophils and bone marrow cells. It is possible that certain of the biophysical properties which characterize blood polymorphonuclear neutrophils and which derive from changes in the cell periphery of immature granulocytes are connected with these differences in membrane lipid composition.

INTRODUCTION

Glycosphingolipids are present as minor constituents of the lipid fraction in most, if not all, types of mammalian cells. Even so, there are wide variations in the amounts of these minor components in different tissues, and, compared with most tissues, the white blood cells are relatively rich in glycosphingolipids. The white cells, which have been examined in a limited number of species (1-3), contained predominantly either dihexosyl or trihexosyl ceramides (GL-2, GL-3). It has been shown that glycolipid biosynthesis occurs in bone marrow cells (2), as well as in normal human leucocytes (4,5), and that in immature leucemic cells it occurs at an accelerated rate (5). Work on liver cells (6,7), intestine (8), and lymph nodes (9) suggests that the glycolipids are located predominantly in the plasma membranes.

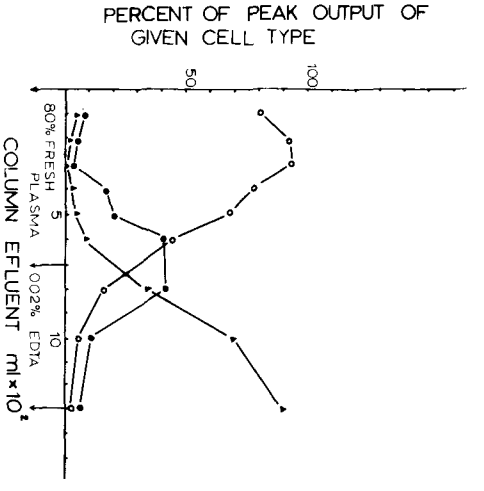

FIG. 1. Separation of isolated leucocytes on a siliconized glass bead column at 37 C. The column was 2 cm x 25 cm and the average diameter of the glass beads 2 mm. A leucocyte suspension in 80% plasma was applied on the column, and the various leucocyte populations were eluted as indicated in the figure. EDTA = ethylenediaminetetraacetic acid, o—o = lymphocytes, ▲—▲ = polymorphonuclear neutrophils (PMN), and ●—● = eosinophils.

Leucocytes are nucleated cells unique among mammalian cells in that they are formed in another part or at least in another environment of the body, different from their functional sites. Therefore, the cells must be adapted for a specific functional role, and, indeed, alteration of the periphery during granulocyte maturation has been reported (10).

The present work is an effort to determine differences in glycolipid composition between blood leucocytes and their parent immature cells.

MATERIAL AND METHODS

Blood was obtained from large white pigs at

the slaughter house and mixed with heparin solution. Total leucocytes were isolated from whole blood and purified as described previously (2). Separation of mixed white cell populations into lymphocytes and granulocytes was achieved on siliconized glass bead columns (2 cm x 25 cm), essentially as described by Rabinowitz (11), except that glass beads were of larger diameter (average diameter 2 mm). A typical scheme of elution is shown in Figure 1. It can be seen that essentially pure lymphocytes were obtained by elution with 80% plasma, and pure polymorphonuclear neutrophils (PMN) were eluted in the last fractions with ethylenediaminetetraacetic acid (EDTA) 0.02%. Thymus and other lymphoid tissues were obtained from pigs up to 60 kg body wt. A partially pure fraction of *thymus lymphocytes* was obtained by chopping the tissue into small pieces with scissors and gently stirring the pieces for 5 min with cold 0.9% NaCl solution after which time the suspension was filtered. Cells were obtained by centrifugation of the filtrate at 50 g for 15 min. Preparations which contained over 60% of lymphocytes were used for further analysis. Bone marrow cells were isolated from young 25 kg pigs, as described previously (2). The differential count of a typical preparation was: myeloblast, 0.5%; promyelocytes, 2.8%; myelocytes, 5.7%; metamyelocytes, 20.6%; polymorphonuclear, 55.4%; lymphocytes, 1.3%; plasma cells, 0.2%; monocytes, 0.6%; reticulum cells, 0.8%; pronormoblasts, 0.5%; and normoblasts, 11.6%. Protein was determined by the method of Lowry, et al. (12). Total lipids were extracted according to Folch-Pi, et al. (13). Lipid phosphorus was determined in duplicate in small portions of the extract by the method of Bartlett (14). Total lipids were subjected to mild alkaline hydrolysis (1), and total glycosphingolipids were isolated by column chromatography on silicic acid (15,16). Methyl esters and nonsaponifiable lipids were eluted with chloroform; ceramides were eluted with ethyl acetate, and glycosphingolipids with acetone:methanol (9:1 v/v). Carbohydrates were determined by gas liquid chromatography (GLC) (17) following methanolysis with 0.7 M HCl in dry methanol for 24 hr at 180 C and preparation of the trimethylsilyl derivatives of the O-methyl-glycosides according to Carter and Gaver (18). The trimethylsilyl O-methyl glycosides were separated and quantitatively estimated on 3% SE-30 at 175 C (support Diatomite CQ 100-120 mesh). An internal standard of mannitol was included in each sample. Analysis was performed either on total glycolipids to obtain quantitative data on total glycolipid content and the glucose/galactose+deacylated galactosamine ratio or on separated glycolipid classes. Results of glycolipid content are given on a molar basis calculated from the glucose peaks. Individual glycolipids were separated by preparative thin layer chromatography (TLC) as described by Gray (19). Thin layer systems for glycolipid analysis used in the present experiments have been described elsewhere by Gray (20). Fatty acid esters were recovered from the methanolysis mixture by extraction with redistilled hexane. The efficiency of the methanolysis procedure was tested with known quantities of Cytolipin H (lygnoceryl derivative, Miles, Laboratory, Elkhart, Ind.) which were treated under the above described conditions. In a triplicate experiment, the recoveries of both carbohydrates and fatty acids, assayed quantitatively by GLC using an internal standard of C_{23}, were in the range of 90-95%. The composition of fatty acid esters recovered after methanolysis was determined by GLC, also on SE 30 at 215 C. Both normal and 2-hydroxy fatty acids were analyzed. To confirm the identity of each class of fatty acids, the total mixture was separated by TLC into normal and 2-hydroxy compounds. The two fractions were recovered from the thin layer plate and subjected separately to GLC.

Identification of the peaks was achieved by comparison with authentic standards of normal fatty acids (Applied Science Laboratories, State College, Pa.) or 2-hydroxy fatty acids isolated from brain glycolipids. When needed, the fatty acid esters also were chromatographed on an 5% EGS column at 170 C (support Diatomite CQ 100-120 mesh), before and after hydrogenation over platinum oxide; the carbohydrate structures of the glycosphingolipids were determined by methods described elsewhere (21).

RESULTS

The various glycosphingolipid fractions from total leucocytes and thymus were separated by TLC, and their carbohydrate compositions are given in Table I. Monohexosyl ceramide (GL-1) from leucocytes was a mixture of glucosyl and galactosyl ceramides.

The fine structure of the major glycosphingolipid of both tissues, GL-3, was identified as galactosyl-(1-4)-galactosyl-(1-4)-glucosyl-(1-1) ceramide. Heamatoside, in measurable quantities, was found only in thymus glycosphingolipids.

The fatty acid composition (Table II) of the four glycosphingolipids, isolated from mixed leucocyte populations and whole thymus from the pig, was similar to those found in most glycosphingolipids from nonnervous tissues.

TABLE I

Glycosphingolipids of Total Leucocytes and Thymus of Pig[a]

Composition	Leucocytes		Thymus	
	Ratio glucosyl:galactosyl	μMoles glucose	Ratio glucosyl:galactosyl	μMoles glucose
Monohexosyl ceramide	3.0:1.0	0.53	1.0:00	0.31
Dihexosyl ceramide	1.3:1.0	0.04	1.1:1.0	0.03
Trihexosyl ceramide	1.0:2.0	1.76	1.0:1.8	0.45
Aminoglycolipid	1.0:1.8	0.05	1.0:2.0	0.09
Heamatoside		Trace	1.0:1.1	0.05

[a]Leucocytes were isolated from the blood of several male white pigs up to 60 kg body wt. Thymuses from the same animals were pooled and a representative sample of the organs free of fat and extraneous tissues was used for analysis. Each glycolipid was isolated by preparative thin layer chromatography and analyzed by gas liquid chromatography.

The presence of a high proportion of 2-hydroxy fatty acids indicated a major difference from the fatty acid compositions of human leucocytes glycosphingolipids (1). The fatty acid compositions of the total glycosphingolipids of blood lymphocytes, thymus and lymph nodes, and thymus ceramides from one small male pig are shown in Table III.

The glycolipid content/mole phospholipid or mg protein of blood lymphocytes was higher than that of *thymus lymphocytes* (Table IV). Though it is understood that circulating lymphocytes derive from more than one lymphoid tissue and that two populations at least are present in blood and the tissues in different proportions, it is suggested that the glycolipid composition of the *thymus lymphocytes* may be considered as ca. representing the glycolipid composition of blood lymphocytes prior to their migration from the thymus. This is substantiated further by the fact that blood lymphocytes are richer in glycosphingolipids than any other of the examined lymphoid tissues, e.g. tonsils and lymph nodes in which the lymphoid population like in thymus makes up ca. 90% of the wt (22).

Of course it is possible that a small proportion of thymus lymphocytes are those which enter the gland from the periphery (22).

Glucosyl ceramide was the predominant glycosphingolipid of blood lymphocytes in all the samples analyzed except one in which the larger glycosphingolipids, incluing aminoglycolipid (globoside), were the major components.

Glycosphingolipid composition of bone marrow cells, platelets, granulocytes, and mixed blood leucocytes is shown in Table IV. Blood leucocytes (50%-60%, PMN), as well as pure PMN, have increased glycolipid content/mole of phospholipids or mg protein as compared to bone marrow cells (>50% PMN). TLC analysis and a decrease in the ratio of glucosyl/galactosyl+galactosamine indicated an increase in the proportion of the higher molecular wt glycolipids in blood leucocytes. Thus, it is evident that the increased ratio of glycolipids to phospholipids found in blood lymphocytes, as compared to lymphoid tissues, also is observed between blood PMN and bone marrow cells. Being rather improbable that such large differences derive only from the glycolipid content of bone marrow PMN which are the immediate precursors of blood PMN, it is suggested that these differences are due to the more immature granulocytes which possibly must comprise only traces of glycosphingolipids. In accordance with the above is the low content in glycosphingolipids of platelets which are produced directly by fragmentation of giant megakaryocytes which constitute a small part of the immature population of bone marrow.

DISCUSSION

The present results have provided information on the carbohydrate structure and fatty acid composition of the glycosphingolipids of both leucocytes and lymphoid tissues. The major component of the glycosphingolipids of pig leucocytes and the other tissues which were studied is a trihexosyl ceramide with a carbohydrate structure similar to that found in many other mammalian tissues (21,23). The monohexosyl ceramide of mixed blood leucocytes was a mixture of glucosyl and galactosyl ceramide, while GL-1 of thymus contained only glucose.

Comparison between the fatty acid composition of glycosphingolipids of mixed leucocytes and thymus revealed that: (A) in both tissues fatty acids with chain length longer than C_{20} were major constituents of GL-3; (B) 2-hydroxy and odd chain fatty acids were found in higher proportion in the glycolipids of blood

TABLE II

Percentage Composition of Fatty Acids in Pig Thymus and Leucocyte Glycosphingolipids

Fatty acid designation	Monohexosyl ceramide		Dihexosyl ceramide		Trihexosyl ceramide		Aminoglycolipid	
	Thymus	Leucocytes	Thymus	Leucocytes	Thymus	Leucocytes	Thymus	Leucocytes
14:0	0.7	1.1	0.9	3.0	1.5	1.0	1.2	1.8
15:0	0.4	0.6	0.4	3.0	0.6	Trace	0.7	0.9
16:0	31.9	10.8	14.0	19.3	11.2	14.8	19.7	24.9
NI[a]	1.2	1.3	4.2	2.0	1.2	---	Trace	---
17:0	---	3.2	---	3.2	---	1.1	---	Trace
16h:0	1.2	5.7	9.7	3.0	0.9	5.0	Trace	16.5
NI	1.3	---	---	---	0.8	---	4.5	---
18:1	0.6	2.9	1.3	8.6	2.7	1.7	3.6	7.4
18:0	5.7	3.7	8.2	7.4	7.2	2.9	7.8	7.5
NI	0.8	---	3.0	---	2.0	---	Trace	---
19:0	1.7	6.2	2.4	9.1	0.5	Trace	2.4	2.5
18h:1	---	0.3	---	1.2	---	---	---	1.7
18h:0	0.9	0.5	0.9	0.6	Trace	---	0.7	---
20:1	---	0.8	---	2.8	---	---	1.2	3.5
20:0	10.5	1.2	6.9	1.8	2.6	1.5	5.1	2.3
21:0	3.4	1.4	3.6	1.8	0.8	0.4	2.4	4.1
22:1	0.4	1.3	2.5	1.0	1.6	0.9	2.1	5.1
22:0	9.6	6.8	9.7	5.1	12.7	7.5	12.6	Trace
NI	0.5	---	1.3	---	0.4	---	0.9	---
23:1	---	0.4	---	---	0.5	0.4	---	---
23:0	1.6	2.0	3.3	0.8	3.0	2.2	3.0	1.1
22h:0	1.7	5.4	---	0.9	1.9	2.2	---	2.0
24:1	3.9	10.1	11.0	7.6	22.8	15.0	7.4	2.1
24:0	13.2	9.4	16.7	6.6	13.2	12.3	15.0	3.9
24h:1	2.9	16.0	Trace	7.3	9.2	21.0	7.9	8.2
24h:0	6.7	9.5	Trace	3.3	2.7	9.2	1.8	4.5
20-24	54.9%	63.1%	55.0%	39.3%	71.4%	73.5%	59.4%	36.8%
Hydroxy	15.9%	37.4%	10.6%	16.3%	13.7%	37.4%	10.4%	32.9%
Unsaturated	7.8%	31.8%	14.8%	28.5%	36.8%	41.2%	22.2%	28.0%
Odd	8.3%	13.8%	9.7%	17.1%	4.7%	4.1%	8.5%	8.6%

[a]NI = nonidentified.

TABLE III

Percentage Composition of Fatty Acid in Glycospingolipids of Blood Lymphocytes, Thymus Lymph Nodes, and Thymus Ceramides[a]

Fatty acid designation	Total glycosphingolipids			Ceramides[b]
	Blood lymphocytes	Thymus	Lymph nodes	Thymus
14:0	6.2	2.2	9.1	3.9
15:0	2.6	0.8		2.4
16:0	24.3	17.6	32.8	
17:0	3.0		6.3	Trace
18:1	Trace	5.0	10.5	16.9
18:0	19.2	9.2	5.2	14.6
19:0	9.4	7.9		3.9
18h:0	4.1	3.0	8.5	---
20:0	3.2	7.7	3.7	10.4
21:0	14.9	2.5		0.3
NI[c]	3.0	1.5		0.6
22:1	Trace			---
22:0	5.8	7.5	7.0	9.3
23:0	1.5	0.8		1.6
24:1	Trace	15.7	5.2	1.7
24:0	2.9	13.9	4.9	1.6
24:1(h)[d]	Trace	1.8	4.5	1.2
24:0	Trace	2.9	2.1	---
Sum 20:0-24:0	31.3	54.3	27.4	26.7

[a]All tissues derived from the same animal (male 25 kg body wt).

[b]Ceramides were isolated during glycosphingolipid separation by elution with ethyl acetate.

[c]NI = nonidentified.

[d](h) = 2- hydroxy fatty acids.

leucocytes than those in thymus, which indicated that they were probably components of granulocytes or alternatively that hydroxylation and α-oxidation of fatty acids (24) were more active in bone marrow than in lymphoid tissues; (C) the proportion of unsaturated fatty acids (especially the C_{24} component) was higher in thymus and leucocyte GL-3 than in GL-2. The significance of this difference is not known, but it may result from a substrate preference of the glycosyl transferases in the pathway from GL-1 to GL-3.

The fatty acid composition of the glycosphingolipids of blood lymphocytes (Table III) was different from that of total leucocytes in which PMN were the predominant cells. It is interesting that the major components of the long chain fatty acids of thymus ceramides and blood lymphocyte glycosphingolipids were C_{20}, C_{21}, and C_{22} and not C_{24} and $C_{24:1}$ which are the usual components of glycosphingolipids from other mammalian tissues. Furthermore, it is evident that the fatty acid composition of blood lymphocyte glycolipids resembles that of the thymus ceramides since both have low proportions of the C_{24} acids. This may suggest that the relative increase in the amount of glycosphingolipids in blood lymphocytes as compared to *thymus lymphocytes* may derive from biosynthesis of glycosphingolipids in the tissues.

Blood PMN comprised much higher quantities of glycosphingolipids than bone marrow cells. It is reasonable to believe that the lipid composition of blood PMN would resemble that of marrow PMN rather than that of more immature proliferating forms which account for ca. 50% of the analyzed bone marrow cells. These cells, therefore, must be poor in glycosphingolipids, most of which should be formed at later stages of proliferation and maturation.

There is evidence that PMN glycolipids derive from metabolic processes occurring in the bone marrow and not in the periphery. Thus, it has been shown that bone marrow cells from the pig can synthesize glycolipids (2), whereas we have been unable to detect any biosynthesis of glycolipids in circulating leucocytes *in vitro* (unpublished data). On the other hand, the fatty acid composition of the pig plasma glycolipids (25) is different from that reported for pig leucocytes in the present study. This would suggest that the increased glycolipid content of blood leucocytes does not derive largely from the glycolipids of the plasma. Also it is worth mentioning that immature cells from chronic myelogenic leukemia comprise higher quantities of gangliosides as

TABLE IV

Glycosphingolipid Composition of Lymphoid Tissues, Bone Marrow Cells, and Blood Leucocytes

Tissues[a]	Glycosphingolipids			
	μmoles[b]			
	Lipid phosphorus/100 μmole	Protein/100 mg	Molar[c] ratio glucosyl:galactosyl	TLC[d] analysis
Thymus (5)	0.90	0.22	1.0:1.1	GL-3, GL-1, Aminoglycolipid heamatoside GL-2
Tonsils (1)	0.95		1.0:1.2	GL-3, GL-1 Aminoglycolipid
Lymph nodes (3)	1.10	1.20	1.1:1.0	GL-3, GL-1 Aminoglycolipid heamatoside GL-2
Thymus lymphocytes (4) 63%-85%	2.20	0.33	1.1:1.0	GL-1, GL-3 Aminoglycolipid
Blood lymphocytes (3) 90%-97%	4.40	4.10	1.8:1.0	GL-1, GL-3 Aminoglycolipid
Bone marrow cells (2)	1.80	1.57	1.0:1.0	GL-1, GL-3 Aminoglycolipid heamatoside
Total leucocytes (7)	7.2	10.4	1.0:1.6	GL-3, GL-1 Aminoglycolipid, GL-2
Granulocytes (2)	6.0		1.0:1.6	GL-3, GL-1 Aminoglycolipid
Platelets (2)	1.5		1.0:1.6	GL-3, GL-1 Aminoglycolipid heamatoside

[a]Figures in parentheses are numbers of sample examined and data are mean values of samples analyzed.

[b]Total glycosphingolipids were isolated as described in the text and their total carbohydrates analyzed by gas liquid chromatography, μmoles were calculated from the glucose peaks.

[c]Molar ratio derives from total glycosphingolipids. Part of the first galactose peak derives from deacylated galactosamine.

[d]Compounds are given in order of intensity of spots on thin layer chromatographic (TLC) plates sprayed with 50% H_2SO_4 and heated at 120 C for 10 min. GL-2, where not noted was present in tracer quantities. GL-3 = trihexosyl ceramide, GL-1 = monohexosyl ceramide, GL-2 = dihexosyl ceramide.

compared to normal mature leucocytes (26) and that biosynthesis of glycolipids in these cells is much more active than in mature PMN (5). Therefore, it is possible that glycolipids of leucocytes are formed in the bone marrow either by biosynthesis or by degradation of gangliosides.

Studies of immature granulocytes and PMN neutrophils from marrow and blood indicated pronounced biophysical differences occurring during granulocyte maturation and which adapt the PMN for its essential cell-extracell interactions (10). Most of these differences, such as the reduction of surface negative charge density, the increase of adhesiveness, and of the degree of surface deformality and mobility rates, are membrane phenomena.

The presented results, for the first time, provide information for a marked difference in lipid composition between a mature cell and its immature precursors. This change must occur in the plasma membrane and probably is more pronounced than in other cell membrane, since glycolipids are particularly plasma membrane lipids.

It is, thus, possible that the biophysical alterations which adapt the cell for its specific circulatory and functional capacities are structurally related with the presented changes in lipid composition. It also remains to be found whether such changes also can effect the outer membrane of the cell to facilitate its exit from the marrow.

The results concerning the lymphocytic series of white cells, though pointing to the same direction, are difficult to discuss in relation to events occurring in the tissue, since, as it is known, circulating lymphocytes and those of thymus and other lymphoid tissues comprise at least two populations in various proportions.

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Studies of Unsaponifiables in Several Vegetable Oils

TAMAR GUTFINGER and **A. LETAN,** Department of Food Engineering and Biotechnology, Technion-Israel Institute of Technology, Haifa, Israel

ABSTRACT

The unsaponifiable fractions of soybean, cottonseed, coconut, olive, and avocado oils have been studied in detail. The oils differed in the contents of total unsaponifiables, squalene, tocopherols, and sterols and also in the composition of the tocopherol and sterol fractions. The presence of absence of individual unsaponifiable components may help in establishing the identity of each of the investigated oils and in detecting of admixture by another oil.

INTRODUCTION

The development of analytical procedures, such as thin layer (TLC) and gas liquid chromatographies (GLC), gave new impetus to the investigation of unsaponifiables and other minor components in various oils (1-3). A recent review in this field was prepared by Fedeli and Jacini (4). Research workers usually were concerned with a particular fraction of the unsaponifiables, such as sterols (5,6), triterpenes (7), or tocopherols (8-10). It was noticed by some of the investigators that the unsaponifiable components furnish a finger-print useful for the identification of oils and that analysis of unsaponifiables may be helpful in detection of foreign admixtures in an investigated oil (11-13).

There were several discrepancies in the published data. For example, Fedeli and Jacini (4) reported that avocado oil was rich in unsaponifiables (6-7%), while Franzke and Henning (14) found that the content of unsaponifiables in that oil was less than 2%. Slover (9) reported that olive oil contained only α-tocopherol, while Losi and Pirette (8) found that γ- and δ-tocopherols also were present in that oil. The reported range for the total tocopherol content in soybean oil was also very wide. Thus, 1680 μg/g oil were found by Lange (15), but only 750 μg/g were reported by Biernoth (16). The differences in the reported data might be due to differences in the variety, origin of the investigated samples, and the procedures used in extracting the oil from the fruit or seed.

Oils of soybean, cottonseed, coconut, olive, and avocado were investigated in the present work, because of their importance and because they are obtained from plants which belong to different families. The authors gathered information on lipid composition of oils, which had been extracted either from the locally grown cottonseeds, olives, and avocados or from the common varieties of the imported soybeans and coconuts. The authors also wished to recommend suitable ways for characterization of the five investigated vegetable oils through their unsaponifiables and for detection of admixtures of a foreign oil to the studied individual oils. In addition in this work, the compositions of the lipid fraction from different anatomical parts of the avocado (flesh and kernel) and the olive (flesh, pit's shell, and pit's kernel) were compared.

EXPERIMENTAL PROCEDURES

Materials

Samples of crude oil from hexane-extracted soybeans (U.S.) were obtained from the industry. Coconut (Singapore) and locally grown cottonseed (variety Acala) were Soxhlet-extracted with petroleum ether (60-80 C). Locally grown avocado (varieties Fuerte and Hass) and olives (variety Suri from different locations in the country) were extracted from the different anatomical parts of the fruits with a chloroform-methanol mixture, as described previously (17).

Standards of fatty acid methyl esters and of sterols were purchased from Applied Science Laboratories, State College, Pa. α-Tocopherol and squalene were purchased from Fluka AG. Standards of γ- and δ-tocopherols were prepared from the soybean oil by TLC.

Methods

The unsaponifiables were separated according to Ames (18) and redissolved in chloroform. Analysis of tocopherols was performed according to Gutfinger and Letan (13); chloroform was used as the developing solvent in the TLC separation of the unsaponifiables, and the Emmerie-Engel colorimetric method for the quantitative determination of individual tocopherols. The contents of total sterols and squalene and the compositions of the sterol fractions were determined by GLC on 3% SE-30 on Gas Chrom Q, as described by Gutfinger, et al., (17). Standards of the sterols and squalene were used to obtain suitable calibration curves. Fatty acid compositions of

TABLE I

Fatty Acid Composition of Several Vegetable Oils

Oil	Component fatty acid (%)					
	16:0	16:1	18:0	18:1	18:2	18:3
Soybean, batch 1	13.2	0.0	4.4	21.7	53.4	7.3
Soybean, batch 2	12.1	0.0	4.2	24.2	51.1	8.4
Cottonseed	23.1	0.8	2.2	17.2	56.7	0.0
Coconut[a]	6.1	0.0	2.1	5.5	1.0	0.0
Olive[b], flesh	15.2	0.9	3.8	68.2	11.4	0.5
Olive[c], flesh	19.3	2.7	2.8	61.2	14.0	traces
Olive[d], flesh	15.3	2.7	3.1	65.1	13.8	traces
Olive[d], pit's shell	14.4	1.3	3.7	67.6	13.0	traces
Olive[d], pit's kernel	9.1	0.6	2.3	68.1	19.9	traces
Avocado[e], flesh	18.2	5.2	1.2	60.4	15.0	traces
Avocado[f], flesh	18.0	8.9	traces	55.9	17.2	traces
Avocado[f], kernel	8.9	traces	2.5	68.5	20.1	traces

[a]Contained also 9.3% caprylic (8:0), 9.6% capric (10:0), 50.7% lauric (12:0), and 25.7% myristic (14:0) acids.

[b]Variety Suri, Golan.

[c]Variety Suri, Carmel.

[d]Variety Suri, Galilee.

[e]Variety Fuerte.

[f]Variety Hass.

TABLE II

Content of Oil in Several Fruits and Seeds and Content of Unsaponifiables and Squalene in Oils

Oil	Oil content in fruit or seed (%)	Unsaponifiables in oil (%)	Squalene in oil (μg/g oil)
Soybean, batch 1	19.2	1.6	137
Soybean, batch 2	19.6	1.5	125
Soybean, batch 3	19.4	1.7	143
Cottonseed	28.2	1.2	91
Coconut	59.0	0.5	16
Olive[a], flesh	32.4	1.5	9,250
Olive[b], flesh	34.9	0.8	2,500
Olive[c], flesh	29.5	1.3	4,500
Olive[c], pit's shell	2.3	4.9	2,350
Olive[c], pit's kernel	43.7	1.5	95
Avocado[d], flesh	14.1	12.2	370
Avocado[e], flesh	19.8	4.8	341
Avocado[e], kernel	1.3	55.5	—

[a-e]See footnotes b, c, d, e, f in Table I.

the oils were determined by GLC, as described by Gutfinger, et al. (17). Fatty acid compositions of the lipids extracted from the avocado kernel were determined after removal of the unsaponifiables.

RESULTS

The fatty acid compositions of the oils are given in Table I. Only avocado and olive oils had similar fatty acid compositions. The data agree with those reported by Hilditch and Williams (19).

The contents of the total lipids, total unsaponifiables, and squalene in the seeds and fruits are given in Table II. Avocado oil which had been extracted from the fruit's flesh contained more unsaponifiables than any other of the investigated oils (4.8-12.2%), but unsaponifiables accounted for over 50% of the lipids which had been extracted from avocado kernel. High content of unsaponifiables in avocado oil from avocado flesh previously was reported by Fedeli and Jacini (4).

The squalene content in olive oil was the highest (2500-9250 μg/g oil), but appreciably lower concentrations of squalene were found in other oils (16-370 μg/g oil). Also Gracian (20)

TABLE III

Sterols in Several Vegetable Oils

Oil	Total sterols (μg/g oil)	Component sterol (%)		
		Campesterol	Stigmasterol	β-Sitosterol
Soybean, batch 1	3,430	23.4	23.3	53.3
Soybean, batch 2	3,640	21.8	23.8	54.4
Soybean, batch 3	3,870	23.3	23.5	53.2
Cottonseed	3,640	7.4	0.0	92.6
Coconut	790[a]	7.1	16.8	76.1
Olive[b], flesh	1,050	1.4	traces	98.6
Olive[c], flesh	2,210	2.5	0.5	97.0
Olive[d], flesh	1,460	2.8	0.6	96.6
Olive[d], pit's shell	6,000	2.8	traces	97.2
Olive[d], pit's kernel	4,200	3.6	0.7	95.7
Avocado[e], flesh	4,860	7.7	0.0	92.3
Avocado[f], flesh	3,770	7.3	1.6	91.1
Avocado[f], kernel	10,720	7.9	2.3	89.8

[a]Contained also traces of cholesterol.

[b-f]See footnotes b, c, d, e, f in Table I.

TABLE IV

Tocopherols in Several Vegetable Oils

Oil	Total tocopherols (μg/g oil)	Component tocopherol (%)		
		α-Tocopherol	γ-Tocopherol	δ-Tocopherol
Soybean, batch 1	1,129	11.0	67.5	21.5
Soybean, batch 2	1,452	8.8	69.9	21.3
Soybean, batch 3	1,132	7.9	65.3	26.8
Cottonseed	864	45.6	54.4	0
Olive[a], flesh	153	94	6	0
Olive[b], flesh	121	90	10	0
Olive[c], flesh	186	93	7	0
Olive[c], pit's kernel	291	75	25	0
Avocado[d], flesh	140	100	0	0
Avocado[e], flesh	153	100	0	0
Avocado[e], kernel	43	100	0	0

[a-e]See footnotes b,c,d,e,f in Table I.

reported differences in the level of squalene in olive oils from different sources.

The contents of sterols and the compositions of the sterol fractions in the oils are given in Table III. Avocado, soybean, and cottonseed contained large amounts of sterols (3430-4860 μg/g oil). Coconut oil, which was poor in unsaponifiables, was also poor in sterols. The lipids extracted from the avocado kernel contained excessive amounts of sterols (over 10,000 μg/g oil). The sterol compositions of olive, cottonseed, and avocado oils were similar. In those oils, β-sitosterol was the dominant sterol (ca. 90%), and it was accompanied by only limited amounts of stigmasterol (0.0-2.3%). Different amounts of sterols were present in avocado and olive oils extracted from either the flesh or the kernel of avocados or olives, but the compositions of the oils' sterol fraction were almost the same. The sterol fractions of soybean and coconut oils had characteristic sterol compositons; they were relatively rich in stigmasterol (23 and 17%, respectively), and the soybean oil was particularly rich in campesterol (ca. 23%).

The sterol content and composition in soybean, cottonseed, and olive oils were in agreement with those reported by Wolff (2) and Gracian and Martel (5). As for coconut oil, a similar sterol composition was reported also by Wolff (2) and Maura Fe (21). Itoh, et al., (6) who used a different stationary phase for GLC analysis than the other workers, found also Δ^5-avenasterol and Δ^7-stigmastenol in the sterol fraction. The coconut oil which was examined by Itoh, et al., (6) contained 2600 μg sterols/g, compared to 760 μg/g found in our sample. Wolff (2) reported a value similar to

TABLE V

Unsaponifiable Components Useful for Characterization of Several Vegetable Oils

Oil	Type of components		
	Presence[a]	Absence	Traces
Soybean	Tocopherols: $\gamma > \delta > \alpha$ Campesterol ∿ 20% Stigmasterol ∿ 20%		
Cottonseed	α-tocopherol ∿ 50% γ-tocopherol ∿ 50%	δ-tocopherol Stigmasterol	
Coconut	Campesterol ∿ 17% Stigmasterol ∿ 7%		Squalene Tocopherols
Olive (flesh)	High content of squalene[b] α-tocopherol ∿ 90% β-sitosterol ∿ 96%	δ-tocopherol	γ-tocopherol Stigmasterol
Avocado (flesh)	High content of unsaponifiables[c] α-tocopherol ∿ 100%	γ-tocopherol δ-tocopherol	Stigmasterol

[a]Percents indicate the component's level in the tocopherol or sterol fraction.
[b]Over 1000 μg/g oil.
[c]Over 40 mg/g oil.

ours for the sterol content in coconut oil (800 μg/g oil). With avocado oil from the flesh of the fruit, Sadir (22) found a similar sterol content (5200 μg/g oil), but Paquot and Tassel (23) and Fedeli, et al., (24) reported the presence of additional unidentified components in the sterol fraction. The sterol fraction of avocado oil was rechromatographied three times by TLC before the GLC separation of the sterols, to eliminate the possibility of appearance of components which were not sterols. It was observed that additional peaks appear when the sterol fraction was subjected to the GLC separation after some delay.

The results for tocopherol content and composition of the oils are summarized in Table IV. Soybean oil was the richest in tocopherols (1130-1450 μg/g oil), while no tocopherols were detected in coconut oil. Slover (9) reported that coconut oil was poor in tocopherols (36 μg/g oils). Lipids extracted from the shell of olive pits contained a single unknown component that gave a positive reaction with the Emmerie-Engel reagent; its R_f was lower than the R_f of δ-tocopherol. α-Tocopherol was present in all the tocopherol containing oils: cottonseed oil had the largest α-tocopherol content (ca. 400 μg/g oil). All the tocopherol containing oils, with the exception of the avocado, contained γ-tocopherol. Soybean and cottonseed were rich in γ-tocopherol (450-1000 μg/g oil). δ-Tocopherol was detected only in soybean oil. The soybean and cottonseed oils, which were rich in tocopherols, differed from the other oils also in the composition of their tocopherol fractions. Each one of these oils had a typical tocopherol pattern. The tocopherol content and composition in soybean, cottonseed, and olive oils generally agreed with the data reported in the literature (4,9). Paquot (25) reported a similar value for the tocopherol content in avocado oil.

DISCUSSION

Characterization of Investigated Oils through Their Unsaponifiables

Tables II, III, and IV show that the oils differed in the contents of total unsaponifiables, squalene, tocopherols, and sterols and also in the composition of their tocopherol and sterol fractions. Despite variations in the absolute amounts of sterols and tocopherols in different samples of the individual oils, the compositions of the tocopherol and sterol fractions were characteristic for each oil. In the three different olive oils, it seems that the location of growth did not affect the composition of the sterol and tocopherol fractions. For the two samples of avocado oils, the differences in the variety of the fruit did not affect the compositions of the sterol and tocopherol fractions. Most of the results for oils extracted from locally grown fruits and seeds agreed with data in the literature ("Results"). Our results for the composition of the tocopherol fraction of cottonseed oil differed from some of the data of Whittle and Pennock (26) and Rao, et al., (27) who found that the tocopherol fraction was composed of 37.7% and 44.6% γ-tocopherol, respectively, but were in good agreement with those of Niderstebruch and Hinsch (28).

Our results indicate that the identities of

individual oils may well be characterized by the composition of their unsaponifiables. Determinations of unsaponifiables also may help in detection of an admixture of another oil to an individual oil. In the soybean and cottonseed oils, no significant differences were observed between the compositions of the tocopherol and sterol fractions in their crude and refined oils. The amounts of unsaponifiables in the refined soybean and cottonseed oils were sufficient for characterization of those oils (29).

The unsaponifiable components which characterize the investigated oils by their presence or absence are summarized in Table V. The presence of certain components in individual oils in excessive amounts can indicate adulteration. Addition of soybean oil to the relatively expensive avocado and olive oils can be recognized by the presence of excessive amounts of stigmasterol and γ- or δ-tocopherol. Admixture of cottonseed oil to olive or avocado oils can be determined by the presence of excessive amounts of γ-tocopherol. Detection of δ-tocopherol and of stigmasterol in cottonseed oil may indicate adulteration of cottonseed oil with the less expensive soybean oil. Avocado and olive oils (which have similar fatty acid, sterol, and tocopherol compositions) can be distinguished through their contents of total unsaponifiables and squalene. Since only two varieties of avocado and only one extraction technique were investigated, a survey of the level of unsaponifiables in avocado fruits of different origins and investigation of avocado oils obtained by different techniques might help to estimate the usefulness of determination of total unsaponifiables for characterization of the avocado oil. From Table V, decisions can be taken which determinations should be made to establish the identity of an investigated oil and to detect adulteration by another oil.

Comparison of Lipids Extracted from Various Parts of Olive and Avocado

Fatty acid compositions of the olive and avocado kernel oils were different from those of the oils which had been extracted from the flesh of the same fruits. The kernel oils were richer in the unsaturated 18 carbon fatty acids (88.0 vs 78.9% for olive kernel and flesh oils, respectively; 88.6 vs 73.1% for avocado kernel and flesh oils, respectively, Table I). Lipids extracted from the olive kernels contained more oleic and linoleic acids than the flesh. The avocado kernel oil contained more oleic and linoleic acids and less palmitic and palmitoleic acids than its flesh oil. Similar results were reported previously by Biale and Young (30).

As for tocopherol determinations, lipids extracted from the avocado flesh and kernel contained only α-tocopherol, but the lipids of the flesh were slightly richer in tocopherols than those of the kernel (153 μg/g oil vs 43 μg/g). Lipids extracted from the olive kernel were richer in tocopherols than the lipids from the flesh (291 μg/g oil vs 186 μg/g); in the former, the relative content of γ-tocopherol was higher (25 vs 7%, Table IV). In both the fruits, lipids extracted from the kernels were also richer in total sterols than the lipids extracted from the flesh, but the sterol composition of the kernel and flesh oils was the same (Table III).

Relationships between Some Components of Lipid Fraction

It seems that the amounts of some of the components of the lipid fraction are correlated. An inverse relationship could be observed between the content of total lipids in the investigated fruits (coconut, olive, and avocado) and the amount of unsaponifiables in the extracted oils. Copra (dried coconut meat) was rich in total lipids (59%) and its oil contained a low amount of unsaponifiables (0.5%). On the other hand, avocado kernel was poor in total lipids (1.3%), but those were rich in unsaponifiables (55.5%). Less oil was obtained from the flesh of avocado variety Fuerte than from the flesh of avocado variety Hass (14.1 vs 19.8%); the Fuerte oil contained, however, more unsaponifiables than the Hass oil (12.2 vs 4.8%). Comparing the lipid fraction of the flesh and pit's shell from olive (Galilee), the flesh was richer in lipids (29.5 vs 2.3%), but the lipids from the shell were richer in unsaponifiables (4.9 vs 1.3%). An inverse relationship exists between contents of the sterols and squalene in the three investigated olive oils (Tables II and III). For example, the oil which had the highest content of sterols (2210 μg/g oil) also had the lowest content of squalene (2500 μg/g oil). The oil that was the richest in squalene (9250 μg/g oil) contained the lowest amount of sterols (1050 μg/g oil). Having in view that squalene is a precursor in the biosynthesis of sterols (31), such a correlation should not be unexpected.

Another connection can be observed between the fatty acid composition of the oils' triglycerides and their tocopherol content and composition (Tables I and IV). No tocopherols were detected by us in the very saturated coconut oil. (According to Slover [9] and Fedeli and Jacini [4], the tocopherol content in coconut oil is low, 36 μg/g oil). Oils rich in unsaturated fatty acids contained considerable amounts of tocopherols (which may act as

antioxidants). The highest concentration of tocopherols (1129-1452 μg/g oil) was found in th easily oxidized soybean oil. Cottonseed oil, whose major fatty acid is the polyunsaturated linoleic acid, also contained an appreciable amount of tocopherols (850 μg/g oil). The unsaturated soybean and cottonseed oils also contained appreciable amounts of γ-tocopherol which is usually considered a better antioxidant than α-tocopherol (32).

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Leaf Wax of *Portulaca oleracea*[1,2]

A.P. TULLOCH, Prairie Regional Laboratory,
National Research Council, Saskatoon, Saskatchewan S7N 0W9

ABSTRACT

Wax coating the leaves and stems of *Portulaca oleracea* consists of hydrocarbons (21%), esters (53%), acids (2%), alcohols (4%), diol monoesters (2%), and unidentified material (15%). Lesser amounts of esterified and free β-amyrin and lupeol, stigmast-4-en-3-one and free diols also are present. The principal component of the hydrocarbons is C_{33}. The C_{40}-C_{56} esters are C_{22}-C_{28} alcohol esters of C_{20}-C_{26} acids; free alcohols are C_{22}-C_{30}; free acids are C_{16}-C_{36}; diols of diol monoesters and free diols are C_{20}-C_{26}.

INTRODUCTION

Portulaca oleracea L., or Purslane (family Portulacaceae) is a common annual weed in North America and also in Europe where it is native. It is a succulent, fleshy plant with dark green, shiny, nonglaucous leaves (1) and is difficult to eradicate by cultivation since uprooted plants can survive for several weeks and readily become reestablished.

Xerophytic characteristics, such as the small thick leaves, are probably mostly responsible for the plant's drought resistance which is demonstrated by the relatively low transpiration ratio (less than half that of spring rye or crested wheat grass [2]).

However, even though the plant is not glaucous like barley or rye, it seemed most likely that it would have a waxy coating to protect the cuticle and to limit cuticular transpiration. As part of an investigation of leaf waxes of plants connected with agriculture, waxes of several types of wheat and oats have been analyzed (3-6). It was useful, therefore, to determine and compare the composition of wax of *P. oleracea*, particularly when the wax was probably a factor contributing to drought resistance.

EXPERIMENTAL PROCEDURES

Flowering plants of *P. oleracea* were collected in mid-July at Saskatoon, Sask., and wax isolated by washing with hexane at room temperature. The wax was a light brown, partly crystalline solid, with melting point (mp) 70-74 C.

NMR spectra were obtained with a Varian HA-100 spectrometer or, for very small samples, with a Varian XL-100-15 spectrometer in the Fourier transform mode at 100.1 M Hz. Mass spectra (MS) were measured, and gas liquid chromatographic (GLC) analyses were performed, as previously described (5). Chain lengths of components were determined by GLC analysis after addition of suitable synthetic compounds (6).

Column Chromatographic Separation of Wax

Wax (4.13 g) was separated on silicic acid (200 g, Biosil A, Biorad Laboratories, Richmond, Calif.). Hydrocarbons (0.86 g) were eluted with hexane (1.5 liters) and most of the long chain esters (2.15 g) were eluted with hexane-chloroform (95:5, 10 liters). The same solvents (90:10, 5 liters) eluted a mixture of long chain esters, triterpene esters, and unidentified material (0.3 g). Further unidentified material (0.1 g) was eluted with the same solvent mixture (500 ml).

The remainder of the wax (0.78 g), consisting of free acids and alcohols, hydroxy esters, and diols, was eluted with hexane-chloroform (80:20, 2 liters) and with chloroform (1 liter). This material was treated with diazomethane and rechromatographed on silicic acid (100 g). Methyl esters and unidentified components (0.12 g) were eluted with hexane-ether (99:1, 2 liters). A complex mixture (0.13 g) was eluted with hexane-ether (98:2, 4 liters), and hexane-ether (95:5, 2 liters) eluted a mixture (0.13 g) of long chain and triterpene alcohols. Hexane-ether (93:7, 2 liters) eluted alcohols and stigmast-4-en-3-one together (0.14 g). Crude hydroxy esters (0.13 g) were eluted with hexane-ether (85:15, 2 liters), and elution with hexane-ether (50:50, 3 liters) gave a mixture (0.13 g) containing free diols and unidentified components.

Fractions were examined by thin layer chromatography (TLC) in chloroform containing 1% ethanol (3) and by GLC.

Esters

Esters (0.25 g) were refluxed with 5% methanolic hydrogen chloride (10 ml) and benzene (10 ml) for 18 hr. The solution then was poured into water and extracted with chloro-

[1]Presented at the AOCS Meeting, Ottawa, September 1972.

[2]NRCC No. 14037.

form. Chromatography on silicic acid gave methyl esters (0.14 g, eluted with hexane-ether 99:1, 2 liters) and alcohols (0.13 g, eluted with hexane-ether 90:10, 2 liters).

Triterpene Esters

The mixture of long chain esters and triterpene esters (0.28 g) was subjected to methanolysis as above. Chromatography, as before, gave a mixture (0.13 g) of methyl esters and unidentified components followed by a brown gum (0.02 g, elution with hexane-ether 95:5, 3 liters). Further elution with the same solvent (1 liter) separated crystalline triterpene alcohols (0.02 g) followed by long chain alcohols (0.06 g, elution with 2 liters solvent). GLC analysis of the acetylated triterpene alcohols showed two equal and almost completely separated peaks with emergence temperatures corresponding to those of acetates of β-amyrin and lupeol (245 and 248 C respectively, α-amyrin acetate had the same emergence temperature as lupeol acetate).

Crystallization of the mixed acetates from acetone separated part (0.002 g) of the β-amyrin acetate as characteristic small rod-like cyrstals; mp and mixed mp with authentic β-amyrin acetate was 242-244 C; NMR spectrum ($CDCl_3$): CH_3 singlets at δ 0.84, δ 0.88(4), δ 0.97(2), and δ 1.14; $COCH_3$ at δ 2.02; $CHOCOCH_3$ at δ 4.50 (triplet); and C=CH at δ 5.19 (triplet). MS: m/e 468 (molecular ion), 249, 218 (base peak), 203, 189, 69, 55, 43. Material recovered from the mother liquors had NMR ($CDCl_3$) with double bond proton signals at δ 4.54 and δ 4.67 (ca. 4 protons) due to $CH_3\overset{|}{C} = CH_2$ and δ 5.14 (ca. 1 proton) due to C=CH. MS: m/e 468 (molecular ion), 249, 218, 205, 204, 203, 189, 81, 69, 55, 43 (base peak). Lupeol acetate (isolated from ouricuri wax) was examined for comparison and had NMR spectrum ($CDCl_3$): CH_3 singlets at δ 0.79, δ 0.85-0.87 (3), δ 0.94, δ 1.03, δ 1.67, and δ 2.02; $CHOCOCH_3$ at δ 4.47 and $CH_3\overset{|}{C}=CH_2$ at δ 4.54 and δ 4.67. MS: m/e 468 (molecular ion), 249, 218, 205, 204, 203, 191, 190, 189, 95, 81, 69, 55, 43 (base peak).

Triterpene Alcohols

Part of the mixture of long chain and triterpene alcohols was converted to trimethylsilyl (TMS) ethers and C_{30} TMS compounds were isolated by preparative GLC (5% SE 30 on acid-washed Chromosorb W at 250 C). GLC showed 2 ca. equal peaks, and NMR and MS results indicated a 1:1 mixture of lupeol and β-amyrin.

Stigmast-4-en-3-one

The mixture of free alcohols and the above ketone was acetylated and rechromatographed on silic acid. Hexane-ether (99:1) eluted alcohol acetates; hexane-ether (95:5) eluted unidentified gum; and the ketone was eluted with hexane-ether (90:10). After purification by preparative GLC, the ketone had NMR ($CDCl_3$): CH_3 singlets at δ 0.69; δ 1.16 and C=CH adjacent to C=O singlet at δ 5.70, MS: m/e 412 (molecular ion and base peak), 229, 124, 95, 57, 55, 43, 41. The NMR spectrum and MS of authentic stigmast-4-en-3-one were similar to the above spectra.

Diol Monoesters

The NMR spectrum (CCl_4) of the fraction containing hydroxy esters had a triplet at δ 3.50 showing the presence of a free primary hydroxyl group. Methanolysis was carried out as above, and products were acetylated and analyzed by GLC without separation by column chromatography.

Diols

The free diol containing fraction (0.12 g) was acetylated and chromatographed on silicic acid. Elution with hexane-ether (95:5, 2 liters) gave a mixture (0.03 g) of acetylated diols and acetates of hydroxy esters which were not the same as those isolated above. The apparent diol liberated by methanolysis of these hydroxy esters did not give a peak (after acetylation) when analyzed by GLC and was not characterized further.

RESULTS AND DISCUSSION

P. Oleracea plants washed with hexane for a very short period lost water much more rapidly than untreated plants (Table I). The increased rate of water loss is probably due mainly to removal of wax from the leaf surface, but hexane also may have penetrated the epidermis and disorganized the membrane structure or even have affected the stomata.

Although *P. oleracea* does not have a waxy bloom, yield of wax (0.44% as a percentage of the dry wt, Table II) is as large as that obtained from glaucous varieties of wheat (3). The composition is given in Table II and chain lengths of components are listed in Tables III and IV. Major components also are seen by GLC analysis of whole wax (Fig. 1).

Hydrocarbons form the second largest group of components in the wax with C_{33} as major component and C_{31}, C_{35}, and C_{29} as minor components. This is an unusual composition since C_{33} hydrocarbon is rarely a major component of plant wax hydrocarbons. Few plant waxes have been analyzed completely, but compositions of the easily separated hydrocarbon

TABLE I

Effect of Hexane Washing upon Wt Loss of Uprooted *Portulaca oleracea* Plants[a]

Time (hr)	Wt of Plants (g)	
	Washed[b]	Unwashed
0	200	200
4	177	183
7	164	175
23	116	146
32	97	137
78	35	107
96	24	97
26 days		33

[a]Plant dry wt was 17.5 g.

[b]Whole plants were swirled in hexane for not longer than 5 sec.

TABLE II

Composition of Leaf Wax of *Portulaca oleracea*[a]

Component	Percent
Hydrocarbons	21
Long chain esters	53
Triterpene esters	1
Free acids	2
Free alcohols	4
Triterpene alcohols	1
Stigmast-4-en-3-one	0.75
Diol monoesters	2
Free diols	0.25
Unidentified fractions	
Eluted between esters and alcohols	10
Eluted after alcohols	5
Yield (% of dry wt)	0.44

[a]Calculated from wt of components obtained by silicic acid column chromatography.

TABLE III

Composition of Major Wax Fractions from *Portulaca oleracea*

Number of carbon atoms	Hydrocarbons	Esters	Hydrolysis products of esters		Free acids	Free alcohols
			Acids	Alcohols		
16	-	-	-	-	4	-
18	-	-	-	-	13	-
20	-	-	16	2	9	3
21	-	-	0.5	-	-	-
22	-	-	47	18	14	32
23	-	-	1	0.5	-	-
24	-	-	31	40	19	23
25	2	-	0.5	1	-	-
26	-	-	4	27	9	23
27	3	-	-	0.5	-	-
28	-	-	-	7	10	12
29	8	-	-	-	-	-
30	-	-	-	2	7	7
31	20	-	-	-	-	-
32	2	-	-	1	9	-
33	48	-	-	-	-	-
34	1	-	-	1	5	-
35	15	-	-	-	-	-
36	-	-	-	-	1	-
37	1	-	-	-	-	-
40	-	1	-	-	-	-
42	-	5	-	-	-	-
44	-	16	-	-	-	-
45	-	1	-	-	-	-
46	-	32	-	-	-	-
47	-	2	-	-	-	-
48	-	27	-	-	-	-
49	-	1	-	-	-	-
50	-	10	-	-	-	-
52	-	3	-	-	-	-
54	-	1	-	-	-	-
56	-	1	-	-	-	-

TABLE IV

Composition of Minor Wax Fractions from *Portulaca oleracea*

Number of carbon atoms	Diol monoesters	Hydrolysis products of diol monoesters		Free diols
		Acids	Diols	
16	-	3	-	-
18	-	2	-	-
20	-	21	17	10
22	-	42	51	44
24	-	26	28	44
26	-	6	4	2
38	3	-	-	-
40	6	-	-	-
42	24	-	-	-
44	38	-	-	-
46	19	-	-	-
48	7	-	-	-
50	3	-	-	-

fractions of waxes from several hundred species have been determined (though in most cases percentage of total hydrocarbons in the wax was not reported). Major hydrocarbons of most waxes are C_{27}, C_{29}, or C_{31} (7-9); waxes with C_{33} as major hydrocarbon have so far been found almost exclusively among members of the family Crassulaceae (also fleshy succulent plants) (10,11) and among the Gymnosperms (12,13).

The chain length range of the esters, C_{40}-C_{56}, is similar to that found for waxes of cereal crops (3-6); 2% of C_{45}-C_{49} esters with an odd number of carbon atoms is also present and can be seen in Figure 1. Acids of the esters, obtained on methanolysis, are mostly C_{20}-C_{26}, but the alcohols are C_{22}-C_{28}, longer by two carbon atoms. Odd carbon acids, C_{21}-C_{25}, and alcohols, C_{23}-C_{27}, also are obtained accounting for the odd carbon esters. Esters differ from esters of cereal waxes in that there are several major alcohols rather than one, and, as a result, the composition is ca. symmetrical about the major peak. Major components of free acids are the same as those of combined acids, but the chain length range is much wider. Free alcohols are similar to combined alcohols.

Small percentages of long chain esters of the common triterpenes β-amyrin and lupeol also are present. These same triterpenes also are found as minor components of the free alcohols; acetates of β-amyrin and lupeol appear, together with triacontyl acetate, as the small incompletely resolved hump between C_{33} and C_{35} hydrocarbon peaks (Fig. 1). Lupeol was identified and estimated from the NMR spectrum, particularly the $C{=}CH_2$ signals at δ 4.54 and δ 4.67; moretenol, the other triterpene with an isopropylene group (14) was absent since there was no high field CH_3 signal at δ

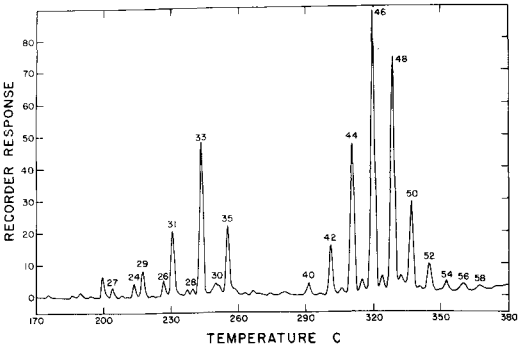

FIG. 1. Gas liquid chromatographic separation of *Portulaca oleracea* wax after treatment with diazomethane and acetylation. Peaks with odd numbers (peak numbers also indicate chain length) from 27–35 are hydrocarbons; peaks with even numbers from 24-30 are alcohol acetates; and peaks with numbers 40-58 are monoesters. Column was 3 ft x 1/8 in. stainless steel packed with 60-80 mesh, acid-washed and silanized Chromosorb W coated with 1.5% Dexsil 300. Temperature was programed from 125-400 C at 3 C min, but results from 170-380 C only are shown.

0.68 (also in the NMR spectrum of moretenol acetate the $C{=}CH_2$ signal is a singlet at δ 4.68 only) (15). α-Amyrin also could be present but could not be identified positively by NMR in the presence of larger amounts of lupeol and β-amyrin. The C_{29} steroid ketone, stigmast-4-en-3-one, is present in small quantity; this ketone has been isolated previously from plant extracts (16).

Other minor components are monoesters of α,ω-diols and free α,ω-diols (Table IV). The major C_{42}-C_{46} components of the diol esters were accounted for by the hydrolysis products: acids and diols both mainly C_{20}-C_{24} components. The acids have almost the same chain lengths as acids of the monoesters, but diols differ from the alcohols in having a chain length

range of C_{20}-C_{24} rather than C_{22}-C_{26}. Larger amounts of appreciably longer chain monoesters of α,ω-diols occur in ouricuri wax (17). A very small amount of free diols, similar in composition to the esterified diols, was isolated. There is 15% of unidentified material in the wax consisting of a large number of very minor components, some long chain and some steroid, distributed over a large number of chromatographic column fractions.

Wax of *P. oleracea* is apparently the only one analyzed so far which has long chain esters as principal components, although it is difficult to make a useful comparison with waxes from other plants because so few, particularly from dicotyledonous plants, have been analyzed completely. Waxes of members of the grass family frequently contain major alcohols (4,5,18) as do waxes of *Chenopodium album* and *Stellaria media* (18). Other frequent major components are hydrocarbons and β-diketones which are often almost entirely of one chain length (C_{29}, C_{31}, or C_{33}) and, perhaps for this reason, are the cause of glaucousness. Thus, C_{31} hydrocarbon is the major component of wax of glaucous peas (19) and C_{29} hydrocarbon of glaucous cabbage (20). Major β-diketones cause glaucousness in *Eucalyptus*, in the grass *Festuca glauca* (21), in the grass *Poa colensoi* (22), in barley (23), and in durum wheat (24).

There has been disagreement about the effect of glaucousness upon culticular transpiration, mainly because it is difficult to remove the bloom mechanically without disturbing the underlying layers (25). Visible bloom always is associated with needlelike projections from the cuticle (26), and it may be that, in some cases, the projections are not useful and that water is better retained by a thicker, less rough layer of wax on the cuticle. Further research is necessary to establish whether the nonglaucous combination of hydrocarbons and long chain esters in *P. oleracea* wax is particularly suited to retain water; electron microscopic studies probably would provide useful information.

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Characterization of Branched Chain Fatty Acids from Subcutaneous Triacylglycerols of Barley-Fed Lambs

W.R.H. DUNCAN, A.K. LOUGH, and **G.A. GARTON,** Rowett Research Institute, Bucksburn, Aberdeen, AB2 9SB, Great Britain, and **P. BROOKS,** Organic Geochemistry Unit, School of Chemistry, University of Bristol, Bristol, BS8 1TS, Great Britain

ABSTRACT

The fatty acids of subcutaneous triacylglycerols (containing ca. 11% of branched chain components) from lambs fed on barley-rich diets were fractionated by treatment with mercuric acetate and by urea adduct formation to yield concentrates rich in the branched chain components, all of which were saturated. The concentrates were subjected to analysis by high resolution gas liquid chromatography in conjunction with mass spectrometry. The branched chain fatty acids consisted of a complex mixture of mono-, di-, and trimethyl substituted components. The greater part of the mixture comprised monomethyl substituted acids of chain length 10-17 carbon atoms. Within each of these molecular species, a number of positional isomers was identified, notably in respect of methyltetradecanoic acid (methyl substituent on carbon 2, 4, 6, 8, 10, and 12) and methylhexadecanoic acid (methyl substituent on carbon 2, 4, 6, 8, 12, and 14). Homologous series also could be recognized of one of which all eight members from 4-methyldecanoic acid to 4-methylheptadecanoic acid were identified; together they accounted for ca. 39% of the branched chain fatty acids which were sampled for mass spectrometry. The dibranched acids identified consisted of five members of a homologous series, ranging in chain length from 11-15 carbon atoms and with substituent methyl groups at positions 4 and 8. Though the identity of only one tribranched acid (2,6,10-trimethyltetradecanoic acid) was established, others also apparently were present in the mixture. The probable involvement of methylmalonate in the biosynthesis of these branched chain acids is discussed briefly, with particular reference to the availability of vitamin B_{12} in relation to the activity of methylmalonyl coenzyme A mutase.

INTRODUCTION

When lambs were reared on pelleted diets having a high content of rolled barley, they produced abnormally soft subcutaneous adipose tissue (1). Analysis of the triacylglycerols of both subcutaneous and perinephric adipose tissue showed the presence of considerably greater proportions (up to 13%) of branched chain fatty acids than are present in the triacylglycerols of conventionally fed lambs, and it was concluded that the softness of the adipose tissue was associated, at least in part, with the lower melting points of branched chain acids relative to their straight chain isomers. In addition to branched chain fatty acids, the triacylglycerols also contained abnormally high proportions of odd numbered n-fatty acids, and further studies with lambs (2-4) showed that the production of both types of fatty acid was related to the availabiltiy of unusual amounts of propionate produced in the rumen by bacterial fermentation of barley carbohydrate. It was concluded (3,4) that, when the capacity for hepatic gluconeogenesis from propionate was exceeded, it was utilized readily as primer unit for the synthesis of long chain fatty acids and that the product of its carboxylation, namely methylmalonate, was incorporated into fatty acids synthesized from either acetate or propionate as primer unit, thereby giving rise to branched chains. Essentially on the basis of equivalent chain length (ECL) values (5) on gas liquid chromatography (GLC), these acids were presumptively identified as consisting, for the most part, of monomethyl substituted 14:0, 15:0, 16:0, and 17:0; preliminary chemical studies (3), based upon identification of methyl n-alkyl ketones produced by oxidative degradation of the mixed acids, indicated that the methyl branch occurred at a number of different positions along the carbon chain of each molecular species.

In this article we report the results of further, detailed investigations which showed the existence of homologous series of positionally isomeric, monomethyl branched fatty acids and which also revealed the presence of novel di- and trimethyl substituted fatty acids.

MATERIALS AND METHODS

Source of Fatty Acid Methyl Esters

The esters were prepared from the subcuta-

neous triacylglycerols of 3 ruminating male lambs which had been fed for 4 months, from weaning at 4 weeks of age, on a diet containing 90% of rolled barley together with white-fish meal and appropriate amounts of vitamins and minerals, including cobalt sulphate (0.1 mg Co/kg diet). These three samples of esters, which previously had been analyzed by conventional GLC (1), were pooled to give a mixture estimated to contain at least 11% of incompletely characterized branched chain components.

Preparation of Concentrate of Branched Chain Esters

The methyl esters (1.42 g) were treated with mercuric acetate (6) to remove the unsaturated components and then with urea (7) which retains a considerable proportion of the straight chain esters as adducts. This enrichment procedure yielded 266 mg which contained ca. 85% of branched chain components. Hydrogenation of the unsaturated esters, followed by GLC, showed that no unsaturated branched chain acids had been present.

Analysis of Concentrate of Branched Chain Esters by GLC

The esters were subjected to high resolution GLC combined with mass spectrometry (MS) using a Varian MAT-CH-7 aerograph; the 100 m open tubular column (internal diameter 0.25 mm), coated with polymerized butanediol succinate, was operated at 170 C with helium supplied at 40 psig and with a filament current of 100 μA.

RESULTS

That the branched chain fatty acids were a complex mixture was evident from the chromatogram resulting from fractionation of the esters on the high efficiency, open tubular column. This chromatogram, which is reproduced in Figure 1, revealed the presence of 125 peaks, some of which represented more than one component, as is apparent from their conformation. Almost all the major peaks were selected for examination by MS, and these together represented 64% of the total peak area; the identities assigned to them are given in Table I. Though it is likely that iso acids were present, no peak corresponding to this structure was sampled for MS.

To derive fatty acid structures, the mass spectra were normalized or, in cases where the base peak was off-scale, ion peaks were compared with the parent ion (M^+). By relating the

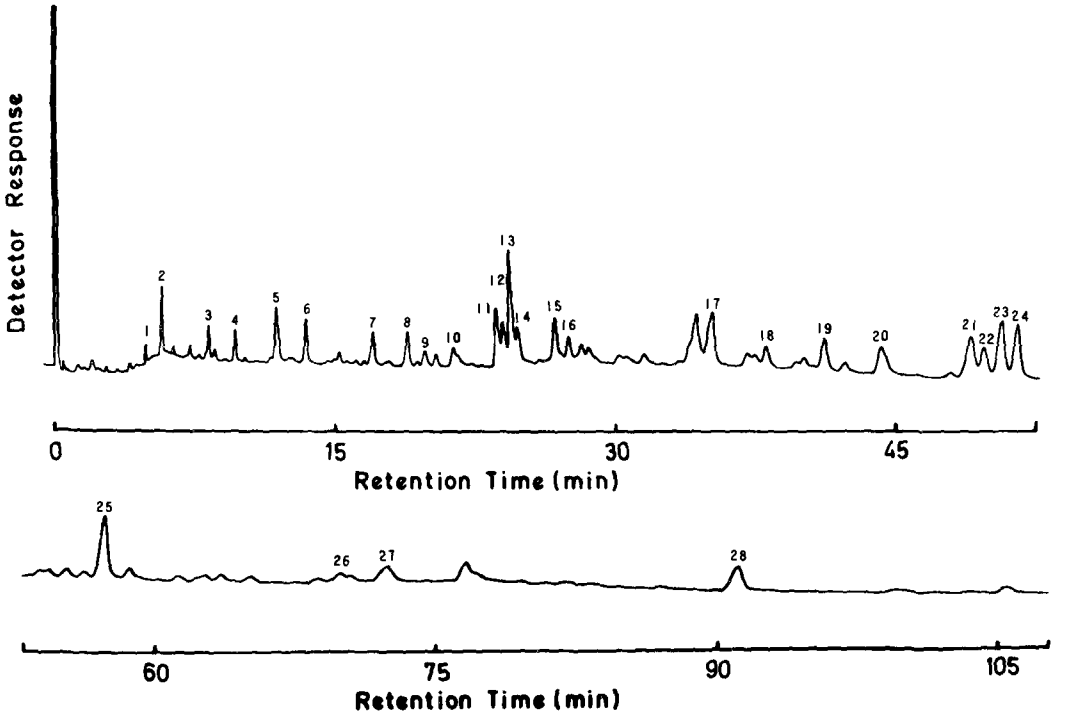

FIG. 1. Gas liquid chromatogram of concentrate of methyl esters of branched chain fatty acids. Open tubular column: polymerized butanediol succinate, 100 m, temperature 170 C. See Table I for identities and equivalent chain length values of numbered peaks.

total ion trace to the chromatogram obtained from the same column but with the MS uncoupled, it was possible to determine ECL values of the components which were examined by MS. Differences in the geometry of the peaks of the total ion trace as compared with that of the peaks on the gas chromatogram indicated that the resolution of the methyl esters was slightly impaired under these conditions, thereby rendering it difficult to obtain mass spectra which represented single components. Correlation of structure with MS evidence was made according to established criteria (8).

Of the 28 chromatographic components sampled for MS, four corresponded to the methyl esters of decanoic, tetradecanoic, hexadecanoic, and octadecanoic acids respectively. The anteiso (ω3) structure of chromatographic component 25 was indicated by the feature M-29 > M-31, together with a prominent peak at M-57 through loss of $CH(CH_3)C_2H_5$ and formation of the ketene at m/e 223 and ketene-H_2O at m/e 205. These peaks, together with the parent ion at m/e 284, are consistent with the structure of methyl 14-methylhexadecanoate. This ester formed part of an isomeric series, as was shown by the presence of a common parent ion at m/e 284; 9 such components were present, with ECL values as follows: 14.42, 15.36, 15.59, 15.80, 16.28, 16.32, 16.36, 16.42, and 16.70 (anteiso). Consideration of the known behavior of branched chain fatty acid esters on open tubular columns coated with polar phases (8,9), together with characterisitic MS features, made possible the identification of eight of these components. Thus, chromatographic component 19 (ECL 15.80) exhibited a major peak at m/e 88 with prominent peaks at m/e 101, m/e 241 (M-43), and m/e 227 (M-57); this pattern of fragmentation is consistent with the structure of methyl 2-methylhexadecanoate which is known (8-10) to be eluted in advance of methyl n-hexadecanoate on a polar phase.

Chromatographic components with ECL

TABLE I

Identity of Components of Chromatogram (Figure 1) Examined by Mass Spectrometry

Component number	Percent wt of total fatty acids of triacylglycerols	Equivalent chain length value	Parent ion m/e (mol wt)	Component(s)
1		10.00	186	Methyl n-decanoate
2	0.21	10.42	200	Methyl 4-methyldecanoate
				Methyl 6-methyldecanoate
3	0.11	11.41	214	Methyl 4-methylundecanoate
4	0.09	11.84	228	Methyl 4,8-dimethylundecanoate
5	0.38	12.40	228	Methyl 4-methyldodecanoate
6	0.19	12.64	242	Methyl 4,8-dimethyldodecanoate
7	0.21	13.39	242	Methyl 4-methyltridecanoate
8	0.21	13.58	256	Methyl 4,8-dimethyltridecanoate
9	0.11	13.80	256	Methyl 2-methyltetradecanoate
10		14.00	242	Methyl n-tetradecanoate
11	0.40	14.30	256	Methyl 6-methyltetradecanoate
12	0.30	14.34	256	Methyl 8-methyltetradecanoate
13	0.78	14.37	256	Methyl 4-methyltetradecanoate
14	0.33	14.42	256	Methyl 10-methyltetradecanoate
			284	Methyl 2,6,10-trimethyltetradecanoate
15	0.41	14.64	270	Methyl 4,8-dimethyltetradecanoate
16	0.22	14.70	256	Methyl 12-methyltetradecanoate
17	0.67	15.36	270	Methyl 4-methylpentadecanoate
			284	Not identified
			298	Not identified
18	0.24	15.59	284	Methyl 4,8-dimethylpentadecanoate
19	0.35	15.80	284	Methyl 2-methylhexadecanoate
20		16.00	270	Methyl n-hexadecanoate
21	0.64	16.28	284	Methyl 6-methylhexadecanoate
22	0.38	16.32	284	Methyl 8-methylhexadecanoate
23	0.73	16.36	284	Methyl 4-methylhexadecanoate
24	0.65	16.42	284	Methyl 12-methylhexadecanoate
25	0.76	16.70	284	Methyl 14-methylhexadecanoate
26	0.21	17.28	298	Methyl 6-methylheptadecanoate
				Methyl 8-methylheptadecanoate
				Methyl 10-methylheptadecanoate
27	0.38	17.35	298	Methyl 4-methylheptadecanoate
28		18.00	298	Methyl n-octadecanoate

values 16.28, 16.32, 16.36, and 16.42 yielded spectra (having a common parent ion at m/e 284) which indicated structures corresponding to methyl 6-methylhexadecanoate (prominent peaks at M-76 and M-50); methyl 8-methylhexadecanoate (ketene at m/e 139 and ketene-H_2O at m/e 121); methyl 4-methylhexadecanoate (base peak at m/e 87 and prominent peaks at m/e 227 [M-57], m/e 115, m/e 211 [M-73]); and methyl 12-methylhexadecanoate (prominent peaks at m/e 227 and m/e 199; ketene at m/e 195 and ketene-H_2O at m/e 177). Similarly, a further array of chromatographic components yielded spectra having a common parent ion at m/e 256 which indicated the presence of a series of esters corresponding to that of the branched C_{17} components but having two carbon atoms fewer. As Table I shows, these comprised methyl 2-methyltetradecanoate, methyl 6-methyltetradecanoate, methyl 8-methyltetradecanoate, methyl 4-methyltetradecanoate, methyl 10-methyltetradecanoate, and methyl 12-methyltetradecanoate, i.e. anteiso.

From the number of components subjected to MS, it was clear that a series of homologues existed involving a range of chain lengths, including the complete series of 4-methyl-substituted fatty acid esters from methyl 4-methyldecanoate to methyl 4-methylheptadecanoate and having ECL values, 10.42, 11.41, 12.40, 13.39, 14.37, 15.36, 16.36, and 17.35, respectively. These 4-methyl branched components collectively account for ca. 39% of the total branched chain fatty esters sampled for MS.

The possibility that fatty acids with more than one methyl substituent also were present was indicated by the presence of components, isomeric with methyl heptadecanoate, and having ECL values 14.42, 15.36, and 15.59. MS of the last of these (ECL 15.59) indicated that it was methyl 4,8-dimethylpentadecanoate; this ester was one of a series of homologues, namely, methyl 4,8-dimethylundecanoate (ECL 11.84), methyl 4,8-dimethyldodecanoate (ECL 12.64), methyl 4,8-dimethyltridecanoate (ECL 13.58), and methyl 4,8-dimethyltetradecanoate (ECL 14.64). Reference spectra (11) for the first two members of the series noted above provided strong evidence in support of these identifications.

Finally, a fraction of ECL 14.42 (parent ion at m/e 284) yielded a spectrum which, from a prominent peak at m/e 88 and well defined ketene peaks, was apparently methyl 2,6,10-trimethyltetradecanoate; the availability of a reference spectrum of this ester (11) enabled the probable structure to be confirmed. The presence of at least one other trimethyl-substituted fatty acid methyl ester is suggested by a component of ECL 15.36, having a mol wt of 298.

DISCUSSION

Long chain fatty acids having one or more substituent methyl groups have been shown to be present in many lipids of natural origin (12-14). Of those with a single methyl branch, the acids of the iso and anteiso series found in sebum and in bacterial lipids are derived indirectly from the branched chain amino acids, valine, leucine, and isoleucine (15-17). In ruminant animals, the normal presence of small amounts of iso and anteiso branched acids in their tissue lipids is attributable to the intestinal digestion and absorption of the structural fatty acids of rumen bacteria (15). That the triacylglycerols of ruminant adipose tissue can contain monomethyl branched fatty acids, other than iso and anteiso acids, was only recently discovered; as noted in the "Introduction," studies in this laboratory (1-4) revealed that such acids were present in the depot fats of barley-fed lambs. Other workers have noted their presence in sheep perinephric fat (9). The present finding that a methyl substituent can be present on any carbon atom from position $\omega 3$ to the carbon atom adjacent to the carboxyl group is consistent with the incorporation of methylmalonate into fatty acids synthesized from acetate and propionate as the primer terminal units of the molecules; iso acids cannot, of course, arise in this way. Hence, in the branched chain acids identified (Table I), each substituent methyl group is located on a carbon atom designated by an even number in relation to the carboxyl group of the molecule. Similarly, exclusively even carbon methyl substitution was found in fatty acids of sheep perinephric fat and bovine butterfat (9) and in nervous tissue lipids of a child who died from congenital methylmalonic aciduria (18), whereas both even and odd carbon substitution have been reported in the fatty acids of bovine butterfat (19) and of human milk fat (20).

The studies reported in this article show that the mixture of methylbranched fatty acids present in the triacylglycerols of lambs fed on diets rich in barley is more complex than was at first thought (3). In these earlier studies, it appeared that only one molecule of methylmalonate was incorporated (in place of malonate)/molecule of branched chain acid formed and that methylmalonate was not utilized to provide the two carbon atoms at the carboxyl end of the chain. While the present detailed studies have shown that monomethyl substi-

tuted acids comprise the greater part of total branched chain acids which are for the presence of small proportions of 5 having the 4,8-dimethyl structure and of 2,6,10-trimethyltetradecanoic acid indicates that the fatty acid synthetase system can incorporate more than one methylmalonate residue/molecule of fatty acid which is produced. Further, the occurrence of a methyl substituent on carbon 2 has been established, not only in the trisubstituted acid referred to above, but also in the series of mono branched tetradecanoic acids and hexadecanoic acids, thus indicating that the carboxyl group with its adjacent substituted methylene group can be derived from methylmalonate.

In one of the first reports from this laboratory (3) concerning the association between propionate metabolism in the lamb and the production of unusual branched chain fatty acids, the involvement of the vitamin B_{12}-dependent enzyme methylmalonyl CoA mutase (EC 5.4.99.2) was discussed. The mutase is required for the metabolism of methylmalonate derived from the carboxylation of propionate. It was considered that the availability of cobalt and hence of the active form of vitamin B_{12} (5'-deoxyadenosylcobalamin) could be of importance in determining whether or not methylmalonate accumulated and became incorporated into newly synthesized fatty acids. However, when groups of barley-fed lambs were given more dietary cobalt (0.9 mg/kg diet) or cyanocobalamin by injection (or both), none of the treatments resulted in a diminished production of branched chain fatty acids (21), suggesting that the lamb has a limited capacity to store and utilize vitamin B_{12}. As mentioned earlier (18), monomethyl branched acids were found in the nervous tissue lipids of a child who died from congenital methylmalonic aciduria, in which vitamin B_{12} is not converted to its deoxyadenosyl derivative; these acids comprised isomers of methylhexadecanoic acid with methyl branches at positions 2, 6, 10, 12, and 14. The evidence adduced for the identities of these isomers is in accord with our observations, except for methyl 2-methylhexadecanoate which, if present, would have emerged on GLC ahead of methyl n-hexadecaoate (9) and not, as is claimed, with the other isomers; it, therefore, appears that this component may have a second methyl branch in the molecule, which would account for its GLC behavior.

It seems possible that, in other disorders associated with impaired uptake or metabolism of cyanocobalamin, methylmalonate may accumulate and become incorporated into fatty acids which could impair the physiological integrity of nervous tissue lipids (22).

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Fatty Acid Composition of Monkey Milk Lipids[1]

LLOYD M. SMITH and **SUHADI HARDJO**[2], Department of Food Science and Technology, University of California, Davis, California 95616

ABSTRACT

Milk from 6 species of monkeys contained 2.2-8.5% total lipids, and 11.5-16.5% total solids. The fatty acid composition of the milks, as determined by an improved gas liquid chromatographic technique, was generally similar among the six species. The predominant fatty acids (by wt) were capric (7.5-14.6%), palmitic (19.4-23.3%), oleic (22.4-30.3%), and linoleic (13.6-15.2%). Small amounts of butyric (0.1-1.2%) and caproic (0.5-0.8%) acids were present in all samples. The averaged data were compared with the fatty acid compositions of primate and cow milks. Milks of the nonhuman primates contained less myristic, but more caprylic and capric acids, than did human or cows' milk.

INTRODUCTION

The use of nonhuman primates as experimental animals in studying factors related to human health is increasing. However, such animals are becoming more difficult to obtain from the wild, making it necessary to place increasing emphasis on domestic breeding programs. Information on the composition of the milk of these experimental animals is important to establish the nutritional requirements of both infant and lactating mother and to determine the extent of the similarities between nonhuman primates and man. In 1941, Van Wagenen, et al., (1) reported that the gross composition of rhesus monkey milk was similar to that of human milk and differed from cows' milk in having lower percentages of protein and ash and a higher percentage of milk sugar. Recently, the gross composition of milk from several primates has been reviewed by Jenness and Sloan (2), Buss (3), and Buss and Cooper (4,5).

Information concerning the lipids and their fatty acid composition in the milk of nonhuman primates is limited. Glass, et al., (6) reported the fatty acid composition of one sample of milk from the rhesus monkey (*Macaca mulatta*) and two from the green monkey (*Cercopithecus callitrichus*) but gave no dietary history of the samples. Buss (7) related the fatty acid composition of milks from 27 baboons to the fatty acid composition of their diet and of their adipose tissue and to stage of lactation. Buss and Cooper (4,5) determined the component fatty acids of 5 milk samples from the talapoin monkey (*Cercopithecus talapoin*) and of 13 samples from the squirrel monkey (*Saimiri sciureus*). Differences in fatty acid composition of samples from nonhuman and human primates were attributed to differences between biosynthetic activities of the mammary glands and to differences in fatty acid composition of the diets.

Milk fats generally contain a wide range of fatty acids (6), and the short chain components are likely to be lost during the preparation or gas liquid chromatographic (GLC) analysis of their methyl esters. With isothermal GLC conditions, the short chain esters are crowded together in the first part of the gas chromatogram, which makes quantitative estimation of the peak areas difficult. In the present study, mixtures of methyl esters were prepared from milk fats by an improved technique which permitted analysis by simple isothermal GLC equipment, instead of more complex, temperature programed instruments. Milk fats from six species of Old World monkeys, maintained under similar dietary and environmental conditions, were analyzed and compared with data for other primates and the cow.

EXPERIMENTAL PROCEDURES

The adult monkey mothers ranged in age from 7-12 years and belonged to the following species: *Cercocebus atys* (sooty mangabey), *Macaca fascicularis* (crab-eating macaque), *M. mulatta* (rhesus), *Macaca nemestrina* (pig-tail), *Macaca radiata* (bonnet), and *Macaca speciosa* (stumptail). The animals were fed a standard ration (modified Christ Hospital Institute of Medical Research diet; composition available on request) twice daily at the rate of 30 g/kg body wt. Average age of the infants at weaning was 27 weeks, at which time they were over 1 kg in wt. Milk was obtained within 48 hr after weaning by a combination of digital manipulation and careful hand expression to remove all

[1]Data taken from thesis of S. Hardjo submitted in partial fulfillment of the requirements for the Masters' degree in food science.

[2]Present address: Bogor Institute of Agricultural Sciences, Bogor, Indonesia.

milk present in the breast. Neither sedation nor oxytocin was used, and 5-25 ml milk were collected from each mother. Milk samples from 3 donors of each species were pooled, pasteurized by heating to 63 C for 30 min, and then cooled to 5 C. Lipids were extracted within 24 hr of milking.

Human milk was a pooled sample from three regular donors certified by the Mother's Milk Bank, San Francisco, Calif. The milk had been heated to 82 C for 20 min before being frozen at -20 C. Bovine milk was a pooled sample from several Holstein cows of the university herd and was prepared in the same manner as the monkey milk.

Reagent grade ethanol and methanol were refluxed and distilled over potassium hydroxide and zinc dust. Hexane was purified by passage through a silica gel column (8).

Total solids of milk were determined by the Mojonnier procedure (9). Lipids were extracted by gently shaking a 25-50 g sample with 80 ml ethanol-ammonium hydroxide (10/1, v/v) for 60 sec in a 500 ml separatory funnel. Then 50 ml hexane was added, and the mixture was shaken for 60 sec, after which it was let stand for 30 min. The lower phase was shaken 3 more times, each time with 50 ml portions of hexane. The pooled extracts were washed once with 50 ml deionized water and then dried with anhydrous sodium sulfate. Most of the hexane was removed under nitrogen by a rotary evaporator before the sample was made up to 50 ml in a volumetric flask. Total lipids were determined by evaporating the hexane from a sample and weighing the residue. This technique extracted 97% of the total lipids extracted by the Mojonnier method (9), which requires much larger volumes of solvents.

Total lipids in the monkey ration were extracted by essentially the same procedure as for milk, except that a Waring blender was used to assist in the extraction. Ration (20 g) was blended with 80 ml ethanol-ammonium hydroxide for 5 min at medium speed. Then, 5 min later, 50 ml hexane was added. The mixture was blended for 5 min,then filtered. The residue was returned to the blender and reextracted with hexane four more times. The combined hexane extracts were washed with 50 ml deionized water and then were dried with sodium sulfate to remove residual water. Most of the hexane was evaporated, and the sample was made up to 50 ml volume with added hexane. Total lipids were expressed as percentage of dry matter in the ration as determined by the Mojonnier method. The results agreed with those obtained by extraction for 16 hr in a Soxhlet apparatus.

Methyl esters of fatty acids were prepared in the apparatus of Endres (10) modified to avoid significant losses of short chain esters. The ground glass joint was replaced with screw threads wrapped with Teflon tape, and a Teflon lined screw cap (Fig. 1). A Pasteur pipette was used to introduce 15-50 mg fat in hexane into the bulb. After the hexane was evaporated with a gentle stream of nitrogen, 0.2 ml 0.1 N sodium methoxide was added with a 2 ml syringe fitted with a 7.5 cm long needle blunted at the tip. The reaction tube was tightly capped and placed in an oven at 60 C for 1 hr. The apparatus was opened after it had cooled in an ice bath, and a drop of 0.04% bromcresol purple indicator was added. By use of a syringe, 0.1 N hydrochloric acid was added to lower the pH below 5.2. Also, previously chilled, saturated sodium chloride was added to bring the level to the bottom of the capillary. The capped

FIG. 1. Apparatus for the preparation of fatty acid methyl esters. Volume of lower bulb is 5 ml, capillary is 3 mm inside diameter and 2.5 cm long, total length of apparatus is 10.5 cm.

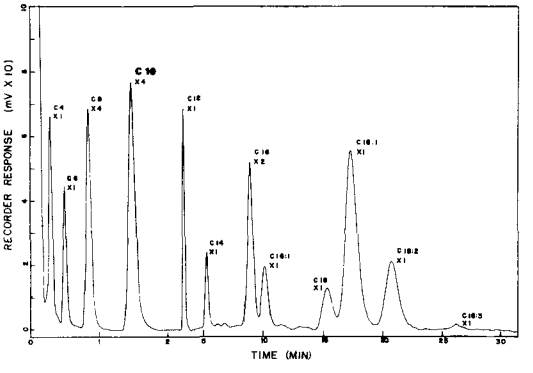

FIG. 2. Typical gas chromatogram of the fatty acid methyl esters prepared from monkey milk fats. Peaks were attenuated 1X-4X as indicated. Recorder speed was 3.05 m/hr for first 2 min, then 0.51 m/hr.

apparatus then was centrifuged at 2,000 rpm for 3 min to float the fatty acid methyl esters into the capillary. Complete conversion of mono-, di-, and triglycerides to methyl esters was confirmed by thin layer chromatography.

The ester mixtures were analyzed immediately by GLC in a Varian Aerograph A90-C chromatograph equipped with a 4-filament thermal conductivity detector and a 1.83 m x 0.64 cm outside diameter stainless steel column packed with 15% diethylene glycol succinate on 60-80 mesh, acid-washed firebrick. Column temperature was 193 ± 2 C, and that of both injector and outlet was 250 C. Filament current was 230 ma, and helium flow rate was 60 ml/min. Sample size was 1-3 μliters. Crowding of initial peaks was prevented by using a chart speed of 3.05 m/hr until the methyl capric (C 10:0) peak had emerged and then switching to 0.51 m/hr (Fig. 2). Separation of esters, including methyl linolenate, was complete in less than 30 min.

Major peaks were identified by comparing their retention times with those of known mixtures of fatty acid methyl esters (Cal Biochem, San Diego, Calif.). Relative peak areas were measured as peak ht x the width at half ht. Response correction factors were obtained by taking known mixtures of methyl esters through both the methanolysis and GLC procedures. The compositions of the standard mixtures were made to approximate those of the lipids being analyzed (11). Fatty acid analyses reported are averages of at least three separate determinations.

RESULTS AND DISCUSSION

The amounts of total solids and total lipids of primate and bovine milks are compared in Table I. Milks from the 6 species of monkeys contained 11.5-16.5% total solids and 2.2-8.5% total lipids, with that of *M. mulatta* having the highest values in both instances. The samples from these species were generally similar in composition to milks from other Old World primates (3). Since the monkeys were kept under the same dietary and environmental conditions, the differences in their milks may be attributed to such factors as normal variations within a species or differences in requirements for natural rate of growth among the species. The averaged total solids and total

TABLE I

Total Solids and Total Lipids in Primate and Bovine Milks

Source	Wt %	
	Total solids	Total lipids
Cercocebus atys	12.4	2.2
Macaca fascicularis	11.5	3.4
Macaca mulatta	16.5	8.5
Macaca nemestrina	14.1	4.0
Macaca radiata	13.8	4.9
Macaca speciosa	12.9	2.3
Monkey (average of above species)	13.5	4.2
Man	12.8	2.9
Cow (Holstein)	12.3	3.4

TABLE II

Fatty Acid Composition of Monkey Milk Fats and Ration

Fatty acid	Wt %						
	Cercocebus atys	*Macaca fascicularis*	*Macaca mulatta*	*Macaca nemestrina*	*Macaca radiata*	*Macaca speciosa*	Ration
4:0	0.1	1.2	0.4	0.1	0.1	0.4	
6:0	0.8	0.7	0.5	0.4	0.6	0.5	
8:0	7.6	6.0	4.5	3.8	7.7	5.8	
10:0	10.8	10.8	7.5	10.8	12.0	14.6	0.2
12:0	4.8	4.1	2.5	5.7	2.9	6.4	0.2
14:0	3.2	2.7	2.0	3.5	2.1	3.4	0.7
16:0	19.4	20.8	23.3	22.8	21.7	20.4	20.7
16:1	7.2	5.8	7.3	7.8	6.9	5.3	2.8
18:0	4.8	5.1	5.1	4.8	4.9	4.5	4.1
18:1	26.8	26.2	30.3	25.0	25.1	22.4	25.1
18:2	13.7	15.1	14.7	15.2	14.8	13.6	42.2
18:3	0.9	1.5	1.7	1.1	1.2	1.5	4.0

TABLE III

Fatty Acid Composition of Some Primate and Bovine Milks

Fatty acid	Wt %				
	Monkey (mean)	Man	Squirrel monkey[a]	Baboon[b]	Cow[c]
4:0	0.4	0.4			3.5
6:0	0.6	0.3	0.4	0.2	2.2
8:0	5.9	0.5	4.3	3.7	1.3
10:0	11.0	1.6	7.9	7.3	2.7
12:0	4.4	7.5	5.7	3.0	3.1
14:0	2.8	6.4	4.6	1.4	10.3
16:0	21.4	20.5	20.0	16.1	28.2
16:1	6.7	4.1	2.4	0.9	3.0
18:0	4.9	5.6	3.3	3.6	10.7
18:1	26.0	39.9	29.3	23.5	25.1
18:2	14.5	11.7	20.6	38.6	3.6
18:3	1.3	1.5	1.3	1.5	2.2

[a]Buss and Cooper (5).
[b]Buss (7).
[c]Smith (11).

lipids in the monkey milks were little different from those in human and bovine milks, considering normal variations expected for these species (12).

Fatty acid composition of the various monkey milk fats and of the standard feed is shown in Table II. There was little variation in the amounts of most of the individual milk fatty acids among the several species of monkeys. The predominant fatty acids were capric (7.5-14.6%), palmitic (19.4-23.3%), oleic (22.4-30.3%), and linoleic (13.6-15.2%). Small amounts of the short chain acids butyric (0.1-1.2%) and caproic (0.4-0.8%) were present. The ration contained 2.4% total lipids, and its fatty acid composition differed from that of the milk fats in having larger amounts of polyunsaturated acids and smaller amounts of myristic and short chain acids. Therefore, the composition of the milk fats at time of weaning only partially reflected the diet of the monkeys.

The fatty acid compositions of milk fats from some primates and the cow are compared in Table III. The average values for the 18 monkeys in this study were reasonably similar to those from the milk of a rhesus and 2 green monkeys (6), except that butyric and caproic acids were present in all our samples. The data were also similar to those reported for squirrel monkey (New World) milk (5) and for baboon milk (7), except for the presence of butyric acid and a lower level of linoleic acid. Milk from these nonhuman primates was higher in caprylic and capric acids, but lower in myristic acid, compared with human milk. The identification of butyric acid in our samples probably reflects the improvement in our analytical techniques. However, differences in the amounts of the other fatty acids in the milks of the monkeys, baboon, man, and cow probably can be attributed to a number of factors, including variations in mammary synthesis, in amount of dietary fatty acids, and in stage of lactation. As discussed by Glass, et al., (6) the actual composition of the fatty acids of milk fats depends upon the relative importance both of dietary acids and those synthesized by mammary gland activity. Results of the present study are consistent with Shorland's thesis (13) that broad phylogenetic differences do occur in the composition of homologous fats.

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Stereospecific Analysis of Triacylglycerols and Major Phosphoglycerides from *Lipomyces Lipoferus*[1]

JAMES E. HALEY and **R. CECIL JACK**, Department of Biology,
St. John's University, Jamaica, New York 11439

ABSTRACT

The stereospecific distribution of fatty acids in triacylglycerols, phosphatidylcholines and phosphatidylethanolamines of *Lipomyces lipoferus* was determined. Position sn-1 of the triacylglycerols had a predominance of unsaturated fatty acids of which $C_{18:1}$ (61%) was the major component. Position sn-2 of the triacylglycerols contained 88% $C_{18:1}$ and was more unsaturated than position sn-1 by 24.6%. Position sn-3 had equal proportions of saturated and unsaturated fatty acids. Phosphatidylcholine had a quantitatively distinctive fatty acid distribution in that position sn-2 was 26.7% more unsaturated than position sn-1. In phosphatidylethanolamine, position sn-2 was 10.8% more unsaturated than position sn-1. Positions sn-1 and sn-2 of these phosphoglycerides had a different fatty acid profile from positions sn-1 and sn-2 of the triacylglycerols. These results suggest a nonrandom distribution of fatty acids in the triacylglycerols and phosphoglycerides. Because triacylglycerols and phosphoglycerides are both derived from 1,2-diacylglycerols, these data suggest two possibilities: some selectivity in utilization of species of diacylglycerols to form triacylglycerols and phosphoglycerides or modification of the triacylglycerols and phosphoglycerides after they are formed or both. This study is the first of its kind in a yeast.

INTRODUCTION

The objective of this study was the stereospecific analysis of the triacylglycerols (TG) and major phosphoglycerides (PG) of the yeast *Lipomyces lipoferus*. Previously, the total lipids of *L. lipoferus* were characterized by Jack (1) and McElroy and Stewart (2); however, information is not yet available on the stereospecific distribution of fatty acids in the glycerolipids of *L. lipoferus* or any other yeast.

L. lipoferus was chosen for this study because it has some unique advantages for stereospecific analyses of glycerolipids. For example, it produces large quantities of TG and PG (1,2), and it can be grown reproducibly under a variety of experimental conditions which influence lipid composition. Moreover, since the organism is grown on a chemically defined medium, the glycerolipids found are those formed from de novo biosynthesis. Thus, the role of dietary lipids, which presents difficulties in the study of glyceride formation in animals, is eliminated by the use of this microorganism. Because its growth conditions can be varied systematically and widely, the use of *L. lipoferus* also can provide a variety of useful experimental systems for investigating the stereospecific distribution of fatty acids among glycerolipids. Such investigations may be expected to yield data pertaining to one of the major unsolved problems of lipid biochemistry: understanding the specificity of fatty acid acylating enzymes.

MATERIALS AND METHODS

Growth of Organism and Isolation of Lipids

Starter cultures of *Lipomyces lipoferus* (American Type Culture Collection 10742) were grown for 9 days in 250 ml Erlenmeyer flasks in the chemically defined nutrient medium previously described (3). Then, 25 ml portions of the starter cultures, with optical densities of 1.5-1.8 at 540 nm, were transferred aseptically to 2 liter Erlenmeyer flasks containing 1 liter of the chemically defined nutrient medium. The 2 liter flasks were incubated with shaking at 150 rpm and 22 C in a Psycrotherm controlled environment incubator shaker (New Brunswick Scientific Co., New Brunswick, N.J.) for 10 days (the later logarithmic phase of growth).

The cells were separated from the medium by centrifugation at 7,000 x g in an International refrigerated centrifuge (model B-20) and were extracted once with chloroform:methanol (2:1, v:v) and twice with chloroform:methanol (1:1, v:v) for a total of 8-9 hr at room temperature with vigorous shaking on a model V shaker (New Brunswick Scientific Co.). The solvents used for extraction were freshly distilled and contained 0.02% (w/v) butylated hydroxytoluene to prevent autooxidation of the isolated lipids.

[1]Presented in part at the AOCS Fall Meeting, Ottawa, Canada, September 1972.

Structural Analysis of Triacylglycerols

The total lipids, ca. 36 ± 7 mg lipids/g cells (wet wt) were separated into classes by silicic acid column chromatography (4) and the TG fraction was checked for purity on Silica Gel H with hexane:diethyl ether:acetic acid (80:20:1, v:v:v) as the solvent system.

The TG were subjected to enzymatic hydrolysis with pancreatic lipase (5,6) and to Grignard lipolysis (7). The sn-2 monoacylglycerols and the 1,2- and 1,3-diacylglycerols (DG) in the reaction mixtures were isolated by thin layer chromatography (TLC) (8); phosphatidylphenols then were prepared from the DG as described by Brockerhoff (6). The synthetic phospholipids were hydrolyzed to lysophosphatidylphenols and free fatty acids with phospholipase A_2, E C 3·1·1·4, and the hydrolysis products were separated and isolated by TLC (8). The stereospecific distribution of fatty acids in the TG was determined from analyses of fatty acids of the appropriate products of the hydrolytic reactions (6,7).

Structural Analysis of PG

The PG, ca. 19.8 ± 2.4% of the total lipids, were obtained by silicic acid column chromatography and were separated into classes by preparative TLC on Silica Gel H (9).

The major PG, namely phosphatidylcholine (PC) and phosphatidylethanolamine (PE), together representing 87% of the PG, were subjected to hydrolysis with phospholipase A_2. The products of the reaction were separated by TLC on Silica Gel H with hexane:diethyl ether (1:1, v:v) as the solvent. Analysis by GLC, of fatty acid methyl esters prepared from the lysophosphatides, gave the fatty acid composition of position sn-1; analysis of methyl esters of the liberated fatty acids gave the fatty acid profile of position sn-2.

Gas Liquid Chromatography (GLC)

When required, the isolated lipids were hydrolyzed with 0.5N methanolic KOH, acidified with 5N sulfuric acid, and the free fatty acids were methylated with boron trifluoride methanol (10). GLC of the fatty acid methyl esters was performed with a Hewlett Packard (model 5750) research chromatograph, as described previously (11). It was determined by the use of a quantitative standard (K102, Applied Science, Inc., State College, Pa.) that the response of the hydrogen flame ionization detector was proportional to the concentrations of fatty acid methyl esters of 14:0, 16:0, 18:0, and 18:1. The relative areas of the peaks were converted to moles percent as described by Brockerhoff (12).

RESULTS AND DISCUSSION

Table I shows the distribution of fatty acids among the three positions of the TG. Each position had a quantitatively unique fatty acid composition, indicating a nonrandom distribution of the fatty acids. At position sn-1 the content of unsaturated fatty acids was greater than the content of saturated fatty acids by ca. 4 to 1, the predominant fatty acid at position sn-1 being C18:1. Position sn-2 had a greater content of unsaturated fatty acids than positions sn-1 or sn-3; the proportion of unsaturated fatty acids to saturated fatty acids was almost 82 to 1 at position sn-2 with C18:1 being the predominant fatty acid. Position sn-3 showed the greatest content of saturated fatty acids, the content of the latter being equal to the content of unsaturated acids at this position. Chi square analysis yielded a probability of greater than 90% that the overall stereospecific analysis of the TG was accurate. In addition, it was established during the stereospecific analysis that the 1,2 (2,3)-DG from the

TABLE I

Structural Analysis of Triacylglycerols[a]

Fatty acid	sn-1	sn-2	sn-3	Total[b] sn-1,2,3	Triacylglycerol original
14:0	2.7	---	6.3	3.0	1.9
16:0	13.9	1.2	29.3	14.8	15.8
16:1	8.0	1.9	12.8	7.6	7.1
18:0	4.1	---	8.7	4.3	2.7
18:1	61.0	87.7	36.7	61.8	62.0
18:2	9.7	8.8	5.8	8.1	9.2
18:3	0.6	0.4	---	0.3	1.1
20:0	---	---	0.4	0.1	0.2

[a]Mole %.

[b]Total of a fatty acid at sn-1,2,3 divided by 3.

TABLE II

Stereospecific Analysis of Major Phosphoglycerides[a]

PC	Position	14:0	16:0	16:1	18:0	18:1	18:2	18:3
	sn-1	0.8	24.3	17.8	0.6	36.5	15.6	4.4
	sn-2	1.9	3.5	5.4	0.5	38.6	31.0	19.1
	Total/2	1.3	13.9	11.6	0.5	37.6	23.3	11.8
	Original	—	12.4	12.8	—	39.4	24.3	11.1
PE	sn-1	2.8	28.7	17.9	3.6	27.8	13.2	6.0
	sn-2	1.7	23.1	14.8	3.3	33.5	17.4	6.2
	Total/2	2.2	25.9	16.3	3.4	30.6	15.3	6.1
	Original	1.7	26.5	15.9	3.3	31.5	15.0	6.1

[a]Fatty acids (mole, %). PC = phosphatidylcholine and PE = phosphatidylethanolamine.

pancreatic lipolysis and the 1,3-DG formed by the Grignard deacylation were representative within rigorously defined limits (13,14).

The stereospecific analyses of PC and PE, together representing 87% of the phospholipids, are shown in Table II. In both classes of phospholipids, positions sn-1 and sn-2 had quantitatively unique distributions of fatty acids suggesting a preference of certain fatty acids for one of the two positions. In PC, position sn-2 was 27% more unsaturated than position sn-1 especially in the predominance of C18 unsaturates. Position sn-2 of PE was 11% more unsaturated than sn-1, this being due, again, to higher proportions of C18 unsaturates at sn-2. Chi square analysis showed a probability of greater than 90% that the data for phosphatidylcholine were accurate; the comparable value for PE was a probability of greater than 99%. The distribution of fatty acids in PC and PE, besides being different from each other, was also different from the distribution of fatty acids at positions sn-1 and sn-2 of the TG; the unsaturated acids were 24.6% higher at position sn-2 than at sn-1 in the TG (Table I).

Because TG and PE are both derived from 1,2-DG, these data suggest two possibilities for explaining the stereospecific patterns of Tables I and II: some selectivity in the utilization of species of DG to form TG and phospholipids or enzyme catalyzed modification of the major PG and TG after they are formed or both.

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Structure and Composition of Aliphatic Constituents of Potato Tuber Skin (Suberin)[1]

P.E. KOLATTUKUDY[2] and V.P. AGRAWAL[3], Department of Agricultural Chemistry and The Graduate Program in Biochemistry and Biophysics, Washington State University, Pullman, Washington 99163

ABSTRACT

Potato tuber skin (suberin), isolated enzymatically, was depolymerized with BF_3-CH_3OH, and the structure and composition of the aliphatic monomers were determined by combined gas chromatography-mass spectrometry. 18-Hydroxyoctadec-9-enoic acid and octadec-9-ene-1,18-dioic acid were the major components. Products of epoxidation and subsequent hydration of the Δ^9 double bond of these compounds, 10,16-dihydroxy hexadecanoic acid, and much smaller quantities of 9,16-dihydroxyhexadecanoic acid and 8,16-dihydroxyhexadecanoic acid also were present. The other significant feature of the monomer composition of potato skin was that it contained substantial quantities of C_{20}-C_{28} fatty acids, fatty alcohols, and ω-hydroxy acids. Based upon these studies, a method of distinguishing between suberin and cutin and a biosynthetic pathway for suberin monomers are suggested.

INTRODUCTION

Living organisms are packaged in an envelope made of a polymeric material. Protein and chitin perform this function in animals while the aerial parts of plants are covered by a hydroxy fatty acid polymer called cutin (1,2). Little is known about the polymeric material which protects the underground parts of plants, even though such materials play an important role in the life of the plant. Furthermore, the covering on tubers, such as potatoes, often referred to as suberin, is widely recognized to be important in the prevention of wt loss (3) and decay (4). However, modern analytical techniques have not been applied to the determination of the structure of this material, except for a recent report on the identification of 18-hydroxyoctadec-9-enoic acid and octadec-9-ene-1,18-dioic acid in potato cork (5). In this article, we report the results of an analysis of the aliphatic monomers of potato skin by means of hydrogenolysis, deuterolysis, and methanolysis in conjunction with combined gas liquid chromatography (GLC) and mass spectrometry (MS).

EXPERIMENTAL PROCEDURES

Materials: Mature potato tubers (Russet Burbank) were harvested from the Othello Experimental Farm, Washington State University, and stored at 38 F. The sources of $LiAlH_4$, $LiAlD_4$ (99 atom %D), bis-N,O-trimethyl silyl acetamide (BSA), pectinase (fungal), and cellulase (from *Aspergillus niger*) were those indicated before (6).

Preparation of potato skin: Whole potato tubers were boiled in water, and the skin was removed by hand. After washing the skin pieces thoroughly with water, they were treated overnight with a solution containing 5 g cellulase and 1 g fungal pectinase/liter of 0.05 M acetate buffer, pH 4.0. The skin slices were washed thoroughly with water and then extracted thoroughly with a 2:1 mixture of chloroform and methanol. The resulting solid was extracted with chloroform overnight in a Soxhlet extractor. The enzyme treatment and extraction procedures were repeated. The resulting material was finely ground in a Wiley mill.

Depolymerization: The skin powder (1 g) was refluxed with 2 g $LiAlH_4$ or $LiAlD_4$ (99.9%) in tetrahydrofuran or with 14% BF_3 in methanol for 48 hr. Excess reagents were decomposed with water; the soluble lipids generated by these treatments were extracted with chloroform; and the solvent was evaporated under reduced pressure.

Chromatography: All thin layer chromatographic (TLC) analyses were done on 0.5 mm or 1 mm layers of Silica Gel G activated overnight. Components were visualized either by charring with dichromate-sulphuric acid or by spraying with a 0.1% ethanolic solution of dichlorofluorescein. The TLC fractions were recovered from silica gel either with a 2:1 mixture of chloroform and methanol or methanol, depending upon the polarity of the fraction.

GLC was done with a glass column (183 x 0.31 cm outside diameter) packed with 5% OV-101 on 80-100 mesh Gas Chrom Q. The

[1]Scientific paper 4199, Project 2001, Agricultural Research Center, College of Agriculture, Washington State University, Pullman, Washington.

[2]The author to whom inquiries should be made.

[3]Fulbright-Hays Fellow on leave from Tribhuvan University, Kathmandu, Nepal.

effluent of the gas chromatograph was passed into a Perkin-Elmer Hitachi RMU 6D mass spectrometer with a Biemann separator as the interphase. Spectra were recorded with 70 eV ionizing voltage at the apex of each peak. They also were recorded at either side of the apex to ensure that each peak did not consist of incompletely resolved mixture of compounds.

Chemical conversions: To locate the double bond, ca. 1 mg component was treated with a 0.1% solution of OsO_4 in dioxane for 1 hr. The reaction mixture was decomposed with aqueous-methanolic Na_2SO_3, and the resulting precipitate was removed by centrifugation, washed once with methanol, and centrifuged. Products were recovered by ether extraction of combined supernatants. Trimethylsilyl ethers were prepared by heating the component with an excess (0.25 ml) of N,O-bis (trimethylsilyl) acetamide. Excess reagent was evaporated with a stream of N_2, and the products were dissolved in a 2:1 mixture of chloroform and methanol for GLC.

RESULTS AND DISCUSSION

Hydrogenolysis ($LiAlH_4$) of the insoluble material obtained from potato tuber skin released ca. 22% of the total wt as chloroform-soluble material. TLC of this soluble material showed one major component and three minor components. The major component had an R_f identical to that of hexadecane-1,16-diol, while the R_f values of the minor components indicated that they were fatty alcohols, C_{16}-triol and C_{18}-triol. Combined GLC-MS of the major TLC fraction (as trimethylsilyl ethers) revealed a major (>90%) component and several minor components. The mass spectrum of the major component had a molecular ion at m/e 428 and significant ions at 413 (M-15), 397 (M-31), 338 (M-90), and at 323 (M-15-90) suggesting that this component was octadecene-1,18-diol. Confirming this identification, a doubly charged ion and its first isotope ion were found at 199 [(M-30)/2] and 199.5, respectively (7,8). The mass spectrum of a similar component obtained from deuterolysis ($LiAlD_4$) showed that dideuterated and tetradeuterated diols were contained in it. For example, doubly charged ions were observed at m/e 200 and at 201. Therefore, the diols must have originated from dicarboxylic acids and ω-hydroxy acids. Since such distinctions are lost by the hydrogenolysis technique, we resorted to a transesterification technique for depolymerization. This method consisted of treating finely powdered polymer with 14% BF_3 in methanol for 24-48 hr. By this method also, 22-25% of the material was recovered as chloroform-soluble lipids. TLC of this soluble fraction gave a pattern shown in Figure 1. Comparison of this distribution pattern with that obtained with the hydrogenolysis technique showed that the transesterification technique provided a suitable depolymerization method for determining the structures of the monomers.

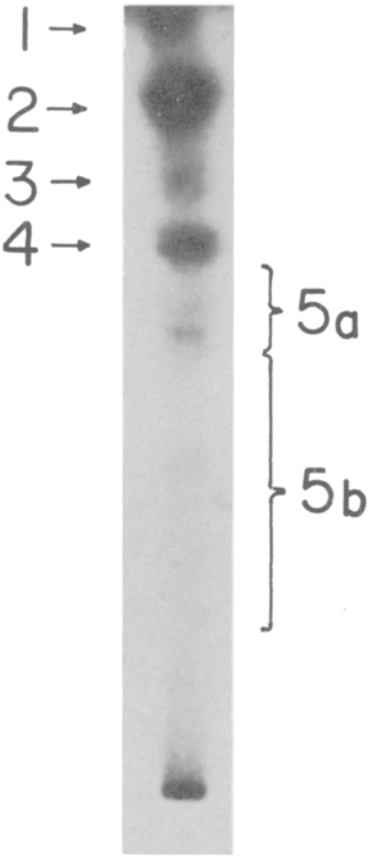

FIG. 1. Thin layer chromatogram of the soluble lipids generated by BF_3-CH_3OH treatment of potato tuber skin. Chromatography on 0.5 mm layer of Silica Gel G with ethyl ether:hexane:methanol (20:5:1 v/v) as solvent system. To resolve the major components more conveniently, hexane:ethyl ether:formic acid (65:35:2 v/v) was used as a solvent system. Under these conditions the R_f values for fatty acid methyl ester, dicarboxylic acid dimethyl ester, primary alcohol, and ω-hydroxy acid methyl ester were 0.6, 0.38, 0.26, and 0.16, respectively. However, the more polar components were not resolved from the origin.

Fraction 1 showed an R_f identical to that of methyl palmitate indicating that it was fatty acid methyl ester fraction. GLC of this fraction revealed that it contained C_{14}-C_{28} fatty acids. These structural assignments, based upon comparison of retention times with those of authentic standards, were confirmed by an examina-

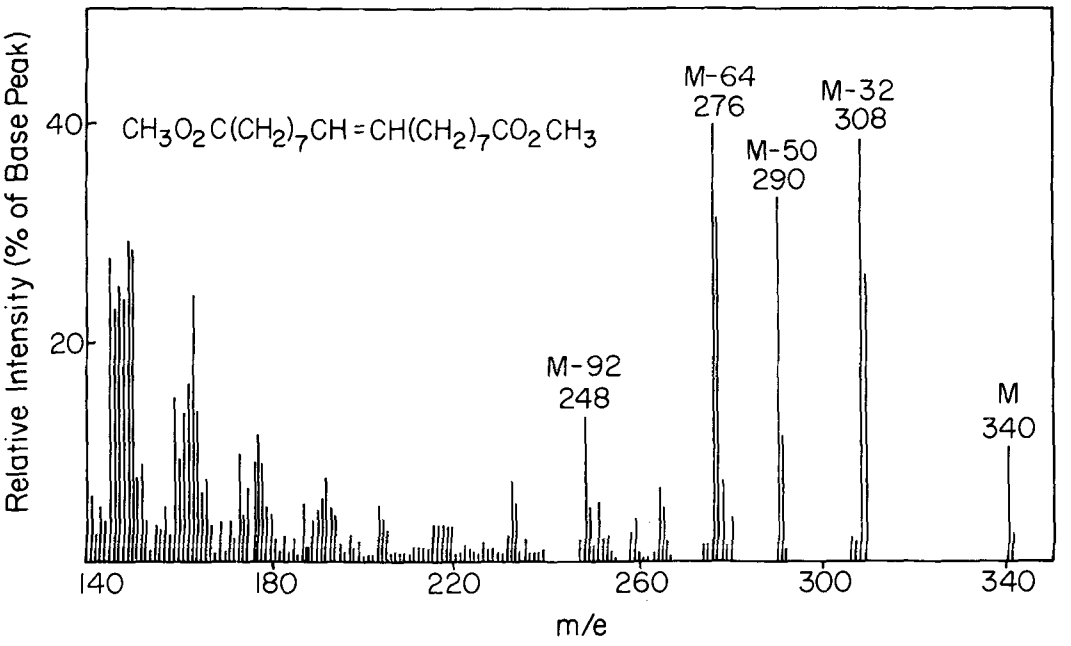

FIG. 2. Mass spectrum of the major component in the gas liquid chromatogram of fraction 2 (dicarboxylic acid dimethyl esters) of Figure 1.

tion of the mass spectrum of each component. The unusual aspect of this fatty acid pattern is that it contained a substantial proportion of very long chain fatty acids.

Fraction 2, which was a major component, had an R_f identical to that of dimethyl hexadecane-1,16-dioate, indicating that this fraction contained dicarboxylic acid dimethyl esters. GLC of this fraction showed that it contained one major component and one minor component. The retention time of the minor component was identical to that of dimethyl hexadecane-1,16-dioate. The mass spectrum of this component showed an extremely weak molecular ion at m/e 314 and a relatively intense ion at 283 (M-31). The other significant ions at m/e 241 (M-73), 222 (M-92), 209 (M-105), and 191 (M-123), as well as those in the lower mass region were identical to those observed in the mass spectrum of authentic dimethyl hexadecane-1,16-dioate.

The mass spectrum of the major component showed a molecular ion at m/e 340. The major ions in the high mass region were at m/e 308 (M-32), 290 (M-50), 276 (M-64), and at 248 (M-92) (Fig. 2). This fragmentation pattern showed that the major component was octadecene-1,18-dioate (9). This identification was confirmed by the fact that the diol obtained by $LiAlH_4$ treatment of this component (as trimethylsilyl ether) gave a mass spectrum identical to that previously obtained for octadecene-1,18-diol (8).

To locate the double bond, the dicarboxylic acid dimethyl ester fraction was treated with OsO_4, and the resulting product was subjected to gas chromatography as the trimethylsilyl ether. As expected, one major component was observed, and its mass spectrum showed no discernable molecular ion. However, significant ions were observed at m/e 455 (M-15), 429 (M-31), 413 (M-47), 370 (M-90), and at 355 (M-90-15). These ions indicate that the compound was a di(trimethylsiloxy)octadecane-1,18-dioic acid dimethyl ester. The base peak of the spectrum was at m/e 259, showing that the two trimethylsilyloxy functions were at C-9 and C-10 positions. The expected trimethylsilyl migration to the carbonyl oxygen (10) also was observed at m/e 332. Thus, the major component of the dicarboxylic acid fraction was shown to be octadec-9-ene-1,18-dioic acid.

Fraction 3 had an R_f identical to that of octadecanol, suggesting that this was a fatty alcohol fraction. Gas chromatography of this fraction, as the trimethylsilyl ethers, showed that it contained C_{16}-C_{26} fatty alcohols. These identifications, based upon comparison of retention times with those of authentic standards, also were confirmed by an examination of the mass spectrum of each component.

Fraction 4 had an R_f value identical to that of methyl 16-hydroxyhexadecanoate, indi-

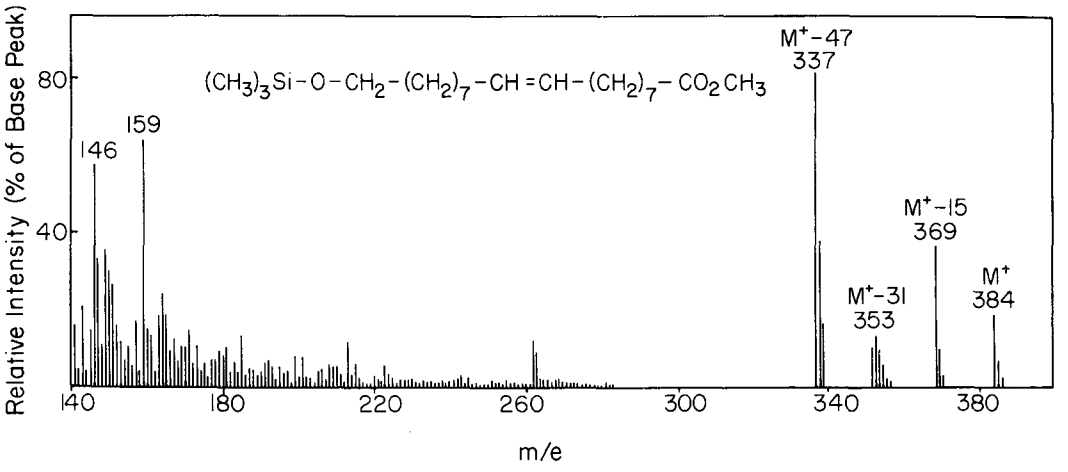

FIG. 3. Mass spectrum of the major component in the gas liquid chromatogram of fraction 4 (ω-hydroxy acid methyl esters) of Figure 1.

cating that this fraction contained methyl esters of ω-hydroxy fatty acids. Gas chromatography of this fraction as trimethylsilyl ethers showed one major component and several minor components. The mass spectrum of the major component showed a molecular ion at m/e 384 (Fig. 3). Moderately strong ions were observed at m/e 369 (M-CH_3), 353 (M-CH_3O), and at 337 (M-CH_3-CH_3OH). The metastable ion representing the transition (M-15) → (M-47) also was observed. This pattern, and the fragmentation in the lower mass range, showed that the major component was methyl 18-hydroxy octadecenoate (10). This identification was confirmed by the fact that the mass spectrum of the trimethylsilyl ether of the $LiAlH_4$ reduction product was identical to the previously observed spectrum of octadecene-1,18-diol (8).

To determine the position of the double bond, a vic-diol function was introduced. The mass spectrum of the product (as trimethylsilyl ethers) showed an extremely weak molecular ion at m/e 562. Significant ions were detected at m/e 547 (M-CH_3) and 531 (M-CH_3O). Two intense α-cleavage ions were observed at m/e 259 and 303. These two ions obviously were derived by cleavage between the two trimethylsiloxy functions, and, therefore, the two hydroxyl groups were at C-9 and C-10 positions. The expected trimethylsilyl migration ion was observed at m/e 332. Thus, the structure of the major ω-hydroxy acid was shown to be 18-hydroxyoctadec-9-enoic acid (10).

The relatively minor components contained in fraction 4 were identified as C_{16}, C_{20}, C_{22}, C_{24}, and C_{26} ω-hydroxy acid methyl esters from their mass spectra (the relatively small

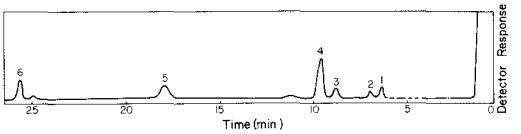

FIG. 4. Gas liquid chromatogram of fraction 5a (as trimethylsilyl ether) of Figure 1. Experimental conditions are described under "Experimental Procedures". Temperature of the column was 240 C, and inlet pressure of carrier gas He was 23 psi. The broken base line indicates locations of peaks due to ω-hydroxy acid contamination from fraction 4.

molecular ion, M-15, M-31, and M-47 together with the metastable ion representing the transition [M-15] → [M-47]).

Components which were more polar than the ω-hydroxy acid methyl esters were relatively minor components, and, consequently, a cluster of weak bands were observed below fraction 4 (Fig. 1). They were, for convenience, split into fractions 5a and 5b, the former being the less polar of the two. GLC of fraction 5a showed that it, invariably, contained some contamination from fraction 4, and the ω-hydroxy acids thus contained in this fraction are omitted from the gas chromatogram shown in Figure 4 (broken base line). Component 1 was not identified, because its mass spectrum did not lend itself to an unambiguous structural assignment. Component 2 gave a mass spectrum shown in Figure 5. The major ions at the high mass region were a molecular ion at m/e 416 and fragment ions at m/e 401 (M-15), 384 (M-32), 369 (M-47), and 337 (M-47-32). A metastable ion representing the transition (M-15) → (M-47) also was observed. These ions are expected from a methoxylated methyl 18-hydroxyoctadecanoate. A set of four moder-

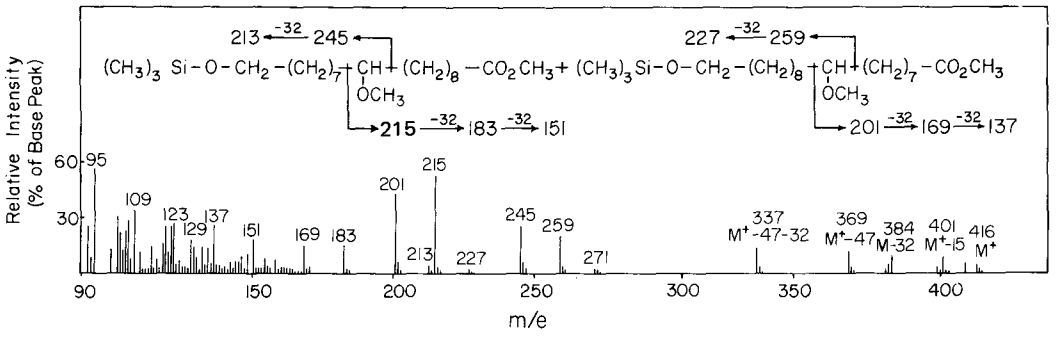

FIG. 5. Mass spectrum of peak 2 of Figure 4.

ately strong α-cleavage ions was observed at m/e 201, 215, 245, and 259. These ions indicate that the component was a mixture of 9-methoxy and 10-methoxy isomers (Fig. 5). In accordance with this structural assignment, these α-cleavage ions were not as strong as those expected to be produced from in-chain hydroxylated compounds. Furthermore, the ion clusters assigned to the fragments derived from the hydroxyl end of the molecule (m/e 245 and 259) showed the expected silicon isotope composition, while those derived from the carboxyl end (m/e 215 and 201) did not show such a pattern. Furthermore, sequential methanol elimination from the α-cleavage ions which were derived from thycarboxyl ends was indicated by the ions at m/e 183 (215-32), 151 (183-32), 169 (201-32), and 137 (169-32). Similar methanol elimination from the α-cleavage ions derived from the hydroxyl end occurred only to a limited extent giving rise to very weak ions at m/e 213 (245-32) and 227 (259-32). As a result of the above interpretation, component 2 was identified as methyl 18-hydroxy-9 or 10-methoxy octadecanoate. It is possible that this compound was derived by a BF_3 catalyzed methanol addition across the double bond of the 18-hydroxy octadec-9-enoate. The fact that equal amounts of 9-methoxy and 10-methoxy derivatives were observed supports this possibility. However, a similar derivative derived from (since then BF_3 catalyzed CH_3OH addition across the Δ^9 of octadec-9-ene-1,18-dioic acid has been detected) octadec-9-ene-1,18-dioic acid was not detected. Component 3 was not readily identifiable from its mass spectrum.

The mass spectrum of component 4 (Fig. 6) had only an extremely weak molecular ion at m/e 470 and a derivative ion at 455 (M-15). However, significant ions were detected at m/e 429 (M-31), 413 (M-47), 397 (M-73), 370 (M-90), and 355 (M-90-15). This pattern indicates that this component was dimethyl methoxy (trimethylsiloxy)octadecane-1,18-dioate. As expected from such a structure, an intense α-cleavage ion was observed at m/e 259 which obviously was derived by cleavage between the carbon atoms carrying the methoxy group and trimethylsiloxy group, with a preferential retention of charge by the fragment carrying the trimethylsiloxy function. The fragment from the other side of the molecule also was detected at m/e 201. Similarly, the α-cleavage ion carry-

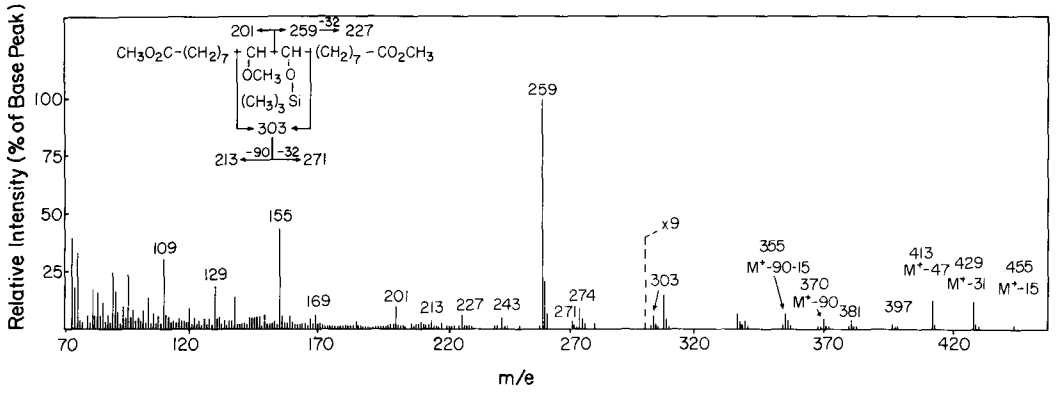

FIG. 6. Mass spectrum of peak 4 of Figure 4.

ing both the methoxy function and trimethylsiloxy function also was detected at m/e 303. Elimination of trimethylsilanol and methanol from this ion was indicated by the relatively weak ions at m/e 213 and 271, respectively. Similarly, methanol elimination from the two α-cleavage ions (m/e 259 and 201) were indicated by the presence of measurable ions at m/e 227 and 169, respectively. Thus, component 4 was identified as the product of methanolysis of the oxirane ring of dimethyl 9,10-epoxy octadecane-1,18-dioate.

Component 5 and component 6 showed very weak molecular ions at 486 and 514, respectively. In both spectra, significant ions corresponding to M-15, M-31, M-90, M-15-90, and M-31-90 were present. Furthermore, the spectrum of component 5 had a doubly charged ion at m/e 228 ([M-30]/2) followed by the first isotope at 228.5, while the spectrum of component 6 showed similar ions at 242 and 242.5. Thus, components 5 and 6 were identified as docosane-1,22-diol and tetracosane-1,24-diol, respectively.

GLC of fraction 5b (as trimethylsilyl ether) showed three major components (Fig. 7). Component 1 had a retention time identical to that

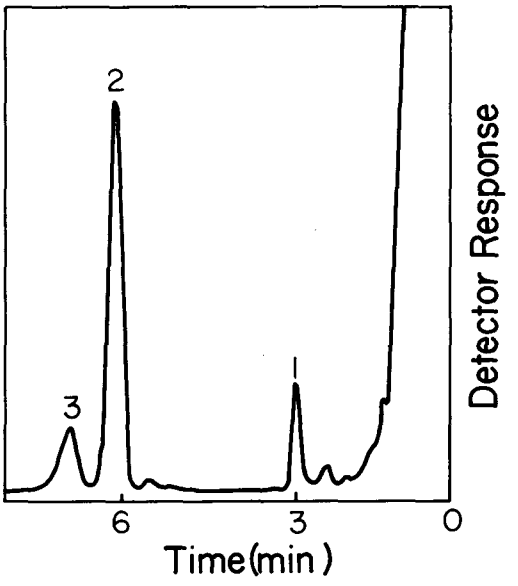

FIG. 7. Gas liquid chromatogram of fraction 5b (as trimethylsilyl ethers) of Figure 1. Column temperature was 240 C, and inlet pressure of the carrier gas He was 23 psi.

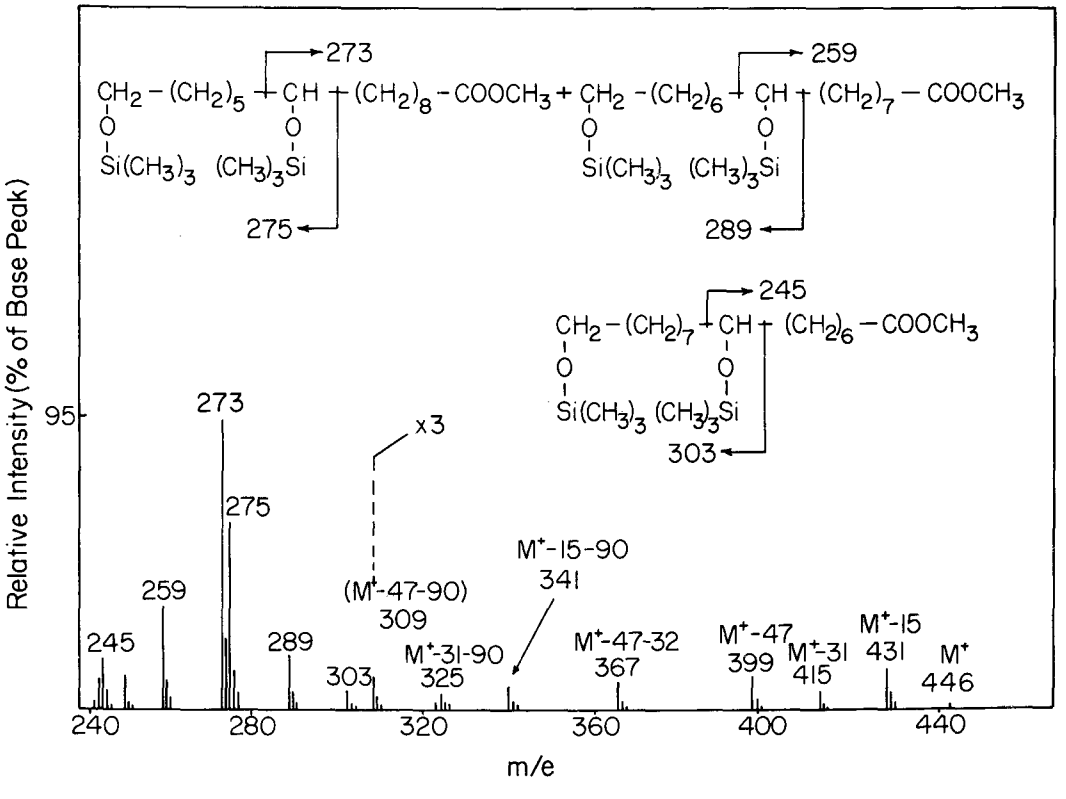

FIG. 8. Mass spectrum of peak 1 of Figure 7.

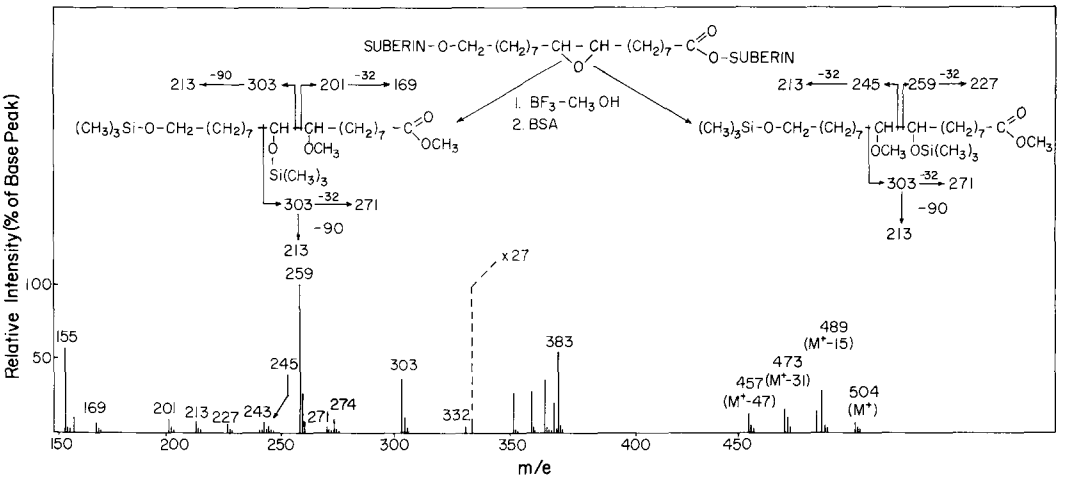

FIG. 9. Mass spectrum of peak 2 of Figure 7.

of methyl dihydroxy hexadecanoate. The mass spectrum of this component (Fig. 8) showed an extremely weak molecular ion at m/e 446 and significant ions at m/e 431 (M-15), 415 (M-31), and 399 (M-47). These are the ions expected from the trimethylsilyl derivative of methyl dihydroxy hexadecanoate (10). The presence of a fairly strong ion at m/e 103 ($CH_2OSi[CH_3]_3$) showed that one of the hydroxyl groups was at the ω-carbon atom. The other hydroxyl group was shown to be at the C-10 position, because a pair of intense ions was observed at m/e 273 and 275, obviously derived by α-cleavage on either side of the in chain trimethylsiloxy function. As expected, the ion derived from the ester end of the molecule (m/e 273) was significantly more intense than that derived from the other end (m/e 275). The presence of smaller quantities of the 9-hydroxy isomer and even smaller amounts of the 8-hydroxy isomer was indicated by the ion pairs at m/e 259, 289, and m/e 245, 303, respectively. Thus, component 1 was identified to be methyl 10,16-dihydroxy (67.3%), 9,16-dihydroxy (22.7%), and 8,16-dihydroxy (10%) hexadecanoate.

Component 2 was the major one of fraction 5b and its mass spectrum had a molecular ion at m/e 504 (Fig. 9). The other significant ions at the high mass region were at m/e 489 (M-15), 473 (M-31), 457 (M-47), and 383 (M-90-31). This fragmentation pattern is consistent with methyl dihydroxy methoxy octadecanoate. A fairly intense ion at m/e 103 placed one of the hydroxyl groups at the ω-carbon. The other two functional groups were placed in the middle of the molecule by the pair of α-cleavage ions at m/e 259 and 303. Since the methoxy and hydroxyl functions most probably originated by methanolysis of the corresponding epoxide, C-9 and C-10 have an equal probability of carrying the hydroxyl function. Therefore, a pair of positional isomers, methyl 9-methoxy-10,18-dihydroxy octadecanoate and methyl 10-methoxy-9,18-dihydroxy octadecanoate, would be produced. Cleavage between the two functional groups in the middle of the two chains should, thus, produce two pairs of ions at m/e 303, 201, 245, and 259. The mass spectrum of component 2 of fraction 5b showed all of these ions. As expected, the ion carrying methyl ester and trimethylsiloxy functions (m/e 259) was more intense than that containing two trimethylsiloxy functions (m/e 303). The other two α-cleavage ions containing trimethylsiloxy, methoxy, and methyl ester functions also would be at m/e 303 (Fig. 9). Loss of methanol and trimethylsilanol from this ion was indicated by the ions at m/e 271 and 213, respectively. Similarly, loss of methanol from the ions at 259 and 201 gave rise to ions at m/e 227 and 169, respectively. The expected trimethylsilyl migration to the carbonyl oxygen of the methyl ester function also was observed at m/e 274 (11). Thus, component 3 was identified to be the product of methanolysis of the oxirane ring of 18-hydroxy-9,10-epoxy octadecanoate of suberin.

The presence of the epoxy acid also was confirmed by deuterolysis ($LiAlD_4$) of the insoluble material followed by combined GLC-MS. Deuterolysis of the epoxy acid would be expected to give rise to the isomeric C_{18}-triols shown in Figure 10. The ions at the high mass region of the mass spectrum of the C_{18}-triol obtained by this procedure showed that trideuterated and pentadeuterated species

were present. The major α-cleavage ions were at m/e 303, 305, 318, and 320 while the corresponding ions in the mass spectrum of the hydrogenolysis products were at m/e 303 and 317. This pattern, particularly the unusual even mass ions, confirmed the presence of the 18-hydroxy-9,10-epoxy octadecanoic acid (8). This acid appears to be quite widespread in the protective hydroxy acid polymers in plants (8, 11-13). Furthermore, the predominance of ions at m/e 305 and 320 over those at 303 and 318 showed that this triol originated from 9,10-epoxyoctadecane-1,18-dioic acid present in the polymer. From the relative intensitites of these ions, it was estimated that 43% of the C_{18}-triol originated from 18-hydroxy-9,10-epoxyoctadecanoic acid and 57% from 9,10-epoxyoctadecane-1,18-dioic acid.

Component 3 of fraction 5b gave a fairly simple mass spectrum on the basis of which a fairly conclusive identification could be made. Although discernable molecular ion could not be detected there were significant ions at m/e 547 ($M-CH_3$) and 531 ($M-CH_3O$) (Fig. 11). The only other significant ion in the high mass region was at m/e 457 [$M-CH_3-(CH_3)_3SiOH$]. These ions indicated that component 3 was methyl tri(trimethylsiloxy)octadecanoate. A significant ion at m/e 103 showed that one of the trimethylsiloxy functions was on the ω-carbon. Two fairly intense α-cleavage ions at m/e 259 and 303 showed that the other two trimethylsiloxy functions were at C-9 and C-10. The expected loss of trimethyl silanol from the α-cleavage ion (m/e 361) containing both the trimethylsiloxy groups and the methyl ester function was noted at m/e 271, and a similar elimination from the ion at m/e 303 was observed at m/e 213. Furthermore, the expected trimethylsilyl group migration to the carbonyl oxygen also occurred as shown by the ion at m/e 332. Thus, component 3 of fraction 5b originated from 9,10,18-trihydroxyoctadecanoate (10).

From the results discussed above, the major aliphatic constituents of the potato skin were identified, and the approximate distribution of these compounds are shown in Table I. Fraction 5a and 5b together constituted ca. 10% of the total aliphatic components, and the individual components within these 2 subfractions were not quantitated. From the results discussed in this article, it is apparent that most of the aliphatic constituents of the skin polymer of the potato tuber may be derived from oleic acid. It appears that ω-hydroxylation of the oleic acid is the first step in this process, just as in the case of cutin biosynthesis (6,8). The next step in suberin biosynthesis appears to be

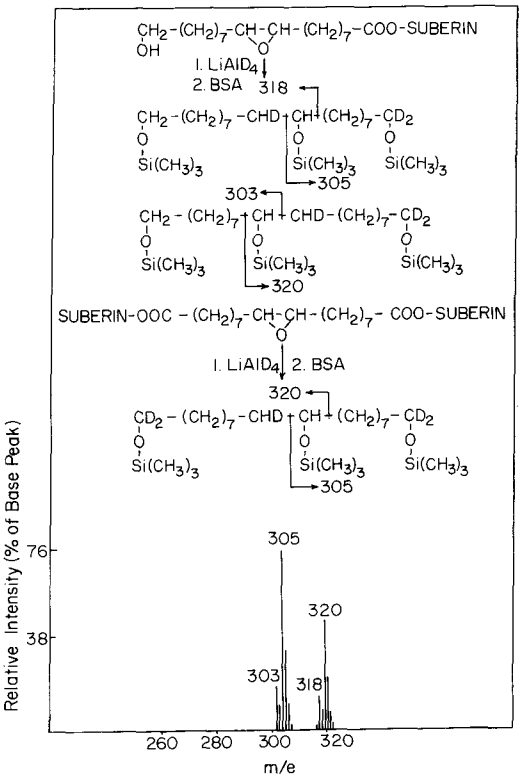

FIG. 10. The α-cleavage reion of the mass spectrum of the trimethylsilyl ether of the C_{18} triol derived from suberin by deuterolysis ($LiAlD_4$).

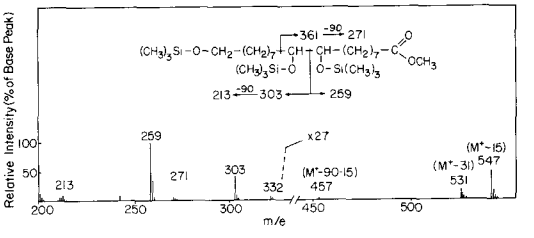

FIG. 11. Mass spectrum of peak 3 of Figure 7.

oxidation of the ω-carbon all the way to a carboxyl group. Recently, we have obtained a cell-free preparation from potatoes which catalyzes this reaction (V.P. Agrawal and P.E. Kolattukudy, unpublished results). Epoxidation of the double bond at C-9 of ω-hydroxyoleic acid followed by hydration, first proposed in 1970 (14) and later demonstrated to be involved in cutin biosynthesis in several plant tissues (15, and R. Croteau and P.E. Kolattukudy, unpublished results), also occurs to a limited extent in suberin biosynthesis. It is quite obvious from the structures identified that either the unsaturated dicarboxylic acid

TABLE I

Chain Length Distribution of Aliphatic Constituents of Potato Tuber Skin (Suberin)[a]

Chain length	Percent of each fraction			
	Fatty acid (8%)	Fatty alcohol (6%)	ω-Hydroxy fatty acid (16%–	Dicarboxylic acid (24%)
16	4.2	3.12	0.59	4.3
18:1	6.95	4.79	95.48	95.6
20	3.47	9.79	---	ND
22	14.20	26.46	1.10	ND
24	26.95	12.91	1.70	ND
26	26.08	18.12	0.81	ND
28	31.73	24.58	0.29	ND

[a]Fatty acids and dicarboxylic acids were analyzed as their methyl and dimethyl esters, respectively. Fatty alcohols were analyzed as their trimethylsilyl ethers and ω-hydroxy acids as trimethylsilyl ethers of their methyl esters. The percentages in parentheses show the approximate quantity of each fraction based upon the total soluble material obtained by BF_3-methanol treatment.

[b]ND = not detected.

TABLE II

Compositional Difference between Cutin and Suberin

Monomer	Cutin	Suberin
Dicarboxylic acids	minor	major
In chain-substituted acids	major	minor
Phenolics	low	high
Very long chain (C_{20}-C_{26}) acids	rare and minor	common and substantial
Very long chain alcohols	rare and minor	common and substantial

can undergo epoxidation of the double bond and subsequent hydration of the epoxide or the ω-hydroxyl group of 18-hydroxy-9,10-epoxy C_{18} acid and 9,10,18-trihydroxy C_{18} acid can undergo oxidation to a carboxyl group. Experimental evidence to distinguish between these two possibilities is not yet available. It is also noteworthy that fatty acid, fatty alcohol, and ω-hydroxy acid fractions contain significant quantities of very long chains (C_{20}-C_{26}), while in the dicarboxylic acid fraction such very long chains could not be detected. This observation suggests that the ω-hydroxy acid dehydrogenase involved in this conversion is specific for C_{18} and shorter carbon chains.

Cutin and suberin were microscopic entities, rather than chemically defined materials, until recently. With the modern analytical techniques, particularly combined GLC-MS, their chemical composition is becoming increasingly clear. The distinctions pointed out below (Table II) are suggested as a means of classifying such hydroxy acid phytopolymers.

The major chemical distinction between cutin and suberin is that the latter contains large quantities of dicarboxylic acids, while they are found only in trace quantities in the former. Oxidation of the ω-carbon of the precursor all the way to a carboxyl group is manifested also in both the 9,10-epoxy aicds and the 9,10-dihydroxy acids. The other major distinction between cutin and suberin appears to be that in-chain substituted compounds constitute the bulk of the monomers of the former, while such compounds are relatively minor components of the latter. Very long chain (C_{20}-C_{26}) fatty acids and fatty alcohols occur rarely and only as very minor components in cutin, while they appear to be common, significant components of suberin (16,17). The high phenolic content of suberin also distinguishes it from cutin. Such distinctions in the monomer composition are bound to be reflected in the overall structure of the polymers, although very little is presently known about these polymers.

It is obviously possible that the composition of some hydroxy acid polymers of plants might fall between suberin and cutin. Therefore, classification of such polymers as cutin or suberin might be somewhat arbitrary. For example, a close examination of a recent analysis of suberin from the cork of *Quercus suber* and *Betula pendula* (16) reveals that the

composition of the so called suberin of *B. pendula* is strikingly similar to that of cutin, while the composition of *Q. suber* fits the criteria for suberin indicated in Table II. Just as in the cutin of many plants, 9,10,18-trihydroxyoctadecanoic acid is, by far, the largest aliphatic component of the cork of *B. pendula*, and the very long acids and alcohols characteristic of suberin appear to be virtually absent. Thus, this material might be more appropriately classified as cutin on the basis of its chemical composition. However, chemical examination of cutin and suberin from a larger number of sources is required before a firm classification can be established.

ACKNOWLEDGMENTS

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Reaction of Cardiolipin and Phosphatidylinositol Antisera with Phospholipid Antigens

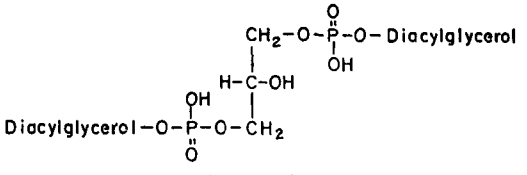
MICHAEL GUARNIERI, Laboratory of Neurochemistry, Department of Neurology, *Johns Hopkins Medical School, Baltimore, Maryland 21205*

ABSTRACT

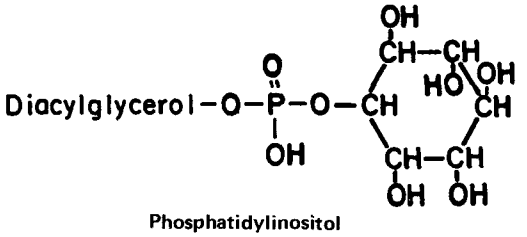
Antisera from rabbits inoculated with cardiolipin react with both cardiolipin and phosphatidylinositol. Antiphosphatidylinositol antisera also react with both phospholipids. These results suggest that antibodies to these phospholipids obtained in certain hyperimmune sera have a limited specificity.

INTRODUCTION

In previous studies, we have shown that antiphospholipid antibodies could be useful tools to study the role of phospholipids in membranes (1). We used anticardiolipin (DPG) or diphosphatidylglycerol antisera to examine the structural and functional role of DPG in mitochondrial membranes. In studies to be reported elsewhere, we have used antiphosphatidylinositol (PI) antisera to study the location of PI in myelin and microsomal membranes.



Cardiolipin



Phosphatidylinositol

Although it has been reported that PI and cardiolipin have little or no capacity to cross react with their reciprocal antisera (2-6), our studies indicated that PI had a significant capacity to react with cardiolipin antisera and the reverse. This condition would restrict the use of the antiphospholipid antibodies in membrane research, because many membranes, for example brain mitochondria and synaptic membranes, have significant amounts of both phospholipids. It seemed important to examine the nature and extent of the potential immunological similarities between the two phospholipids. We also explored the possibility of adsorbing PI and cardiolipin activity from their reciprocal antisera.

MATERIALS AND METHODS

Cardiolipin and phosphatidylinositol were obtained from Supelco (Bellefonte, Pa.). Cholesterol and β-γ-dipalmitoylphosphatidylcholine (PC) were obtained from Sigma (St. Louis, Mo.). The purity of the phospholipids was verified by 2-dimensional thin layer chromatography (TLC) (7). The lipid phosphorus of the PI was determined as 3.4% (theoretical 3.6%), and, upon deacylation, glycerol phosphorylinositol was shown to be homogeneous by paper chromatography (7).

Production of Antisera

Antisera to cardiolipin were obtained by the method of Inoue and Nojima (8). Antisera to PI were obtained by the method of Kataoka and Nojima (2).

White male rabbits weighing 3-5 kg were obtained from several different suppliers and fed a standard laboratory chow. Serum from each animal was tested for its capacity to react with the phospholipid antigen suspensions. Animals having sera which showed a preinoculation positive reaction (5 rabbits) and animals which developed ear lesions during the inoculation schedule (10 rabbits) were discarded. Over a 3 year period, 27 rabbits were used for the production of anticardiolipin antisera; 19 rabbits were used for the production of anti-PI antisera. Control serum was obtained from each rabbit before the rabbit was inoculated and from two rabbits inoculated with PC-cholesterol-methylated bovine serum albumin suspensions. All sera were tested with a blank antigen suspension containing only cholesterol and PC.

The concentration of the antibodies in the antisera were measured by a microflocculation test, as previously described (1). A negative reaction, indicated by the absence of flocculation was scored as 0; positive reactions were graded on a 1+ to 4+ scale dependent upon the amount of flocculation. The concentration of the antibodies to phospholipids in the antiserum was graded in antibody units by determining the

maximum dilution of antisera, 50 μliter of which produced a 4+ flocculation reaction with 20 μliter of antigen suspension. The antigen suspension contained 0.6 μg cardiolipin (or PI)/20 μliter. Thus, if 50 μliter of a 1:16 dilution of cardiolipin antiserum was just sufficient to produce a 4+ reaction, that antiserum contained 16 x 0.6 antibody units/50 μliter serum or 192 antibody units/ml, and the concentration of the antiserum in antibody units would be expressed as 192 μg cardiolipin equivalents/ml serum.

RESULTS

The data in Figure 1 show that rabbits inoculated with either cardiolipin or PI alone (both prepared in phosphatidylcholine-cholesterol adjuvant) can produce antisera that react with both phospholipids. Although the capacity of the individual antisera to react with one or both of the phospholipid antigens varied greatly, the sera of the PI and the cardiolipin-inoculated rabbits were combined into 3-4 groups for the purpose of comparison. Eleven sera, nine from the cardiolipin group and two from the PI inoculated animals, produced sera which contained less than forty-eight antibody units/ml. That is, the titer of these sera was less than 1:4 against either antigen. Ca. half (14 of 27) of the rabbits immunized with cardiolipin produced antisera which contained, on the average, ca. 270 cardiolipin antibody units and 48 PI antibody units/ml. Fourteen of the nineteen rabbits immunized with PI produced antisera which contained 270-380 PI antibody units. These sera were divided into two groups depending upon their relative proportions of PI and cardiolipin antibody units. In one group, the average PI antibody units were fourfold greater than the cardiolipin activity. In the second group, the average PI activity was two-

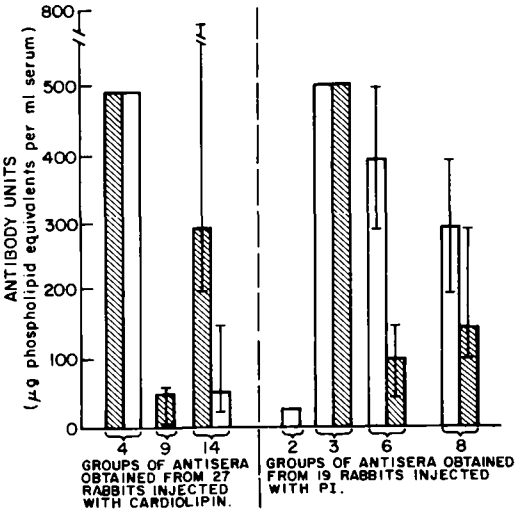


FIG. 1. Cross reactions of cardiolipin and phosphatidylinositol (PI) antisera with cardiolipin and PI. The bar ht represent the average value for the concentration of antibody units, cardiolipin, or PI in each group of antisera. The lines represent the range of values. The average value was determined from 5-6 tests with each serum with antigen suspensions prepared from 2-3 different stock solutions of cardiolipin and PI antigen. Antiphospholipid antibodies in antisera ▨ = cardiolipin and □ = PI.

fold greater than the cardiolipin antibody units. The range of cardiolipin activity in several sera from the latter group exceeded the PI units. These sera did not react with blank antigen suspensions.

The cardiolipin and PI antisera that precipitated equal amounts of both antigen suspensions, four in the cardiolipin series and three in the PI series, (Fig. 1) precipitated the blank antigen suspensions which contained only the adjuvants, cholesterol, and PC. Such blank reactions were a reflection of the PC content of

TABLE I

Titer of Antiphospholipid Antisera by Complement Fixation Test[a]

Serum against[b]	Antigen[b]	Titer (reciprocal dilution) 128	192	256	320	384	448	512
DPG 112271	DPG	+	+	+	+			
	PI	+						
DPG 062471	DPG			+	+	+		
	PI			+				
PI 110270	PI			+	+	+	+	
	DPG			+				
PI 1122270	PI						+	+
	DPG						+	

[a]Antibody titers were measured by the Kolmer (complement fixation) test (9). The data shown above were obtained with 1:200 dilutions of the cardiolipin and phosphatidylinositol antigen suspensions.

[b]DPG = cardiolipin and PI = phosphatidylinositol.

TABLE II

Adsorption of Anticardiolipin Antiserum with Phosphatidylinositol and Cardiolipin

Antiphospholipid activity in antiserum (Antibody units)	Adsorbed with antigen[a]	Antiphospholipid activity remaining after adsorption[b] (Antibody units)
96 μg DPG and 48 μg PI	96 μg PI	10-20 μg DPG
	48 μg PI	20-40 μg DPG
	96 μg DPG	None
	48 μg DPG	10-20 μg DPG

[a]Adsorption experiments used the indicated wt of antigen plus the proportional amount of auxiliary lipid for 48 hr at 5 C. Control reactions contained the auxiliary lipids alone to verify the absence of nonspecific precipitation of the antiserum.

[b]The concentrations of antibody units in the antigen-treated antiserum are expressed as the range of six determinations.

TABLE III

Adsorption of Antiphosphatidylinositol Antiserum with Cardiolipin and Phosphatidylinositol

Antiphospholipid activity in antiserum (Antibody units)	Adsorbed with antigen	Antiphospholipid activity remaining after adsorption (Antibody units)
192 μg PI and 64 μg DPG	64 μg DPG	60-120 μg PI and 5-10 μg DPG
	192 μg PI	None
	96 μg PI	5-40 μg PI and 20-60 μg DPG

the blank antigen. The greatest amount of flocculation was produced with blank suspensions containing 0.9% cholesterol and 0.12% PC. Because of the possibility that these antisera contained anti-PC or anticholesterol activity, they were not used for further study.

We measured the reaction of the cardiolipin and PI antigen with antisera from the various groups described in Figure 1 by a complement fixation test. Results from a typical experiment are shown in Table I. The sensitivity of the complement fixation test was 2-10-fold greater than the microflocculation test. The fixation tests revealed a limited pattern of specificity between the two phospholipid antisera which was similar to the pattern observed by the microflocculation tests.

The results of adsorption studies of phospholipid antiserum with their reciprocal antigens are shown in Tables II and III. For these studies, antisera were selected which contained significant amounts of activity against the reciprocal antigen. The results of an experiment with an anticardiolipin (DPG) antiserum are shown in Table II. In these tests, it was necessary to use the cardiolipin (or PI)-phosphatidylcholine-cholesterol antigen suspension to adsorb antibody. Cardiolipin (or PI) alone or when mixed with either cholesterol or with phosphatidylcholine had no capacity to react with the antisera. The cross reaction of the two phospholipids is shown clearly by the capacity of the cardiolipin antigen to adsorb PI activity and the reverse. Based upon the wt of the phospholipids in the antigens, cardiolipin had a greater capacity to adsorb PI activity compared to the adsorption of cardiolipin activity by PI.

The results of adsorption tests with the anti-PI antiserum are shown in Table III. Both the PI and the cardiolipin antigens adsorbed the reciprocal phospholipid antibodies activity. However, cardiolipin antigen did not quantitatively adsorb the anticardiolipin activity. Although 64 μg cardiolipin antigen was used to titrate the 64 μg cardiolipin activity in the antiserum, 5-10 μg cardiolipin activity was detected in subsequent tests with the antiserum. This finding, namely, that cardiolipin antigen did not quantitatively adsorb cardiolipin activity from PI antiserum suggested that cardiolipin also reacts with PI antibody.

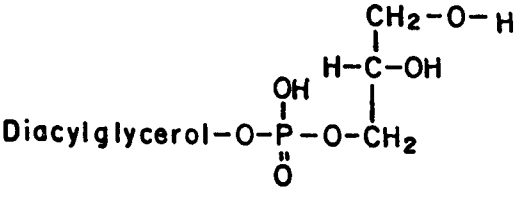
DISCUSSION

The results of this study show that rabbits inoculated with a cardiolipin antigen suspension produced antibodies which adsorbed cardiolipin and PI. Rabbits inoculated with a PI antigen also produced antibodies to both phospholipids. Several factors may reconcile the apparent discrepancy between these results and previous studies which have observed a high degree of specificity in PI and cardiolipin antisera (2-6). For example, rabbit species may vary in their response to phospholipid antigens. The preinoculation history of the animals also may be important. Many rabbits have some naturally acquired anticardiolipin antibody. A high proportion of rabbits may have a primary immune response to cardiolipin regardless of what their pre-PI immunization serum showed. In this case it would be possible to obtain an anamnestic response to cardiolipin following PI immunization.

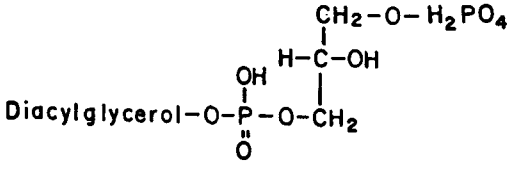
A further issue in studies on phospholipid immunochemistry concerns the role of the auxiliary lipids, phosphatidylcholine and cholesterol. The requirement or function of these lipids is poorly understood. Both the inoculation and the test antigens require the addition of phosphatidylcholine and cholesterol. As previously noted (2), antigens for the adsorption studies also require auxiliary lipids. Nonspecific

interactions may occur between various serum proteins and lipids (10,11). Such interactions with rabbit serum have been described for phosphatidylcholine, sphingomyelin, and cholesterol-antigen suspensions in complement-dependent antibody reactions (12,13).

The limited specificities of the cardiolipin and PI antisera may be related to structural similarities between the two phospholipids. Studies which involved the chemical synthesis of phospholipids having structures similar to cardiolipin have revealed that the antigenic activity cardiolipin is associated with its polar head, consisting of the two phosphodiester groups and the interior glycerol moiety (14-16). Since phosphatidylglycerol and phosphatidyl-



Phosphatidylglycerol



Phosphatidylglycerol Phosphate

glycerol phosphate also react with anticardiolipin antibody, it appears that the minimum molecular structure recognized by the antibody to cardiolipin is the portion of the molecule outlined in Figure 2. One of the hydroxyl groups of the inositol moiety of PI may, thus, be antigenically similar to the free hydroxyl group in cardiolipin. This suggestion is consonant with the findings that cardiolipin or PI antigen adsorbed both phospholipid antibodies.

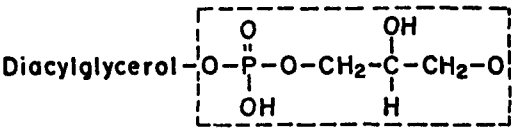


FIG. 2. Antigenic determinants of cardiolipin.

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Homolytic Decomposition of Linoleic Acid Hydroperoxide: Identification of Fatty Acid Products[1]

H.W. GARDNER, R. KLEIMAN, and **D. WEISLEDER,** Northern Regional Research Laboratory[2], Peoria, Illinois 61604

ABSTRACT

An isomeric mixture of linoleic acid hydroperoxides, 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (79%) and 9-hydroperoxy-*cis*-12,*trans*-10-octadecadienoic acid (21%), was decomposed homolytically by Fe(II) in an ethanol-water solution. In one series of experiments, the hydroperoxides were decomposed by catalytic concentrations of Fe(II). The 10^{-5} M Fe(III) used to initiate the decomposition was kept reduced as Fe(II) by a high concentration of cysteine added to the reaction in molar excess of the hydroperoxides. Nine different monomeric (no detectable dimeric) fatty acids were identified from the reaction. Analyses of these fatty acids revealed that they were mixtures of positional isomers identified as follows: (I) 13-oxo-*trans,trans*- (and *cis,trans*-) 9,11-octadecadienoic and 9-oxo-*trans,trans*- (and *cis,trans*-) 10,12-octadecadienoic acids; (II) 13-oxo-*trans*-9,10-epoxy-*trans*-11-octadecenoic and 9-oxo-*trans*-12,13-epoxy-*trans*-10-octadecenoic acids; (III) 13-oxo-*cis*-9,10-epoxy-*trans*-11-octadecenoic and 9-oxo-*cis*-12,13-epoxy-*trans*-10-octadecenoic acids; (IV) 13-hydroxy-9,11-octadecadienoic and 9-hydroxy-10,12-octadecadienoic acids; (V) 11-hydroxy-*trans*-12,13-epoxy-*cis*-9-octadecenoic and 11-hydroxy-*trans*-9,10-epoxy-*cis*-12-octadecenoic acids; (VI) 11-hydroxy-*trans*-12,13-epoxy-*trans*-9-octadecenoic and 11-hydroxy-*trans*-9,10-epoxy-*trans*-12-octadecenoic acids; (VII) 13-oxo-9-hydroxy-*trans*-10-octadecenoic and 9-oxo-13-hydroxy-*trans*-11-octadecenoic acids; (VIII) isomeric mixtures of 9,12,13-dihydroxyethoxy-*trans*-10-octadecenoic and 9,10,13-dihydroxyethoxy-*trans*-11-octadecenoic acids; and (IX) 9,12,13-trihydroxy-*trans*-10-octadecenoic and 9,10,13-trihydroxy-*trans*-11-octadecenoic acids. In another experiment, equimolar amounts of Fe(II) and hydroperoxide were reacted in the absence of cysteine. A large proportion of dimeric fatty acids and a smaller amount of monomeric fatty acids resulted. The monomeric fatty acids were examined by gas liquid chromatography-mass spectroscopy. Spectra indicated that the monomers were largely similar to those produced by the Fe(III)-cysteine reaction.

INTRODUCTION

The decomposition of linoleic acid hydroperoxide (LOOH) by enzymes or systems postulated to be enzymes has been studied extensively. Among the products identified were oxooctadecadienoic acid (1); 13-oxotridecadienoic acid, n-pentane (2); dimers (3); isomeric oxohydroxyoctadecenoic acids (4,5); trihydroxyoctadecenoic acid, hydroxyepoxyoctadecenoic acid (6); hydroxyoctadecadienoic acid; (6,7) and an unsaturated ether, 9- (nona-1′,3′-dienoxy)-non-8-enoic acid (8).

In contrast to all this enzymatic research, complete structural determination of products from the decomposition of LOOH by non-enzymatic reactions has been less thoroughly investigated, except for studies concerning the identification of pentane (9), addition products with α-tocopherol (10), and 11-hydroxy-12,13-epoxy-9-octadecenoic acid (11). Most investigators indicate that the overall mechanism of decomposition is probably homolytic. In some studies, fatty ester hydroperoxides were decomposed, neat, both thermally (12) and at low temperatures (13). Products in both instances were primarily dimers, whose structural details were not determined completely.

Transition metal ions are especially effective in catalyzing free radical reactions in the presence of peroxides or hydroperoxides. In their lower valency state, metal ions readily produce alkoxy radicals (RO·) from hydroperoxides (ROOH), whereas higher oxidation states promote peroxy radical (ROO·) formation (14). O'Brien (15) observed that LOOH degraded with Fe(II) yielded numerous products, but he did not identify them. O'Brien also discovered that Fe(III) was much less effective in decomposing LOOH, but, when electron donors like cysteine were present, the reaction resembled the decomposition caused by Fe(II)

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[2]ARS, USDA.

according to his parameters. This similarity can be attributed to the reduction of Fe(III) to Fe(II) by cysteine.

We examined nonvolatile products after homolytic degradation of LOOH in dilute solution. When equimolar Fe(II) was added to the LOOH solutions, we observed facile formation of dimers, which complicated the isolation of the monomeric fatty acids also formed. Catalytic amounts of Fe(III) (10^{-5} M) in the presence of cysteine in molar excess of LOOH moderate decomposition enough that only monomeric fatty acids can be detected. We identified the major fatty acid products of this reaction and compared them with equimolar-Fe(II) products.

METHODS

Hydroperoxides

LOOH was prepared by soybean lipoxygenase ([EC 1.13.1.13] lipoxygenase type 1, Sigma Chemical Co., St. Louis, Mo., 134,000 units/mg) oxidation of linoleic acid. The method of oxidation, including the concentration of enzyme (units/ml), and the isolation of LOOH were the same as used previously (10). By thin layer chromatography (TLC) densitometry (16), LOOH was determined to be an isomeric mixture of 13-hydroperoxy-*trans*-11,*cis*-9-octadecadienoic (79%) and 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic (21%) acids.

Reaction Conditions

Fe(III)-cysteine: The final reaction solution was 3.2 mM LOOH, 12.8 mM cysteine (free base, Nutritional Biochemicals Corp., Cleveland, Ohio) and 10^{-5} M $FeCl_3$ in 80% ethanol. The reaction was initiated by the addition of a small volume of $FeCl_3$ solution (10^{-3} M), while stirring vigorously for 1 hr at room temperature in the presence of air. In certain experiments, the reaction proceeded under either pure O_2 or N_2. The reaction mixture was extracted once with a 1.6 times larger volume of chloroform.

Equimolar Fe(II): The reaction solution was 3.2 mM LOOH and 3.2 mM ferrous ammonium sulfate in 80% ethanol. The other conditions were the same as those used with Fe(III)-cysteine, except that the reaction time was 10 min.

Chromatography

Fatty acids initially were isolated with two different chromatographic columns. The first column, serving as a preparative procedure, was packed with silicic acid, as described previously (5). The column was equilibrated with chloroform before the crude product (ca. 0.7 g) was applied. Elution was stepwise with 100 ml chloroform, 150 ml 2% methanol, and 250 ml 10% methanol in chloroform. The free fatty acids were eluted in the first 320 ml, except for trihydroxyoctadecenoic acid which eluted mixed with other minor components between 320-380 ml. The fatty acids (less than 260 mg) collected in the first 320 ml were applied to a second column (inside diameter 2.5 cm) packed with 50 g Mallinckrodt SilicAR CC-4 in isooctane. Stepwise elution was with 40 ml 20% ether, 260 ml 24% ether, 250 ml 30% ether, 250 ml 40% ether, 300 ml 50% ether, 250 ml 70% ether in hexane, 200 ml ether, and 200 ml methanol.

Column fractions found to be mixtures were purified further by TLC (10). Solvent systems were: hexane-ether-acetic acid 50:50:1 and 30:70:1 for most fatty acids and dihydroxyethoxyoctadecenoic acid, respectively; chloroform-methanol-acetic acid 65:10:1 for trihydroxyoctadecenoic acid; hexane-ether 80:20 for methyl oxostearate; hexane-ether 60:40 and 50:50 for most methyl esters; hexane-ether 30:70 for methyl dihydroxyethoxyoctadecenoate; and chloroform:methanol 95:5 for methyl trihydroxyoctadecenoate.

Silyl derivatives of fatty esters were separated by gas liquid chromatography (GLC), as described by Kleiman and Spencer (17). Column temperature was programed from 175-375 C at 4 C/min. Products from oxidations with periodic acid were separated by GLC with a 4 ft x 1/4 in. glass column packed with Silar 5 CP on Gas Chrom Q (Applied Science Laboratories, State College, Pa.). The column temperature was programed from 50-230 C at 4 C/min. Calculated carbon number of the dimer was determined by GLC using a standard wax ester (jojoba oil). The column for this determination was 3 ft x 1/8 in. and packed with 3% OV-1 on Gas Chrom Q. The column was temperature programed from 200-400 C at 2 C/min.

Spectroscopy

Mass spectrometry (MS) was employed in tandem with GLC, as described by Kleiman and Spencer (17). Multiple spectra were recorded throughout the GLC elution of each fatty ester to detect positional isomers. NMR spectra were determined as before (5). IR spectra were recorded by a Perkin-Elmer model 621 with 0.1 mm thick NaCl cells containing 10% solutions in CCl_4 and CS_2.

Derivatives

Diazomethane was used to esterify and H_2-Pd or $NaBH_4$ to reduce (10). Hexamethyldisilazane-trimethylchlorosilane-pyridine 2:1:1

TABLE I

Yield of Fatty Acids Produced by Decomposition of LOOH[a] by Fe(III)-Cysteine as Separated by Column Chromatography[b]

Compound[c]	Wt, mg[d]	Eluant volume, ml
Unknown	4.1	0-210
I	29.4	210-590
II	40.1	590-750
III, IV	12.9	710-810
V	9.4	810-910
VI	16.4	870-1010
Unknown	8.0	980-1080
VII	12.4	1080-1210
VIII[e]	51.5	1150-1260
IX[f]	116	320-380
Other	40.2	Minor components and polar material

[a]LOOH = linoleic acid hydroperoxide.

[b]Crude product (720 mg) was applied to a silicic acid column and eluted with chloroform-methanol. VIII was collected as tabulated. The other components studied (253 mg) were collected as a mixture in the first 320 ml eluted from the column. This mixture was applied to a second silicic acid column and eluted with hexane-ether as tabulated above.

[c]Key to the Roman numerals is given in Figure 1.

[d]Wt determined from actual wt of fractions. Those fractions composed of a mixture of two components were analyzed by thin-layer chromatography densitometry so that individual wt could be calculated.

[e]Two partially separated isomeric forms.

[f]IX plus other minor components as detected by thin layer chromatography.

was selected to silylate fatty esters with more than one hydroxyl group and bis(trimethylsilyl)-trifluoroacetamide (Regis Chemical Co., Chicago, Ill.) for monohydroxylated esters.

Diols and epoxides were oxidized with periodic acid (18), except that epoxides required 1/2 hr at 60 C instead of 15 min at room temperature used for diol oxidation. Quantitation of the molar ratio of the oxidation fragments, hexanal and methyl 9-oxo-nonanoate, was made possible by comparison with fragments from a standard, methyl 9,10,12,13-tetrahydroxystearate, oxidized under the same conditions. Methyl tetrahydroxystearate was prepared by alkaline $KMnO_4$ oxidation of linoleic acid (19) at 0 C reaction temperature followed by esterification and isolation by TLC.

RESULTS

Fe(III)-Cysteine Reaction

Catalytic quantities of Fe(III) ions in the presence of excess cysteine rapidly decompose LOOH to a number of chloroform-ethanol-soluble products. The reaction is complete within 20 min. Fatty acids are 52% of the mixture of lipid products. In addition to fatty acids, a number of ninhydrin-positive lipids are produced and account for the remainder of the total lipid. Other highly polar material was extracted into the chloroform layer, which was not recovered from the silicic acid column, and was subsequently assumed to be nonlipid. Preliminary data indicated that the ninhydrin-positive lipids arose from an additon reaction between cysteine and LOOH. Isolation and characterization of these adducts will be detailed in a subsequent publication. First, we concentrated on identifying the fatty acids. We did not examine the volatiles produced, but surmise their presence from the unusual almost breadlike aroma emanating from the reaction mixture. When Fe(III) was not added to the reaction mixture, LOOH failed to decompose substantially after 1 hr.

Fatty acids were isolated by column chromatography in quantities sufficient for structural work (Table I). Yields of individual fatty acids fluctuated somewhat from one experiment to another, presumably because the free radical reactions taking place were so complex. Although yields shifted for each product in replicate experiments, the identity of fatty acids predominating remained nearly the same, as far as we could ascertain.

Structures of the fatty acids identified and labeled by Roman numerals are summarized in Figure 1.

In certain experiments, the reactions were made to proceed either under a N_2 atmosphere or under pure O_2, instead of under air. Comparison of products from reactions under the three conditions were strikingly different. Compared to the products from reaction in air, the products from the pure O_2 reaction were observed to have the following changes in product distribution: (A) production of II and III was enhanced and (B) formation of the cysteine-fatty acid addition compounds was inhibited. On the other hand, compared to the air reaction, the N_2 experiment resulted in: (A) no detectable formation of II and III and (B) an enhanced production of cysteine-fatty acid addition compounds.

Structure Determinations

Product I: Its UV spectrum compared favorably with that reported by Binder, et al., (20); λ_{max} (methanol) = 277 nm, ϵ_{max} = 20,300.

An IR spectrum (I methyl ester) also compared with that reported by Binder, et al., (20) for methyl 9-oxo-*trans,trans*-10,12-octadecadienoate, except there was a small absorption at 955 cm^{-1} due to the presence of some *cis, trans*-oxodiene.

TABLE II

Mole Percent of Isomers as Determined by Thin Layer Chromatography or Periodic Acid Oxidation[a]

Compound[b]	Carbons of hydroperoxy-, or oxo-group		Carbons of epoxide- or diol-group	
	9	13	9,10	12,13
LOOH	21	79	—	—
I	26	74	—	—
II	—	—	22	78
III	—	—	51	49
V	—	—	12	88
VI	—	—	29	71
			23	77
IX	—	—	40	60

[a]The appropriate derivatives of linoleic acid hydroperoxide (LOOH) and I were determined by TLC separation of isomers followed by char densitometry. The other compounds were oxidized by periodic acid and their cleavage products quantitated by GLC.

[b]Key to the Roman numerals is given in Figure 1.

Some features in the NMR spectrum were: methylene protons α to the conjugated carbonyl at δ 2.54 (t, 2 H); olefinic proton β to carbonyl centered at δ 7.51 (dd J = 15 Hz, J = 11 Hz, 1 H); olefinic protons centered at δ 6.10 (m, 3 H); methylene protons α to the carboxylic acid at δ 2.34 (t, 2 H); and methylene protons α to the conjugated olefin at δ 2.20 (m, 2 H).

MS confirmed the proposed structure on the basis of a trimethylsilyl oxooctadecadienoate derivative. Ions with normalized intensities greater than 35% were: 67; 73, $(CH_3)_3$ Si+; 75, $(CH_3)_2$SiOH+; 81; 95; 151, cleavage of C-8 and C-9; 166, cleavage of C-7 and C-8 with rearrangement (18); 276, M - 90; 295, fragmentation between C-13 and C-14; 341, M - 15; and 366, M.

Percentages of 13- and 9-oxo isomers, as determined by TLC char-densitometry (21) after hydrogenation and esterification, are reported in Table II. Identities of the corresponding spots scraped from TLC plates were ascertained by MS compared with MS of authentic methyl 13- and 9-oxostearates described by Ryhage and Stenhagen (22).

Product II: The two isomers of II were inseparable by TLC or column chromatography in all solvents tested. On TLC, II reacted readily with a 2,4-dinitrophenylhydrazine spray (1) yielding a yellow-orange spot.

The UV spectrum of II was characteristic of spectra of α,β-unsaturated carbonyls; λ_{max} (ether) = 229 nm, ϵ_{max} = 16,500.

Characteristic IR absorptions (II methyl ester) were 1635 cm^{-1}, olefin α,β to a carbonyl; 1680 and 1700 cm^{-1}, conjugated carbonyl; 1740 cm^{-1}, ester carbonyl; 973 cm^{-1}, *trans*-

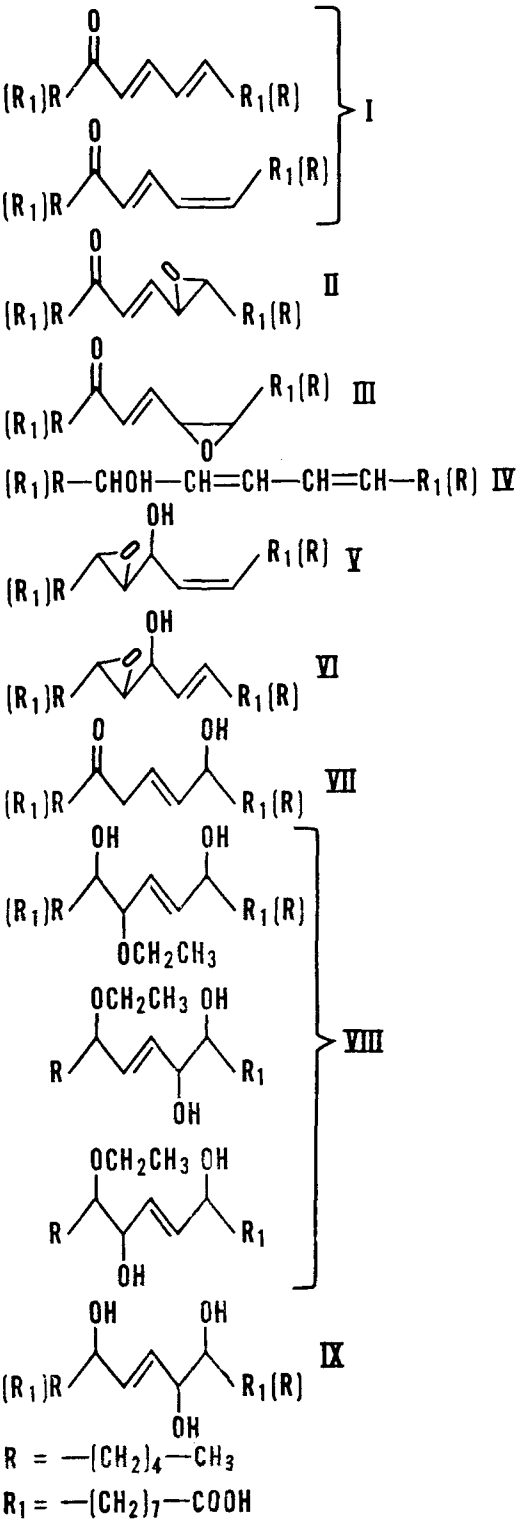

FIG. 1. Numerical key and structures of fatty acids isolated as products of the Fe(III)-cysteine reaction.

monoene; and 885 cm^{-1}, *trans*-epoxide (23).

NMR delineated the sequence of the substituents as follows: epoxide proton α to methylene at δ 2.90 (m, 1 H); epoxide proton α to unsaturation at δ 3.20 (dd, J = 6 Hz, J = 2 Hz, 1 H); olefinic proton α to the epoxide centered at δ 6.57 (dd, J = 16 Hz, J = 6 Hz, 1 H); olefinic proton α to carbonyl centered at δ 6.36 (d, J = 16 Hz, 1 H); methylene α to conjugated carbonyl at δ 2.52 (t, 2 H); and methylene α to carboxylic acid carbonyl at δ 2.34 (t, 2 H). Double irradiation of the epoxide proton at 3.20 δ confirmed its position α to the double bond. The epoxide protons were assigned a *trans*-configuration because of their upfield absorption compared to the corresponding *cis*-epoxide, product III. The difference in chemical shift between the *cis*- and *trans*-epoxide protons α to methylene and α to olefin were 0.24 and 0.27 δ, respectively, which correspond closely to 0.22 δ difference reported for *cis*- and *trans*-epoxides of saturated fatty esters (24). Reduction of II with $NaBH_4$ yielded the corresponding hydroxyepoxyoctadecenoic acid, which was examined by NMR. The epoxide protons α to methylene and α to olefin absorbed at 2.78 and 3.05 δ, respectively, upfield from the corresponding *cis*-epoxide reported by Graveland (6).

MS of the trimethylsilyl ester of II resulted in poor spectra which could not be interpreted readily. However, $NaBH_4$ reduction of II, esterification and preparation of the trimethylsiloxy derivative yielded a compound that fragmented into intense ions. MS compared favorably with the reported by Graveland (6) who also examined a mixture of methyl 13-trimethylsiloxy-9,10-epoxy-11-octadecenoate and methyl 9-trimethylsiloxy-12,13-epoxy-10-octadecenoate. Our spectra differed from Graveland's in that the cleavage between the epoxide and the double bond was reduced greatly, which resulted in small intensitites of m/e 199 and 285.

Periodic acid oxidation showed that the epoxide was mostly at C-12,13 (Table II).

Product III: III migrated in TLC with a slightly lower R_f compared with II, as expected for *cis*- vs *trans*-epoxides (25). III eluted from column chromatography mixed with IV but was isolated almost completely from IV by esterification followed by TLC.

The UV spectrum of III was similar to the one reported for II, λ_{max} (ether) = 229 nm.

An IR spectrum (III methyl ester) had features similar to the spectrum of II. The notable difference was a shift in epoxide absorption from 885 cm^{-1} in II to 825 cm^{-1} in III, indicating that III was a *cis*-epoxide (23).

Assignments of an NMR spectrum of III (methyl ester) are as follows: epoxide proton α to methylene at δ 3.14 (m, 1 H); epoxide proton α to unsaturation at δ 3.47 (dd, J = 6 Hz, J = 4 Hz, 1 H); olefinic proton α to epoxide at δ 6.63 (dd, J = 16 Hz, J = 6 Hz, 1 H); the other olefinic proton at δ 6.34 (d, J = 16 Hz, 1 H); carbomethoxy methyl at δ 3.62 (S, 3 H); methylene α to ester carbonyl at δ 2.27 (t, 2 H); and other assignments identical to those observed for II. Double irradiation experiments confirmed the structure.

MS (III methyl ester) was less than satisfactory; however, a small molecular ion (m/e 324), M - 18, and M - 31 were observed. After $NaBH_4$ reduction and trimethylsilylation, the MS was similar to the comparable derivative of II.

III was shown to be a nearly equal mixture of positional isomers by periodic acid oxidation (Table II).

Product IV: IV was present in trace quantities compared to the other constituents and thus was difficult to isolate. After preparative TLC of the column fraction containing a mixture of III and IV, a partially purified sample of IV was obtained. The TLC fraction was separated further by GLC after trimethylsiloxy derivatization. Replicate MS sampled over the appropriate GLC peak gave spectra comparable to trimethylsiloxy derivatives of methyl 9-hydroxy-*trans,trans*-10,12-octadecadienoate and others more comparable to methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate, as indicated by a shift in ratios of m/e 225 to 311 (17). The geometric configuration of the diene was not determined.

Product V: Product V separated from the analogous product VI both by column chromatography and TLC. Although the difference in structures reported here is proposed to be *cis*- vs *trans*-unsaturation, this difference would not be expected to result in chromatographic separation with the techniques used in this study. We did not examine these products for either erythro or threo isomerism. This type of isomerism may be the additional criterium that caused the separation of V from VI.

Characteristic IR absorptions (V methyl ester) were 890 cm^{-1}, *trans*-epoxide, and 3480 cm^{-1}, hydroxyl. Absorptions at 970 cm^{-1}, indicative of a *trans*-double bond, and at 1660 or 710 cm^{-1}, indicative of *cis*-unsaturation, were weak and not definitive enough to assign either geometry to the unsaturation.

NMR assignments are as follows: carbinol methine proton at C-11, δ 4.63 (dd, J = 4 [epoxide] and 8 [olefin] Hz, 1 H); epoxide proton α to carbinol methine, δ 2.77 (dd, J = 2 [epoxide] and 4 [carbinol methine] Hz, 1 H); epoxide proton α to methylene, δ 2.98 (m, 1

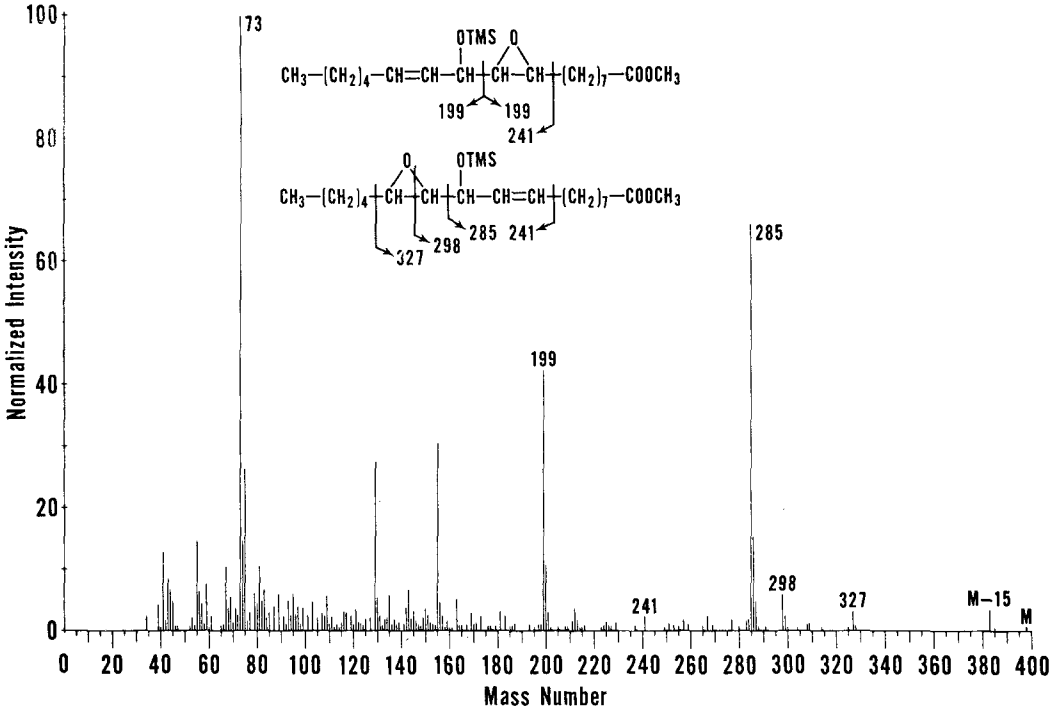

FIG. 2. Mass spectrum of VI; methyl ester-trimethylsiloxy derivative.

H); olefinic proton α to carbinol methine, δ 5.32 (dd, J = 11 [olefin] Hz, 1 H); olefinic proton α to methylene, centered at δ 5.60 (m, J = 11 [olefin], 1 H); methylene α to olefin, δ 2.06 (m, 2 H). Double resonance experiments determined the sequence of substituents, as well as the J values. The coupling between epoxide protons of 2 Hz indicates that the epoxide is *trans-* which agrees with the coupling constant of similar epoxides (26). The coupling observed between the olefinic protons of 11 Hz suggests that the double bond is probably *cis-*.

MS of V clearly supported the proposed structure and were identical to the MS obtained with VI (Fig. 2). Replicate MS taken over the GLC peak showed first a spectrum of the one isomer, the derivative of 11-hydroxy-12,13-epoxy-9-octadecenoic acid, characterized by m/e 285 ion. At later elution times, the former isomer mixed with the derivative of 11-hydroxy-9,10-epoxy-12-octadecenoic acid was observed as characterized by both m/e 285 and 199. The m/e 285 ion predominated in intensity relative to the 199 ion in all the spectra taken pointing to a preponderance of the one positional isomer. The observation on positional isomerism was confirmed by periodic acid oxidation (Table II), i.e. a preponderance of 12,13-epoxide.

Product VI: IR absorptions were largely similar to those observed with V. Relative to V, VI had a more intense absorption of a *trans-* epoxide at 890 cm^{-1}; however, a weak absorption at 840 cm^{-1} indicated the possibility of a minor amount of *cis-*epoxide. Like V, the absorptions characteristic of unsaturation were not definitive for VI.

NMR was most helpful in determining the difference between V and VI. The olefinic coupling constant could not be determined, but the narrow absorption at δ 5.54 (m, 2 H) indicates that the double bond is *trans-* rather than the *cis-* in V. Absorption of the C-11 carbinol methine proton was at δ 4.25 (dd, J = 6 [epoxide] and 8 [olefin] Hz, 1 H). The difference in chemical shifts of the C-11 methine in products V and VI is 0.38 ppm. This difference in shift is of the magnitude and direction expected for a difference in geometry (*cis-* vs *trans-*) of the α-unsaturation (27). Other absorptions are similar to those observed in the spectrum of V, except that the proton of the epoxide α to methylene was slightly upfield at δ 2.93 (m, 1 H). Coupling of the epoxide protons is the same as in V, i.e. J = 2 Hz, and, therefore, the epoxide is predominantly *trans-*.

MS of VI is shown in Figure 2. A shift in ion intensities during the GLC elution of VI suggested a separation of the two positional

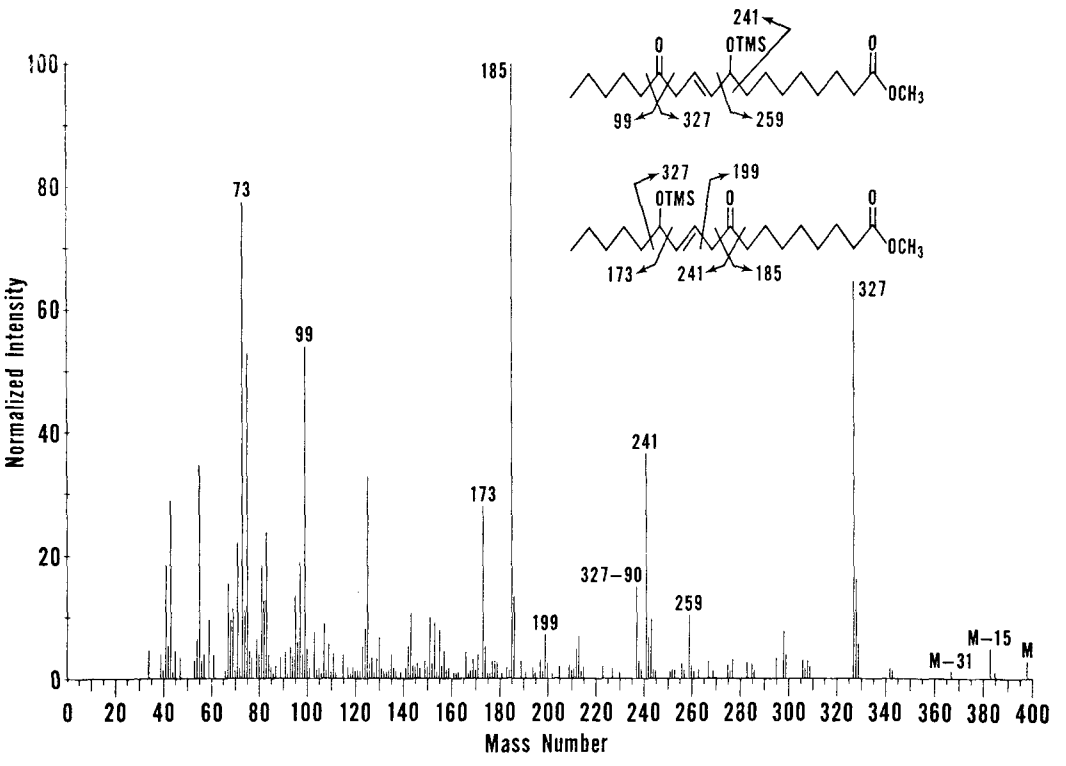

FIG. 3. Mass spectrum of VII; methyl ester-trimethylsiloxy derivative.

isomers as described for V. The 12,13-epoxide predominated over the 9,10-epoxide, Table II.

Product VII: The positional isomers of VII were not chromatographically separable by our methods. On TLC, VII reacted with 2,4-dinitrophenylhydrazine spray forming orange-yellow spots.

Features of the IR spectrum (methyl ester) are 970 cm^{-1}, isolated *trans*-olefin; 1717 cm^{-1}, oxo-carbonyl; 1740 cm^{-1}, ester carbonyl; and 3460 cm^{-1}, hydroxyl.

The NMR spectrum is interpreted as follows: olefinic proton α to carbinol C-H at δ 5.56 (m, J = 16 Hz, 1 H); olefinic proton α to methylene centered at δ 5.7 (m, 1 H); carbinol proton at δ 4.08 (m, 1 H); methylene groups α to ester and ketone carbonyl at δ 2.33 (t, 4 H); and methylene protons between the carbonyl and oiefin at δ 3.11 (d, J = 6 Hz). Integration of the absorption at δ 3.11 yielded 1.1 H, instead of the 2 expected. Another NMR study, (H.W. Gardner and D. Weisleder, unpublished results) of 12-oxo-13-hydroxy-*cis*-9-octadecenoic acid showed that similar protons at C-11 usually integrated considerably less than two. Double irradiation experiments completed the NMR studies and confirmed the positions of the functional groups.

MS (Fig. 3) demonstrated fragment ions characteristic of two positional isomers. MS scans of the GLC peak showed a shift in ion intensities indicating a partial separation of one isomer from the other.

Product VIII: There was a partial separation in two isomers of VIII that could be discerned both in column chromatography and TLC. We do not have sufficient information, especially of the minor component, to understand the reason for the separation. Identification of VIII was based upon the major component; however, spectral data for the minor component were similar to the major one. There is the possibility of geometric isomerism (erythro-threo), as well as three possible positional isomers each of 9,10,13-dihydroxyethoxy-*trans*-11-octadecenoic and 9,12,13-dihydroxy-ethoxy-*trans*-10-octadecenoic acids that depend upon the positions of the hydroxyls relative to the ethoxy. Certain isomers were considered to be less probable on the basis of some of the NMR and MS evidence.

Characteristic absorptions of the IR spectrum are: 978 cm^{-1}, isolated *trans*-olefin; 1090 cm^{-1}, ether-alcohol C-O stretch; and 3420 cm^{-1}, hydroxyl.

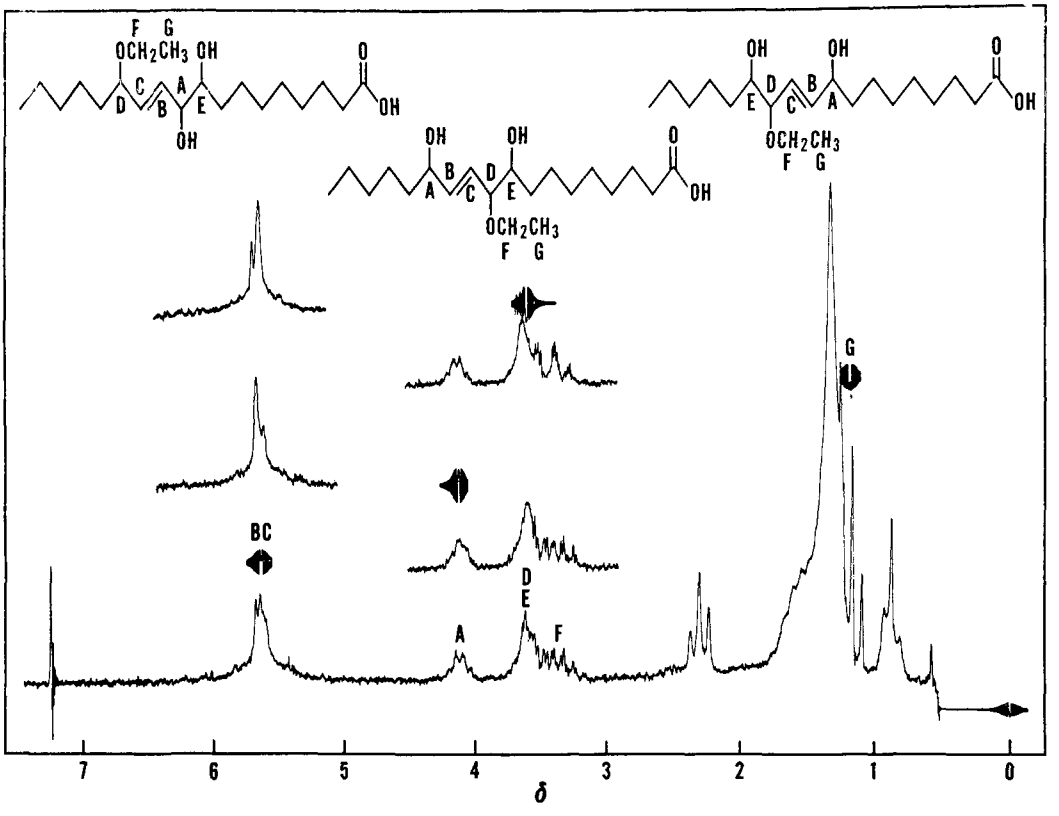

FIG. 4. NMR spectrum of VIII.

As shown in Figure 4, absorptions in the NMR are: methyl of ethoxy group at δ 1.16 (t, 3 H); methylene of ethoxy group centered at δ 3.44 (m, showing splitting due to two or more isomeric forms, 2 H); carbinol C-H proton α to olefin at δ 4.12 (m, 1 H); ether C-H α to olefin and carbinol C-H at δ 3.62; carbinol OH at δ 5.60 (2 H); and olefinic protons at δ 5.63 (m, 2 H). Double irradiation (Fig. 4) indicated that the double bond is situated between a secondary alcohol and an ether C-H, at least in a significant proportion of the total isomeric mixture. Thus, 9,12-dihydroxy-13-ethoxy-*trans*-10-octadecenoic and 10,13-dihydroxy-9-ethoxy-*trans*-11-octadecenoic acids were probably not the prevalent isomers.

Replicate GLC-MS were made on both chromatographically separable components of VIII. MS of both yielded similar fragment ions. One of these spectra is (Fig. 5) sampled from the center of the GLC peak. The fragment ions observed would be characteristic of four different isomers (Fig. 1), including 9,12-dihydroxy-13-ethoxy-*trans*-10-octadecenoic acid, although NMR data are not in accord with this structure. The two isomers with a 9-ethoxy group were eliminated from consideration because the characteristic expected ions, m/e 215, 215-46, or 215+73, were absent in all the MS. Because two pairs of the remaining four isomers have fragmentation ions that are identical, one cannot select which are prevalent. However, the varying intensities of m/e 343 or 416 vs 257 from one spectrum to another suggest that both 9,13-dihydroxy-12-ethoxy-*trans*-10-octadecenoic and 9,13-dihydroxy-10-ethoxy-*trans*-11-octadecenoic acids are present. The existence of 9,10-dihydroxy-13-ethoxy-*trans*-11-octadecenoic acid was considered to be unlikely, since the rearrangement ion, m/e 332 (TMSO-CH-$(CH_2)_7$-COTMS-OCH_3^+), was not present in the MS (17). On the basis of NMR, one might assume that 9,12-dihydroxy-13-ethoxy-*trans*-10-octadecenoic also was not present; however, decoupling experiments by NMR would probably not be sensitive to minor components. Even though the four isomers shown in Figure 1 must be considered as possible, we conclude that 9,13-dihydroxy-12-ethoxy-*trans*-10-octadecenoic and 9,13-dihydroxy-10-ethoxy-*trans*-11-octadecenoic acids are the major components of product VIII on the basis of all the

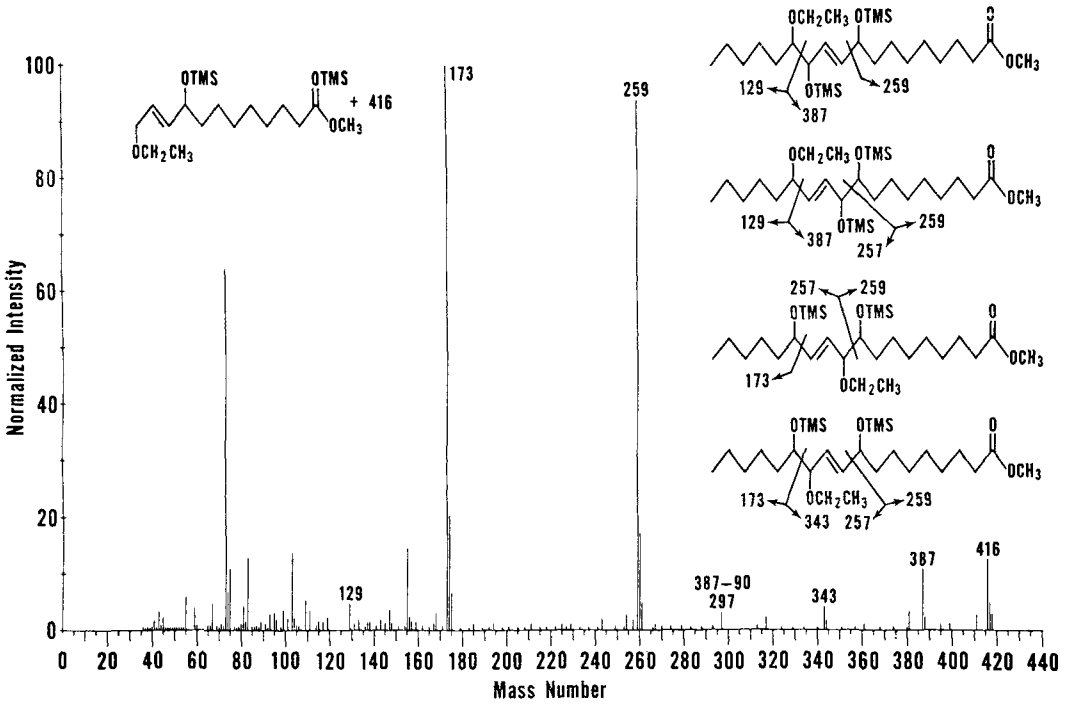

FIG. 5. Mass spectrum of VIII; methyl ester-trimethylsiloxy derivative.

available information.

Product IX: IR, NMR, and MS were essentially as described by Graveland (6), who also examined a mixture of 9,12,13-trihydroxy-10-octadecenoic and 9,10,13-trihydroxy-11-octadecenoic acids. We found a few minor differences in MS data; for example, the intensity of the rearrangement ion, m/e 460 (TMSO-CH-CH=CH-CHOTMS-$[CH_2]_7$-COTMS-OCH_3), was much reduced. Also, the MS we obtained of hydrogenated IX presistently had more fragment ions, especially at m/e less than 173 and a persistent ion of 213 (303-90) in all spectra taken. We found m/e 389 to be virtually absent, but m/e 389-90 was intense. Three oxidation products of IX (methyl ester) were identified by GLC-MS after periodic acid treatment: hexanal, methyl 9-oxononanoate, and 4-hydroxy-2-nonenal. The molar quantities of hexanal and methyl 9-oxononanoate are reported in Table II.

Equimolar Fe(II) Reaction

LOOH was decomposed with an equimolar quantity of ferrous ammonium sulfate. The reaction occurred immediately as expected and could be assessed visually by the formation of brown Fe(III). Products were separated by column chromatography, and fractions from it were separated further by GLC. Among the products, we detected substances giving MS and chromatographic properties identical to I, IV, V (and VI), VIII, and IX, as well as many compounds other than those resulting from the LOOH-cysteine-Fe(III) reaction. Because of the complexity of the Fe(II) system, the presence of trace quantities of II, III, and VII could easily have been missed. A determination of the geometry of the epoxides or unsaturation was not possible, except for oxooctadeca-(*trans, trans*)-dienoic acid which was isolated relatively pure in ca. 15% yield.

Other products detected were compounds yielding MS and chromatographic data that indicated they were ethoxyoctadecadienoic acid and diethoxyhydroxyoctadecenoic acid. Additional IR data confirmed the identity of ethoxyoctadecadienoic acid. Other components, including a large amount of compounds tentatively identified as dimer, were not researched in detail. Typically, the silylated methyl ester of the dimer eluted from GLC as one peak centered at calculated carbon number of 41.4 calculated from the standard wax esters. The 41.4 value is consistent with a structure of dimeric C-18 esters containing some oxygenated functional groups. Detailed structure determinations of the dimer were not made, except that a few MS recorded had fragments up to m/e 706. In the more polar

fractions eluting from a hexane-ether silicic acid column (over 800 ml), dimers accounted for 69-92% of the total as determined by GLC. The percentage dimer decreased with decreasing elution volumes.

DISCUSSION

The homolytic decomposition of LOOH by a catalytic concentration of Fe(III), 10^{-5} M, was made possible by keeping Fe(III) reduced as Fe(II) by cysteine in molar excess of LOOH. According to present theories, the homolytic breakdown of LOOH by Fe(II) proceeds through the alkoxydiene radical (14). Supposedly, the variety of products formed results from further reactions of the alkoxydiene free radical. More evidence is needed to determine whether the final products are formed entirely by a free radical mechanism or by participation in part or total by ionic mechanisms. Cysteine in the absence of 10^{-5} M Fe(III) was relatively ineffective in decomposing LOOH, an observation that indicates the reaction was at least initiated homolytically.

Many of the same products were produced with Fe(II) equimolar to LOOH without cysteine. The major difference between equimolar Fe(II) and the Fe(III)-cysteine system was formation of dimer by the former. Apparently, the rapid reaction with equimolar Fe(II) caused a high concentration of radicals that promoted termination reactions.

Information provided by quantitative analyses of positional isomers gave clues as to the origin of the products. When the 9- to 13-oxo ratio of I is compared to the 9- to 13-hydroperoxy ratio of LOOH (Table II), it can be concluded that I was derived from LOOH by a net loss of one molecule of water. The *cis, trans*-diene of LOOH was only partially retained in the formation of I as the *trans,trans*-diene was a significant portion of the mixture of isomers found. In a previous study (10) of the decomposition of LOOH in the presence of α-tocopherol, I was observed as one of the major products, except that only the *trans, trans*-diene was detected.

Because IV was only a trace product, the 9- to 13-hydroxy ratio of isomers could not be determined readily, but IV is assumed to be formed from the alkoxydiene radical, possibly through gain of a hydrogen radical from sulfhydryl. It was surprising that the yield of IV was so low, leading us to believe IV may have been utilized in secondary reactions.

Epoxides were located predominantly at carbons-12,13 in a molar percentage corresponding to the percentage of 13-hydroperoxide used as a reactant (Table II). The exception was III, which was a minor product compared to II, V, and VI. Apparently, the predominant reaction is cyclization of the hydroperoxy group to the α-unsaturation. These epoxides may have formed either through a free radical mechanism similar to that proposed by Gardner, et al., (10) or through an ionic mechanism as outlined by Hamberg and Gotthammar (11). Even though epoxide formation by the addition of peroxide oxygen across a double bond is a well documented reaction, the epoxides found by us do not appear to be produced in this manner, because the positional isomers largely do not correlate.

At this time little can be said of the formation of products VII, VIII, and IX, as well as the mechanistic details of the formation of the other compounds. We currently are attempting to sort out the various pathways and plan to report further on the details of these reactions in the future.

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Studies on Lipid and Fatty Acid Composition of Human Hepatoma Tissue

EIJI ARAKI,[1] F. PHILLIPS, and O.S. PRIVETT, The Hormel Institute, University of Minnesota, Austin, Minnesota 55912

ABSTRACT

Studies are reported on the composition of the lipids of human liver and hepatoma tissues from male adults. Liver tissues were obtained from individuals who died from causes other than liver disease or cancer. The hepatoma tissues were obtained from individuals shortly after they succumbed to cancer. The total lipid of each tissue was fractionated quantitatively by silicic acid column chromatography into neutral lipid, glycolipid, and phospholipid fractions. These fractions were analyzed by thin layer chromatography and converted to methyl esters for analysis of their constituent fatty acids by gas liquid chromatography. In comparison to liver tissue, the total amount of lipid in the hepatoma tissues was generally higher and more variable; the lipid of one hepatoma was ca. 92% of the dry wt of the tissue. The greater lipid content of the hepatoma tissues was due to the high percentage of neutral lipid. Except for one specimen, there was ca. the same amount of glycolipid in the hepatoma as in the liver tissues, but the composition of the glycolipid fraction of the hepatoma lipid differed considerably, particularly in the ganglioside fraction. The phospholipid fraction of hepatoma lipid was much lower than that of liver but exhibited only quantitative differences in composition. No glyceryl ether diesters and only traces of plasmalogens of phosphatidyl choline or phosphatidyl ethanolamine were detected in the liver and hepatoma lipids. The levels of monoenoic acids were higher and those of linoleic and polyunsaturated fatty acids lower in the hepatoma lipids. Positional isomers of trienoic acids not normally present in liver tissue were detected in hepatoma lipids. The abnormalities observed in lipid composition indicated interferences in the regulatory processes of lipid metabolism in human hepatoma similar to those observed in animals.

[1]Present address: National Cancer Center Hospital, 5-Chome, Tsukiji, Chuo-ku, Tokyo, Japan.

INTRODUCTION

Studies (1-6) on the lipid of liver carcinoma of animals show that the composition varies widely not only from that of normal liver tissue but also among themselves. Differences also have been observed in the composition of the lipid of human liver and hepatoma tissue (7-10), but much less information is available on the effect of cancer upon lipid composition in human tissues than in animal tissues. Reported here is a comparison of the lipid and fatty acid composition of the tissue of three liver and five hepatoma specimens obtained from human subjects.

MATERIALS AND METHODS

The liver and hepatoma tissues were provided by the National Cancer Center Hospital in Tokyo. The liver tissues were obtained from 3 adults, a female 54 years of age and 2 males, one 50 and the other 58, 3-4 hr after they died of massive gastrointestinal bleeding. Histological examination of the tissues indicated that the livers of these adults were normal. The hepatoma tissues were obtained from 5 adult males between the ages 44-61 within 6 hr after death. Histopathological examination showed that these tumors were of the hepatocellular type. The subjects had been treated with dosages of vitamins B and C but no special drugs as might conceivably alter lipid composition. Immediately after removal of the tissues, they were sliced, wiped with filter paper, and the necrotic areas separated after dipping them in physiological saline. The tissues were quick frozen on dry ice and stored at -20 C until processed. The lipids were extracted from the tissues at room temperature under an atmosphere of nitrogen by homogenization with chloroform-methanol, 2:1 and the nonlipid impurities removed by aqueous extraction of a solution of the lipid in chloroform and petroleum ether, as previously described (11). The amount of total lipid in each tissue was determined from an aliquot of this solution by gravimetric analysis.

Fractionation of the lipid into neutral lipids, glycolipid, and phospholipid was carried out by column chromatography, as previously described (11), except that a commercial grade of silicic acid was used instead of acid-washed

TABLE I

Analysis of Liver and Hepatoma Tissue Lipids

Tissue	Total lipids[a]	Neutral lipids	Glycolipid fraction	Phospholipid fraction	Thiobarbituric acid reaction[b]
Hepatoma					
1	16.8	70.1 (11.8)[c]	2.4 (0.4)	27.5 (4.6)	0.31
2	12.8	73.3 (9.4)	4.2 (0.5)	22.5 (2.9)	0.20
3	18.2	62.9 (11.5)	8.3 (1.5)	28.7 (5.2)	0.51
4	92.7	82.7 (76.7)	4.4 (4.1)	12.9 (12.0)	0.29
5	25.9	84.3 (21.8)	2.7 (0.7)	13.3 (3.4)	0.22
Liver					
1	20.7	40.6 (8.4)	2.9 (0.6)	56.5 (11.7)	0.94
2	21.8	34.6 (7.5)	2.6 (0.6)	62.7 (13.7)	0.79
3	19.9	34.7 (6.9)	4.2 (0.8)	61.0 (12.1)	0.55

[a]g/100 of dried tissue.

[b]Millimicromoles malonaldehyde/10 mg of total lipid.

[c]The figure in parenthesis is g/100 g of dried tissue. The figure above parenthesis is the composition of each lipid fraction expressed as percentage of the total lipid.

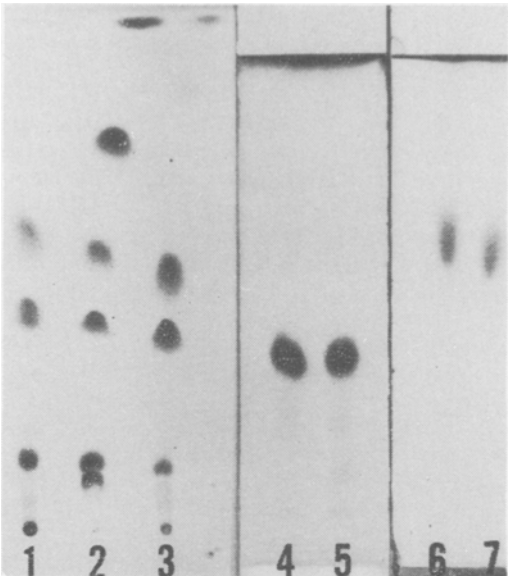

FIG. 1. Thin layer chromatographic (TLC) analysis. 1. Normal liver neutral lipids. 2. Neutral lipid standards (top to bottom) cholesterol oleate, glyceryl 1,2-dioleyl-3-hexadecyl ether, triolein, oleic acid, cholesterol, 1-3 diolein. 3. Hepatoma neutral lipids. 4-7. Plasmalogen analysis by TLC after exposure of sample to HCl. 4. Normal liver, phosphatidyl choline. 5. Hepatoma, phosphatidyl choline. 6. Normal liver, phosphatidyl ethanolamine. 7. Hepatoma, phosphatidyl ethanolamine. Solvent systems: 1-3 developed in petroleum ether-ethyl ether-acetic acid, 90:10:0.5; 4-7 exposed to HCl vapors for 5 min, dried under nitrogen for 15 min, then developed in chloroform-methanol-ammonium hydroxide, 65:35:5.

Florisil. In this procedure, 60-65 mg lipid was fractionated on a 39 x 1 cm column of Silic AR CC-7 (Mallinckrodt, St. Louis, Mo.) (17.5 g) treated with ammonium hydroxide (12). The neutral lipids were eluted with 500 ml chloroform, followed by the glycolipids with 500 ml acetone and then the phospholipids with 800 ml methanol. There was no overlapping of fractions as determined by gravimetric and thin layer chromatographic (TLC) analyses of aliquots of eluant taken at the end of each elution. A new column of adsorbent was used for each analysis. Under these conditions, recovery of total lipid was quantitative (104-107%) and the separations were reproducible within an error of ±5%.

Fatty acid composition of each column fraction, as well as the total lipid, was determined by gas liquid chromatography (GLC) of methyl esters prepared by interesterification with methanol at 95 C for 4 hr in sealed glass tubes containing an atmosphere of nitrogen, using HCl as a catalyst. The GLC analyses were carried out with an Aerograph model 600 D gas chromatograph equipped with 6 ft x 1/8 in. column packed with 10% EGSS-X on Gas Chrom P and a flame ionization detector at 195 C with a carrier gas (N_2) flow rate of 40 ml/min. Fatty acid composition was determined by comparison of retention volumes of the individual components with authentic methyl esters before and after fractionation of

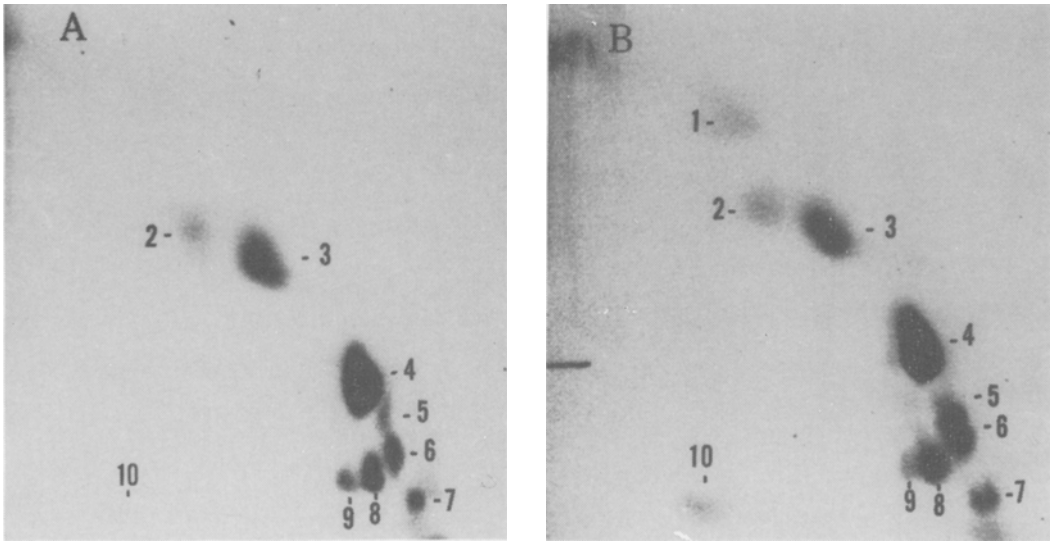

FIG. 2. Two dimensional thin layer chromatography of phospholipid fractions. A. Normal liver. B. Hepatoma. 1. Lyso bis phosphatidic acid. 2. Diphosphatidyl glycerol. 3. Phosphatidyl ethanolamine. 4. Phosphatidyl choline. 5. Lyso phosphatidyl ethanolamine. 6. Sphingomyelin. 7. Lyso phosphatidyl choline. 8. Phosphatidyl inositol. 9. Phosphatidyl serine. 10. Phosphatidic acid. Solvent systems: y-dir (first) chloroform-methanol-ammonium hydroxide, 65:35:5; x-dir (second) chloroform-acetone-methanol-acetic acid-water, 5:2:1:1:0.5.

the sample by argentation-TLC to confirm the degree of unsaturation and, before and after hydrogenation, to confirm chain length. Quantitative analysis was made on the basis of the peak areas.

TLC of the lipid classes was carried out using Silica Gel G or H (A.G. Merck, Darmstadt, Germany) coated plates. The specific conditions used for these analyses and the components identified are described in the legends to the figures. Plasmalogens were analyzed by the method of Viswanathan, et al. (14).

RESULTS

Total lipid content of the three liver and five hepatoma tissues and the results of the column fractionation are shown in Table I. These results show that differences in the total lipid/dry wt of the tissue for the three liver specimens were relatively small. In contrast, the amount of lipid in the hepatoma tissue was highly variable and generally much greater due to the large content of neutral lipid. One tissue contained over 90% lipid. Except for one specimen, the hepatoma tissue contained ca. the same amount of glycolipids, but the composition of this fraction was much different from that of liver tissue. However, there was less phospholipids in the hepatoma than liver tissues. Table I also shows that TBA values of hepatoma lipid were generally lower than those of liver tissues.

In spite of the large differences in the relative amounts of the neutral, glycolipids, and phospholipid fractions, no qualitative differences were observed in either the neutral lipid or phospholipid fractions of liver and hepatoma tissues, as illustrated in Figures 1 and 2. Glycerol ether diesters could not be detected in the neutral lipids of either the liver or hepatoma lipids, as illustrated in Figure 1. Traces of plasmalogens were detected in both liver and hepatoma phosphatidyl choline and phosphatidyl ethanolamine, as evidenced by the detection of minor amounts of lyso compounds by the method of Viswanathan, et al. (14). However, these compounds were not elevated in the hepatoma lipids (Fig. 1). Only minor differences existed in the composition of the phospholipids of liver and hepatoma lipids, as illustrated in Figure 2.

Major differences were observed in the composition of the glycolipid fractions of the liver and hepatoma lipids, as shown in Figure 3. The fractions of both hepatoma and liver tissues contained unknown compounds, some of which did not give a positive orcinol test. None of the spots gave a phosphorous test. Hence, the identification of these compounds must await further experimentation. A major difference between the composition of the hepatoma and liver fractions was in the less polar gangliosides that are extracted with chloroform-methanol and which separate with the glycolipid fraction.

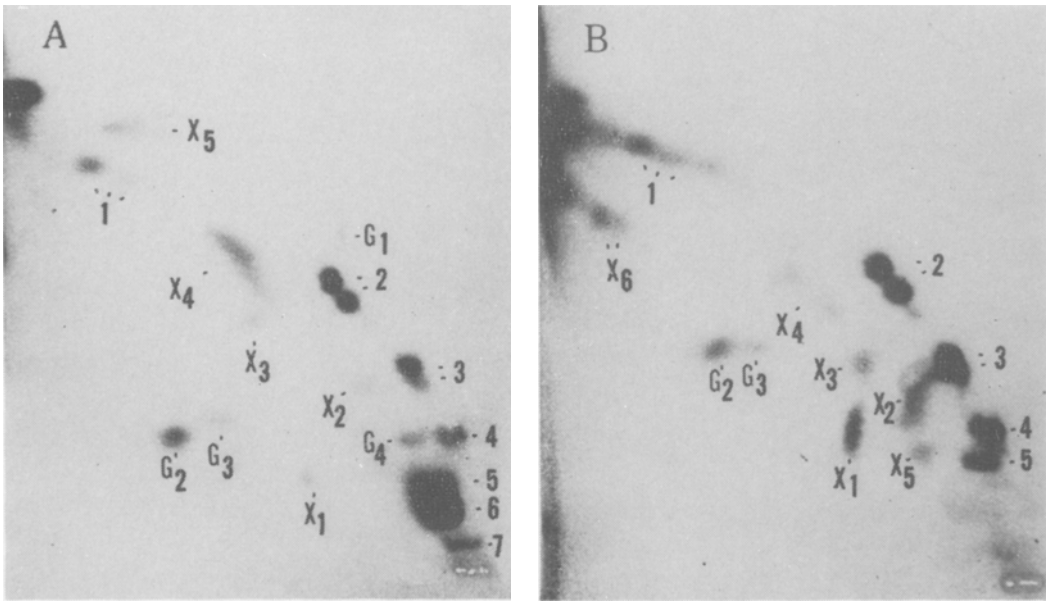

FIG. 3. Two dimensional thin layer chromatography of glycolipid fractions. A. Normal liver. B. Hepatoma. 1. Ceramide monohexoside. 2. Ceramide dihexoside. 3. Ceramide trihexoside. 4. Ceramide tetrahexoside. 5-7. Gangliosides; unidentified glycolipids–G_n; unidentified polar lipids–X_n. Solvent systems: y dir (first) chloroform-methanol-water, 65:25:4; x-dir (second) chloroform-acetone-methanol-acetic acid-water, 5:2:1:1:0.5

Not only were there quantitative differences in composition, but the hepatoma lipids were completely devoid of some of these constituents, as illustrated in Figure 3.

Differences also were observed in the fatty acid composition of the lipids of liver and hepatoma tissues, as shown in Table II. These differences were not confined to any one fraction, as illustrated by comparison of the composition of the lipid groups separated by column chromatography. In general, the hepatoma lipids contained higher levels of monoenoic acids and lower levels of linoleic and polyunsaturated fatty acids. In addition to the fatty acids listed in Table II, virtually all of the lipid fractions contained traces or small amounts of other fatty acids. Among those detected, based upon relative retention times, were 12:0, 15:0, 17:0, 20:0, 18:3ω6, 20:1, 18:3ω3, 20:2, 20:0, 20:3ω9, 20:3ω6, 20:5ω3, 22:3, 24:1, 22:5ω6, and 22:5ω3. Fatty acids that could not be identified precisely by their retention time also were detected. Some of these acids appeared to be uncommon positional isomers. To determine if fatty acids of this type existed, the trienoic acid fractions were isolated from several hepatoma and liver tissues by argentation-TLC and examined in more detail, as shown in Figure 4.

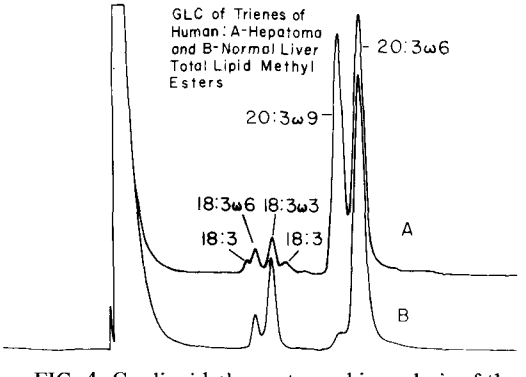

FIG. 4. Gas liquid chromatographic analysis of the methyl esters of the trienoic acid fractions isolated by $AgNO_3$-thin layer chromatography from human liver and hepatoma tissue lipids.

These analyses showed that hepatoma lipid contained positional isomers of both the 18- and 20-carbon chain acids not normally found in liver lipids. Confirmation of the isomeric composition of these acids (Fig. 4) must await structural analyses. Positional isomers of other unsaturated fatty acids, particularly the monoenoic acids, also might be present in hepatoma tissue, but no attempt was made to examine other fractions in detail. However, no *trans*-isomers appeared to be present in the hepatoma lipids as determined by IR spectral analysis.

DISCUSSION

The small variation in the analyses of the

TABLE II

Fatty Acid Analyses[a]

Tissues and lipid fraction	14:0[b]	16:0	16:1	18:0	18:1	18:2	20:4	22:4	22:6
Hepatoma 1									
TL	2.1	38.4	6.8	8.3	25.4	9.2	2.4	0.3	2.7
NL	3.1	40.9	7.2	6.2	26.6	8.1	1.7	tr	2.3
PL	0.6	27.1	3.4	16.6	17.4	13.6	5.1	2.2	6.5
Hepatoma 2									
TL	1.5	21.6	4.6	9.4	40.8	9.8	2.9	1.6	1.7
NL	1.8	20.8	5.3	8.8	42.6	9.8	2.4	0.7	1.5
PL	0.8	23.0	2.8	15.9	20.8	12.3	7.1	5.0	4.1
Hepatoma 3									
TL	1.2	25.4	5.5	10.6	26.9	10.9	4.9	1.5	3.1
NL	2.0	26.7	6.6	8.1	30.1	10.3	4.0	0.6	2.9
PL	0.6	24.9	3.7	17.3	16.3	13.5	6.2	3.2	3.1
Hepatoma 4									
TL	1.4	24.1	5.8	10.6	27.7	10.9	5.0	1.4	4.0
NL	1.5	23.8	6.7	10.5	28.1	11.8	5.2	1.0	4.5
PL	1.0	30.0	3.7	16.6	20.7	8.2	4.4	3.4	1.4
Hepatoma 5									
TL	3.2	30.1	6.8	7.9	29.5	10.4	3.6	0.9	1.7
NL	3.5	30.2	7.2	6.6	32.4	9.1	3.3	1.2	1.6
PL	0.9	29.4	2.8	19.0	19.6	14.4	4.7	0.5	0.8
Liver 1									
TL	0.9	23.3	2.9	15.0	17.5	14.9	6.8	1.2	9.1
NL	1.9	19.5	3.2	8.6	22.0	17.0	7.8	0.7	11.2
PL	0.8	25.5	1.9	23.8	13.1	14.7	4.9	2.1	5.9
Liver 2									
TL	1.5	20.7	3.2	16.8	16.5	16.9	8.6	1.9	7.5
NL	2.1	19.5	3.7	6.2	26.7	17.6	8.4	1.3	8.3
PL	1.0	19.8	2.7	37.7	14.4	8.5	5.0	2.7	3.6
Liver 3									
TL	0.5	23.2	2.7	13.7	18.8	18.4	8.4	1.6	7.3
NL	1.3	24.8	8.3	5.8	31.3	18.2	3.4	0.8	2.6
PL	0.2	21.8	1.7	19.2	10.4	18.1	11.9	2.0	9.3

[a]Percent wt.

[b]Shorthand designation of fatty acids: carbon chain length: number of double bonds. TL = total lipid, NL = neutral lipid, PL = phospholipid, and tr = trace, less than 0.1%.

three liver tissues indicates they are representative of normal liver tissue and serve as valid comparisons for hepatoma tissue. The wide difference, not only between hepatoma and liver tissue, but among the individual hepatoma tissue lipids themselves demonstrates the marked effect of malignancy upon lipid metabolism. The differences in the relative amounts of the major lipid groups and particularly the wide differences in the composition of glycolipid fraction of liver and hepatoma tissue lipids affirm the conclusion drawn from animal studies (15,16) that tumor growth affects the normal regulatory mechanisms of lipid metabolism. Differences in fatty acid composition of tumor, normal, and host tissues also have been reported in other investigations (16-18), but there does not appear to be a consistent pattern to the differences, unless it be that they are dissimilar. The 5,8,11-eicosatrienoic acid (ω-9 20:3) detected in this study probably is produced from the chain elongation and desaturation of oleic acid, as it is in the essential fatty acid deficient rat. The production of this acid normally is suppressed by the preferential interconversion of linoleic acid.

The mechanism that underlies the production or accumulation of large amounts of neutral lipid in the hepatoma tissue is not known. Possibly there is an effect upon lipoprotein synthesis that affects the secretion of triglycerides from hepatoma cells.

Although ether lipids occur in elevated levels in many tumors (19-22), these compounds did not appear to be present in abnormal concentrations in human hepatoma tissues. A similar observation has been made by Wood (23) with a minimum deviation Morris hepatoma.

The glycolipid fraction, although small by comparison to the neutral and phospholipids, differed considerably in composition in liver and hepatoma lipids. A major difference appeared in the gangliosides that separate with this fraction. The composition of the gangliosides was not analyzed, although it is probable

that they consist mainly of the GM_3 species of these compounds inasmuch as they are the least polar and are extracted with chloroform-methanol (24). Brady, et al., (24) and Mora, et al., (25) also observed differences in the ganglioside composition of hepatoma cells, but they found that, in general, the more polar gangliosides, GD_{1A} and GM, were lower than GM_3. That changes in the gangliosides are peculiar to tumor cell growth, in general, is indicated further by the observation of virtually identical differences in the ganglioside composition of the glycolipid fraction of rat mammary tumor and mammary gland in other work performed in this laboratory (W.C. Tan, C. Chapman, and O.S. Privett, unpublished results). Some of the unknown compounds observed in the glycolipid fraction shown in Figure 3 may be degradation products of the missing gangliosides and present in blood, as well as in the hepatoma cells. Hence, it is evident that the serum lipids of subjects with cancer should be examined for similar compounds. Conceivably, the detection of compounds in the blood arising from aberrations in the metabolism of gangliosides could serve as an indication of a precancerous condition. Studies along these lines embodying the isolation and characterization of the glycolipid fraction of blood and a variety of tumors obtained from humans and from animals are in progress in this laboratory.

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Intramolecular Fatty Acid Distribution in Milk Fat Triglycerides of Monkeys[1]

LLOYD M. SMITH and **SUHADI HARDJO**[2], Department of Food Science and Technology, University of California, Davis, California 95616

ABSTRACT

Pancreatic lipase hydrolysis was used to determine the distribution of fatty acids in the milk triglycerides of four species of monkeys and of human milk. The patterns of the major fatty acids were generally similar in all species examined, but there were some differences in the relative concentrations of individual fatty acids esterified at either the 2 or 1,3 positions. Caprylic, stearic, oleic, and linoleic acids were found predominantly at the 1,3 positions; in contrast, lauric, myristic, palmitic, and palmitoleic were concentrated at the 2 position. Monkey milk fats had greater proportions of these acids at the respective positions than did bovine milk fat. Also, the monkey fats were relatively uniform both in total unsaturated fatty acids (41-48%) and in the proportion of these esterified at the 2 position (19-26%). In general, both the fatty acid composition and the specific distribution of fatty acids in the monkey milk fats more closely resembled the patterns in human milk fat than did those in ruminant milk fats.

INTRODUCTION

Both fatty acid composition and specific structure of the triglycerides influence absorption and metabolism of fats, as well as their technology and preservation. The triglyceride structure of milk fats of various species continues to be an active area for research (1). By using pancreatic lipase hydrolysis of triglycerides, the general distribution of fatty acids between the 2 position and the 1,3 positions has been determined for human milk and for milks from several species of ruminants (2,3). More complex enzymatic and chromatographic techniques have made it possible to identify the fatty acid esterified on each of the three hydroxyl groups of glycerol (3).

Because of the importance of nonhuman primates as experimental animals in research significant for human welfare, we have compared the intramolecular fatty acid distribution in four species of monkeys with the distributions in human and ruminant milks. This research is a continuation of our comparative studies of the specific distribution of fatty acids in the triglycerides and diacylglycerophospholipids of milk lipids of interest in human health and nutrition (2, 4-6).

MATERIALS AND METHODS

Milk was obtained as described by Smith and Hardjo (6) from three individuals of each of the following monkey species: *Cercocebus atys* (sooty mangabey), *Macaca fascicularis* (crab-eating macaque), *Macaca mulatta* (rhesus), *Macaca nemestrina* (pigtailed macaque). Human milk was a pooled sample from three mothers and was donated by the Mother's Milk Bank, San Francisco, Calif. Heat treatment of all samples and isolation of the milk lipids have been described (6).

The semimicromethod used for the pancreatic lipase hydrolysis of milk triglycerides was essentially similar to that of Luddy, et al. (7). To 50 mg of triglycerides in a 10 ml screw-cap tube were added 0.1 ml 22% calcium chloride solution and 0.25 ml 0.1% sodium taurocholate solution. Lipid impurities in pork pancreas lipase powder (Mann Research Laboratories, New York, N.Y.) were extracted with diethyl ether (1/10, wt/v), then with acetone (1/5, wt/v), and the purified powder was dried under nitrogen. Enzyme solution (1 ml) containing 9 mg lipase/ml 1 M tris buffer (pH 8), was added to the tube at 40 C. The tightly capped tube was shaken mechanically in a water bath for 2 min. Then the contents were mixed with 0.5 ml 6 N hydrochloric acid and extracted twice with 5 ml diethyl ether. The extracts were combined in a pointed 10 ml tube, and the solvent was removed with a gentle stream of nitrogen.

The lipolytic products were separated on two thin layer plates (20 x 20 cm) coated with a 250 μ layer of Silica Gel G, as described by Luddy, et al. (7). Zones identified as monoglycerides or triglycerides were scraped off the plates, and the lipids were eluted with diethyl

[1]Data taken from thesis of S. Hardjo submitted in partial fulfillment of the requirements for the M.S. Degree in Food Science.

[2]Present address: Bogor Institute of Agricultural Sciences, Bogor, Indonesia.

TABLE I

Pancreatic Lipase Hydrolysis of Triglycerides of Primate and Bovine Milks

Source	Fatty acid, mole %									
	6:0	8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2
Cercocebus atys										
Original triglycerides	1.6	12.2	14.5	5.5	3.2	17.6	6.5	4.5	22.0	11.3
Residual triglycerides	1.3	9.6	11.9	4.8	2.9	17.8	8.0	3.4	22.6	14.9
Monoglycerides	4.4	6.1	15.7	10.5	4.7	24.3	7.9	1.1	6.4	16.9
Percent acid at 2 position[a]	91	17	36	64	49	46	41	8	10	49
Preferential esterification	2–	1,3–	none	2–	2–	2–	2–	1,3–	1,3–	2–
Macaca fascicularis										
Original triglycerides	1.4	9.6	14.6	4.7	2.7	18.9	5.2	4.1	21.6	12.5
Residual triglycerides	1.2	8.6	12.0	3.9	2.4	19.7	8.5	3.7	21.6	14.7
Monoglycerides	2.3	4.9	18.2	9.6	5.9	33.3	7.7	1.1	7.8	8.0
Percent acid at 2 position	44	17	42	68	73	59	49	9	12	21
Preferential esterification	2–	1,3–	2–	2–	2–	2–	2–	1,3–	1,3–	1,3–
Macaca mulatta										
Original triglycerides	1.1	7.7	10.5	3.1	2.1	22.2	7.0	3.9	26.1	13.6
Residual triglycerides	0.9	6.0	8.6	2.9	1.9	20.4	9.6	3.8	23.7	16.5
Monoglycerides	1.6	3.4	14.6	7.2	4.8	39.5	9.4	1.3	9.9	8.7
Percent acid at 2 position	48	15	46	77	76	59	44	11	13	21
Preferential esterification	2–	1,3–	2–	2–	2–	2–	2–	1,3–	1,3–	1,3–
Macaca nemestrina										
Original triglycerides	0.8	6.3	14.1	6.8	3.7	21.5	7.4	4.0	21.3	13.0
Residual triglycerides	0.7	4.8	13.7	6.9	3.5	20.5	8.5	3.9	20.5	13.2
Monoglycerides	0.5	2.3	7.4	13.2	6.9	42.2	11.8	0.9	5.8	8.0
Percent acid at 2 position	21	12	14	65	62	65	53	7	9	21
Preferential esterification	1,3–	1,3–	1,3–	2–	2–	2–	2–	1,3–	1,3–	1,3–
Man										
Original triglycerides	0.4	0.9	2.3	9.7	7.2	20.6	4.1	5.1	36.3	10.7
Residual triglycerides	1.6	1.0	1.4	6.5	5.6	18.8	10.7	6.9	38.2	9.8
Monoglycerides	0.3	0.1	1.0	10.4	12.7	45.9	8.2	0.7	11.0	7.2
Percent acid at 2 position	25	4	14	34	59	74	67	5	10	22
Preferential esterification	1,3–	1,3–	1,3–	none	2–	2–	2–	1,3–	1,3–	1,3–
Cow[b]										
Original triglycerides	3.8	1.9	3.3	3.4	10.6	24.3	4.1	9.6	21.0	3.1
Residual triglycerides	4.2	1.9	3.2	3.3	11.5	25.2	3.2	9.8	22.0	2.7
Monoglycerides	1.3	2.1	3.4	4.6	16.5	28.7	5.6	5.0	15.3	1.4
Percent acid at 2 position	11	37	34	45	52	39	45	17	24	15
Preferential esterification	1,3–	none	none	2–	2–	2–	2–	1,3–	1,3–	1,3–

[a]Calculated from $\frac{M}{3T} \times 100$ = proportion (percent) of fatty acid type esterified at the 2 position, where M is mole percent of the acid in the monoglycerides and T is mole percent of the same acid in the original triglycerides.

[b]Freeman, et al. (2).

ether. Original and residual triglyceride and residual monoglyceride fractions were methylated and analyzed by gas liquid chromatography by the procedures described earlier (6).

RESULTS AND DISCUSSION

The specificity of pancreatic lipase in preferentially hydrolyzing fatty acids esterified at the 1,3 positions of triglycerides generally is accepted (7,8). However, to apply pancreatic lipase hydrolysis to determine the specific positions of fatty acids in triglycerides containing short chain fatty acids, Jack, et al. (9) concluded there must be nonpreferential hydrolysis of a triglyceride species, absence of a substantial amount of complete hydrolysis to free glycerol, and absence of a significant amount of acyl migration during the hydrolytic reaction. Freeman, et al., (2) adopted a short hydrolytic period of 2·5 min to minimize acyl migration in the 2-monoglyceride and destruction of the enzyme. Luddy, et al., (7) also recommended a rapid (1-2 min) reaction time with intense agitation for their semimicrotechnique. In the present work, mixing was less intense during a 2 min digestion period and the degree of hydrolysis was ca. 33% compared to 50% for Luddy, et al.

The results obtained when the milk fat

TABLE II

Distribution of Unsaturated Fatty Acids in Triglycerides of Milk Fats of Various Species as Determined by Pancreatic Lipase Hydrolysis

Source	Unsaturated fatty acids, mole %		Proportion[a] of unsaturated acids at 2 position, %
	In triglycerides	In residual monoglycerides	
Cercocebus atys	41	32	26
Macaca fascicularis	41	25	21
Macaca mulatta	48	28	19
Macaca nemestrina	43	27	21
Man	52	28	18
Man[b]	53	27	17
Cow[b]	33	27	28
Buffalo[b]	31	32	34
Sheep[b]	20	23	38
Goat[b]	27	24	30

[a]Calculated from $\frac{M}{3T} \times 100$ = proportion (percent) of unsaturated fatty acids esterified at the 2 position, where M is mole percent of unsaturated fatty acids in the residual monoglycerides and T is the mole percent of unsaturated fatty acids in the original triglycerides.

[b]Freeman, et al. (2).

triglycerides of primates were analyzed by the above lipolytic technique are indicated in Table I. For each species, the composition of the triglycerides remaining after lipolysis agreed reasonably with that of the original triglycerides. These results indicate there was no significant preferential hydrolysis among the various types of triglycerides present (2,9).

The proportion of each fatty acid at the 2 position in the triglycerides of each milk fat, as calculated by the method of Mattson and Volpenhein (8), also is indicated in Table I. Theoretically, a value of 33.3% would indicate no preferential esterification of a particular acid at either the 2 position or 1,3 positions; but this makes no allowance for experimental error. We have considered that values within the range 28-38% indicate random distribution, as suggested by Smith, et al. (10). Less than 28% of a particular fatty acid esterified at the 2 position indicates that the fatty acid is located preferentially at the 1,3 positions; and, conversely, more than 38% at the 2 position indicates preferential attachment at the 2 position. The 28-38% range is arbitrary and may not fully cover the variability due to use of semi-microsamples and the partial loss of caproic acid or ester during the hydrolytic and analytic steps. The amounts of butyric and linolenic acids were too low for reliable calculation of the proportions present at the 2 position.

The distribution patterns of the major fatty acids in the monkey and human milk fats were similar (Table I). In all species, caprylic acid ($C_{8:0}$) was esterified predominantly at the 1 and 3 positions. It was reasonable to expect that caproic acid ($C_{6:0}$) would follow the same trend, but this was true only for *M. nemestrina* and human milk fats. Lauric, myristic, palmitic, and palmitoleic acids were esterified preferentially at the 2 position, whereas stearic, oleic, and linoleic acids were positioned predominantly at the 1,3 positions. There was no consistent pattern for capric acid ($C_{10:0}$) among the primates.

The results for human milk closely agree with those found previously in our laboratory by Freeman, et al. (2), who used larger samples and different hydrolytic and separation techniques. Their data for bovine milk fat are included in Table I for comparison. There was a generally similar distribution pattern in all the milk fats, but some differences between the monkey and bovine fats were evident in the relative concentration of individual fatty acids esterified at either the 2 or the 1,3 positions of the triglycerides. Monkey milk fat had greater proportions of caprylic, stearic, and oleic acids in the 1,3 positions and of lauric, myristic, and palmitic in the 2 position than did bovine milk fat.

The fat of human milk is absorbed better by human infants than is fat from cows milk. Filer, et al., (11) related digestibility of fats to the arrangement of fatty acids in triglyceride and monoglyceride molecules. They concluded that the relatively large proportion of palmitic acid in the 2 position of human milk fat, as compared to bovine milk fat, was responsible for larger amounts of 2 monopalmitin in the lumen of the intestinal tract and more efficient digestion of human milk fat. It is reasonable to assume that glyceride structure also would be important in determining the absorption and

utilization of fats fed to nonhuman primates.

The monkey milk fats were relatively uniform both in total unsaturated fatty acids (41-48%) and in the proportion of these esterified at the 2 position (19-26%) (Table II). Human milk fat had more unsaturated fatty acids in the triglycerides (52-53%) than did monkey milk fat, but ca. the same proportion was in the 2 position (17-18%). The ruminant milk fats contained less unsaturated fatty acids in their triglycerides (20-33%) but higher proportions at the 2 position (28-38%), compared to the primate milk fats. In general, both the fatty acid composition and the specific distribution of the major fatty acids in the monkey milk fats resembled the patterns in human milk fat more than those in ruminant milk fats.

The pancreatic lipase hydrolytic technique does not differentiate among the fatty acids esterified at the 1 and 3 positions. Techniques to determine the stereospecific distribution of fatty acids in the major molecular species of triglycerides have been reviewed recently by Kuksis, et al. (3). These techniques are difficult and time consuming, but further work in this areas may be justified to explain the complex mechanisms involved in the biosynthesis of milk fat triglycerides in different species of mammals.

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Cholesterol and Bile Acid Turnover in Miniature Swine

JACQUELINE DUPONT, SUK-YON OH, LOUISE O'DEEN, and **MARY ANNE MCCLELLAN,** Department of Food Science and Nutrition, **WILLIAM V. LUMB** and **ARTHUR B. BUTTERFIELD,** Department of Clinical Sciences, and **DUANE J. CLOW,** Department of Mathematics and Computer Science, Colorado State University, Fort Collins, Colorado 80523

ABSTRACT

Miniature swine were fitted with indwelling cannulae at two sites in the gut and catheters in the aorta and portal vein. Ring labeled cholesterol was administered via jejunum and portal vein and various parameters of disappearance measured over 17-66 days. Conversion of cholesterol to bile acids and their subsequent disappearance from gut lumen were measured. Cholesterol disappearance was found to follow a two component exponential in serum and three components in gut. The serum curves were similar to those reported for humans. It was concluded that the minipig is an advantageous model for cholesterol turnover studies and that serum and gut cholesterol dynamics are different.

INTRODUCTION

Goodman and Noble first proposed a model for cholesterol metabolism in humans based upon the kinetics of disappearance of isotopically labeled cholesterol from serum (1). They postulated two pools based upon a two component disappearance curve. Later Goodman, et al., (2) and Samuel and Lieberman (3) reported experiments conducted over longer periods of time which indicated the possibility of three pools based upon a three component disappearance curve. The pools are postulated to represent tissues having different rates of equilibrium of cholesterol with serum cholesterol.

The models of cholesterol metabolism derived from serum cholesterol disappearance assume no loss of cholesterol, except from the fast turnover pool (pool A or 1) (1,2). Conversion of cholesterol to bile acids is considered as loss of cholesterol from pool A. Bile acid metabolism responds to effects of diet (4). Secretion and excretion of bile acids have been found to be greater on a polyunsaturated than saturated fatty acid diet (5,6). The kinetics of conversion of cholesterol to bile acids in the whole animal and enterohepatic circulation (7) may have an influence upon interpretation of cholesterol disappearance data. There is evidence that a substantial bile acid concentration occurs in tissues other than liver and gut (8). In the experiments reported herein, cholesterol disappearance from the serum and gut and bile acid appearance and disappearance in the gut have been monitored in the miniature swine. To obtain these measurements, surgical implantation of indwelling gut cannulae and a vascular catheter were needed. The minipig, similar to man in digestive and cardiovascular systems (9), was large enough for necessary surgical adaptation. The costs of use of a relatively large animal and of surgery necessitated limitation of replication. The objectives of this study were: (A) to determine whether the minipig is an advantageous animal model for studies of turnover of cholesterol; (B) to compare disappearance of cholesterol from serum with the disappearance of cholesterol from the gut; and (C) to observe the time pattern of conversion of cholesterol to bile acids and subsequent disappearance of bile acids from the gut.

METHODS

Hormel derived miniature swine, bred and reared on the Colorado State University campus were used. The original stock was obtained from the University of Missouri (10). The minipigs were kept in outdoor pens and fed a corn-soy swine ration twice a day at 8:00 a.m. and 5:00 p.m. A week before scheduled surgery, each minipig was brought into a barn with controlled temperature and remained there penned individually throughout the rest of the experiment. Three 10-12 month old females were used for the experiments reported herein.

Preanesthetic medication consisted of 0.4 mg fentanyl and 20 mg deoperidol/14 kg (Innovar-Vet, Pitman Moore Co., Washington Crossing, N.J.) and 1 mg atropine/20 kg. Anesthesia was induced and maintained with nitrous oxide-halothane in a semiclosed system. A two stage surgical procedure was employed for chronic intestinal, vascular, and lymphatic sampling. A 30 cm ventral midline incision provided exposure of gut cannulation sites during first stage surgery. Eliptical sections of gut wall were excised and Thomas-type kynar (Kynar [vinylidene fluoride resin], Pennwalt Chemical Corp., Philadelphia, Pa.) cannulae inserted just distal to the duodenojejunal junction and ca. 10 cm proximal to the ileocecal valve. Following insertion, a 5-0 silk whipstitch

brought cut margins of gut wall into apposition with implanted cannulae. A rectangular strip of autogenous peritoneum was sutured over implant sites to prevent leakage. Gut cannulae exited through the ventral abdominal wall such that collection of ingesta could be performed with the animal in standing or recumbant positions. The abdomen was closed with 00 surgical steel sutures placed in a 3-layer simple interrupted pattern. Second stage surgery was performed 14-21 days after gut cannulation. Animals were positioned in left lateral recumbency; the cysterna chyli and vessels to be catheterized were approached through a parabolic incision in the right lateral abdominal wall. The abdominal aorta, portal vein, posterior vena cava, hepatic vein, and cysterna chyli were catheterized in step-wise fashion and the polyvinyl catheters stabilized at vessel wall with surgical silk. A large metal trocar was driven from abdominal cavity to emerge at the dorsal midline. Implanted catheters were passed through the trocar, trocar withdrawn, and exteriorized catheters placed in a plastic pouch sutured to the pig's back. Abdominal closure was identical to that described for first stage surgery.

When injections and sampling were conducted, the pig was penned in a cage which did not permit walking or turning, but allowed standing, lying, eating, and other motion. The pigs entered the pen voluntarily and were under no stress during the sampling periods.

The three minipigs weighed 60-68 kg at the time of surgery. They returned to preoperative wt within 10 days, and radioactive die-away experiments were begun at 10, 18, and 23 days postsurgery for the three animals. Their wt remained stable or showed a slight gain for the duration of the experiment.

Cholesterol-4-^{14}C (0.15 mCi/mg) was dissolved in ethanol, and 250 μCi was given via the gut. Cholesterol 1,2-^{3}H (130 mCi/mg) was dissolved in 0.2 ml ethanol and incubated at 37 C overnight in 2 ml plasma from the recipient pig (11), and 1 mCi was given via the portal vein. Radioisotopic compounds were purchased from New England Nuclear Corp., Boston, Mass., and used without further purification.

Blood samples were centrifuged without heparin, and serum was removed and stored at -20 C for analysis. Lipids were extracted by the Folch-Pi procedure (12), using an aqueous $CaCl_2$ wash, saponified, and digitonin precipitable sterols (13) were quantitated using the Zak reagent (14). Aliquots of the sample were dissolved in methanol and ^{14}C and ^{3}H determined by differential counting on a Nuclear-Chicago Unilux II liquid scintillation system equipped with external standardization. The scintillation solution consisted of 6 g Omnifluor (New England Nuclear Corp.) in 1 liter toluene. Appropriate corrections were made for quenching and dual label counting, when indicated.

Total cholesterol and bile acids of gut contents were determined by the procedure described by Manes and Schneider (15). The sampled jejunal and ileal contents were dried under air flow and ground in a mortar. The dried samples were extracted with 0.5N HCl in absolute ethanol. The pooled extract was dried under air flow and then saponified in 20% KOH in ethylene glycol at 220 C. The neutral sterols (nonsaponifiable material) were extracted with petroleum ether. Cholesterol was analyzed in the same manner as used for serum cholesterol.

The saponified residues were acidified to pH 2 with 6N HCl and extracted 3 times with diethyl ether into a preweighed small vial. The bile acid content was determined gravimetrically, and radioactivity was determined in the same manner as for cholesterol.

The raw data were fit using a computer program which utilized the stripping process developed for fitting multicomponent exponential curves. This program uses the criteria of least squares, and the program is capable of evaluating the model:

$$\text{Specific activity} = \sum_{i=1}^{n} B_i e^{-\lambda_i t},$$

where n refers to the number of exponential components present (n=1,2,3) in the data; β_i is the ordinate intercept of the i^{th} component; and λ_i is estimated by the slope of the linear portion of the i^{th} component and further $\lambda_i = 0.693/T_{i½}$ where $T_{i½}$ is the half-time of the i^{th} component.

RESULTS AND DISCUSSION

The paucity of data in the literature made it necessary for us to conduct trial and error experiments on sampling time.

In one experiment (Pig 1) cholesterol-1,2-^{3}H and cholesterol-4-^{14}C were administered simultaneously via the portal vein and the gut, respectively. The peak serum activity for ^{14}C administered via gut occurred at 32 hr. Cholesterol administered via portal vein (^{3}H) peaked at 30 min. Subsequent 12 day die-away curves (Fig. 1) computed with data for time $t>32$ hr yielded single component curves with half lives of 95.9 and 101.4 hr for ^{14}C and ^{3}H, respectively. These values are not significantly different from each other and appear to be descrip-

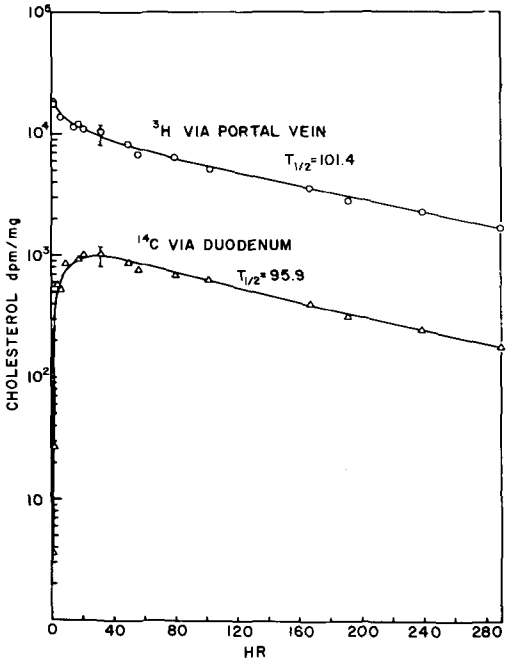

FIG. 1. Disappearance curves for aortic serum cholesterol administered simultaneously as 1,2-^{3}H-cholesterol via the portal vein and 4-^{14}C-cholesterol via the jejunum. Ratio of ^{3}H to ^{14}C was 4.73; actual values are reported.

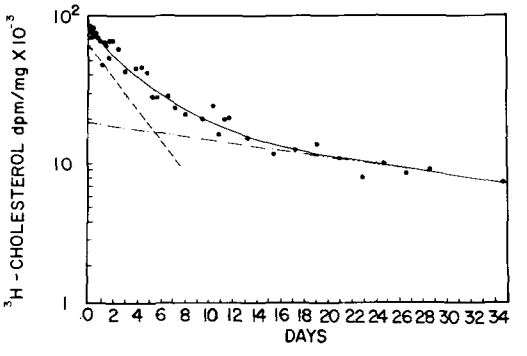

FIG. 2. Disappearance curve for serum cholesterol administered via the portal vein.---- $t_{1/2}$ = 72.2 hr and -●●-●●- $t_{1/2}$ = 605.5 hr.

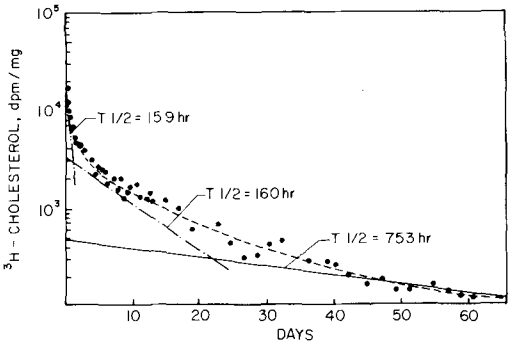

FIG. 3. Disappearance curve for jejunal cholesterol administered via the portal vein.

tive of a homogeneous pool. This same experiment revealed that, if sampling were begun within 30 min of administration of the tracer and continued at 30 min intervals for 48 hr, a rapidly disappearing exponential was defined. This is illustrated in Figure 1 (^{3}H via portal vein) and jejunal cholesterol had exponentials of 0.12 and 0.32 hr for ^{3}H and ^{14}C, respectively. When sampling was not begun until 3 hr postinjection, no such exponential was defined. We concluded that the very rapidly disappearing component was indicative of equilibration of the tracer with the blood and gut pools of cholesterol. Subsequently, sampling was begun 3 hr postinjection to eliminate this component.

Another aspect of sampling time is meal eating. In human studies (1-3), sampling was conducted at weekly intervals with fasting subjects. Venipuncture is stressful to the minipig, hence the use of an indwelling catheter which made sampling at frequent intervals possible. Frequent sampling made it undesirable to fast the animals prior to sampling. Each die-away experiment was begun 2 hr postprandially. Sampling was conducted every 3 hr for 48 hr, every 12 hr-14 days, then every 2 days until termination of the experiment. Following the 48 hr intensive sampling period, the time of sampling was 10:00 a.m. and 10:00 p.m. The pigs were meal fed to maintain wt, so these samples were taken 2 and 5 hr postprandially. Later samples were taken at 10:00 a.m. The data shown indicate greater variation from sample to sample than in the human studies (1-3), and this may be attributed to the more frequent sampling and variation due to digestion.

An illustration of a serum die-away curve is shown in Figure 2. The solid line is a least squares computer fit and the broken lines are drawn to show the half lives of the various components as shown in Table I (Pig 3). Table I shows four serum die-away analyses. The data from the 12 day curves were not for a long enough period to show two components. The mean of 4 observations of the faster component of the serum cholesterol die-away was 3.47 days (Table I, exponential 2). This compares quite closely with the mean of 3.33 days by Goodman, et al., (2) for three normal and three type IV hyperlipidemic human subjects. The range of values which they reported (2.02-4.09 days) also compares well with our minipig values

TABLE I

Die-Away of Serum, Jejunal, and Ileal Cholesterol in Miniature Swine

Pig	Duration of sampling	Tracer	Half-time of exponential 1	2	3
	Days			Hr	
		Serum cholesterol			
1	12	4-^{14}C		95.9	
1	12	1,2-^{3}H		101.	
2	37	1,2-^{3}H		63.9	343.
3	35	1,2-^{3}H		72.2	606.
		Mean ± standard error		83.2 ± 9.0	
		Jejunal cholesterol			
1	17	4-^{14}C	12.2	123.	
1	17	1,2-^{3}H	8.1	96.6	
2	42	1,2-^{3}H	10.3	65.3	441.
3	66	1,2-^{3}H	15.9	160.	753.
		Mean ± standard error	11.6 ± 1.6	111 ± 20.1	
		Ileal cholesterol			
1	17	4-^{14}C	5.3	160.	
2	15	1,2-^{3}H	10.4	88.	
3	66	1,2-^{3}H	14.8	74.1	823.
		Mean ± standard error	10.2 ± 2.7	107 ± 26.6	

(2.66-4.21). When sampling was continued for 35 and 37 days, a second serum component was defined, (exponential 3, Table I). The number of observations was insufficient for comparison to human data, but the values are reasonably close to those for humans (2).

An additional observation which can be made from these data is an estimate of cholesterol absorption. Zilversmit (16) has shown that in an experiment in which ^{3}H and ^{14}C cholesterol are administered simultaneously, the ratios (adjusted for equal radioactivity) of ^{3}H/^{14}C in serum from 24 hr to several days later indicate adsorption. Such a calculation using the data shown in Figure 1 indicates 41.5 ± 3.8% absorption by this pig. The variation indicates values for the 12 days of the experiment. In rats, Zilversmit reported a mean absorption of 48.5% determined by the plasma iostope ratio and 54.9% determined by fecal isotope analysis. The minipig was consuming a diet practically devoid of cholesterol, so this absorption represents enterohepatic cycling.

Disappearance of tracer cholesterol from the gut is illustrated by the die-away curve in Figure 3. The components of this curve are different from those shown by the serum. There were 3 components within the time period 3 hr-66 days. One of these components differed significantly from any values observed for serum (Table I, exponential 1 [1-3]). This component had a mean half-life of 11.6 hr when measured in jejunal contents and 10.2 hr for the ileal contents. Samples were obtained at the same time intervals as for the serum, so the fast component was not overlooked in serum by failure to obtain samples.

There appears to be no difference in the specific activity of cholesterol obtained from the jejunum and ileum. The two slower components of the gut cholesterol resemble in half-life the two components observed in serum (Table I, exponentials 2 and 3). The major difference lies in the time of intersection of the two exponentials. For serum this was ca. 5 days and for gut ca. 22 days.

The experiment with Pig 1, in which cholesterol-1,2-^{3}H and cholesterol-4-^{14}C (4.73 ratio) were administered simultaneously via portal vein and gut, respectively allowed a test of radiohomogeneity of the tracers in conversion of cholesterol to bile acids. The ratio of ^{3}H/^{14}C of serum cholesterol was constant from 3-12 days. In bile acids the ratio also was constant from 3-12 days. The mean ratio in bile acids was 1.77 ± 0.17 and serum cholesterol 1.96 ± 0.18, indicating that the bile acids were derived from the absorbed cholesterol, as would be expected. This indication of radiohomogeneity suggests that the harsh hydrolysis procedure

for isolating bile acids did not affect the stability of the 1,2-^{3}H label.

Appearance of ^{3}H-bile acids derived from 1,2-^{3}H-cholesterol in the jejunum and their subsequent disappearance is illustrated in Figure 4. In a preliminary experiment when jejunal sampling was begun 15 min following intravenous injection of cholesterol, substantial bile acid synthesis had occurred, and the bile acids had appeared in the jejunum. In Figure 4 are shown bile acid labeling patterns in which sampling began 3 hr after administration of tracer. Following the point of reaching maximum specific activity, there was a plateau for ca. 5 and 15 days before die-away began. This indicates conversion of cholesterol to bile acids at the same rate as disappearance of the bile acids. The specific activity of serum cholesterol (Figure 2) remained higher than that of jejunal bile acids throughout the time of the experiment. When die-away commenced, the curve exhibited only one component with a half-life of 8 and 19 days for the 2 pigs, respectively. Half-life values were somewhat longer than those previously reported (4,17,18) which were determined by using labeled bile acids. Lack and Weiner (19) have shown a two component die-away curve for bile acids, suggesting a rapidly equilibrating pool representing enterohepatic circulation and another relatively slowly equilibrating pool representing colonic bile acids. With only two observations and the use of tracer cholesterol rather than bile acids, the nature of the curves cannot be interpreted conclusively.

The conclusions drawn from these studies are: (A) the miniature swine is a desirable animal model for the study of cholesterol turnover; (B) disappearance curves for cholesterol from serum and gut are not the same; and (C) bile acid synthesis from tracer cholesterol occurs within 15 min after administration, reaches a maximum within 6 hr, then maintains an equilibrium for several days before die-away commences.

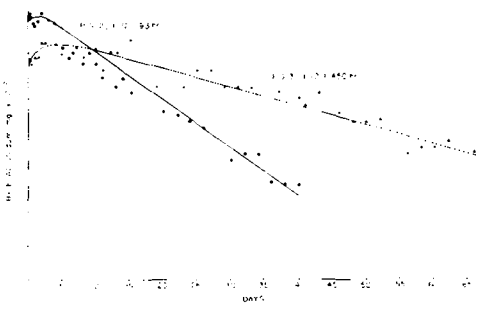

FIG. 4. Appearance and disappearance curves for jejunal bile acids formed from 1,2-^{3}H-cholesterol administered via the portal vein.

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Biosynthesis of Anacardic Acids from Acetate in *Ginkgo biloba*

JOANNE L. GELLERMAN, WAYNE H. ANDERSON, and **HERMANN SCHLENK,**
The Hormel Institute, University of Minnesota, Austin, Minnesota 55912

ABSTRACT

Anacardic acids synthesized from [1-^{14}C] and [2-^{14}C] acetates by *Ginkgo biloba* were subjected to chemical degradation to locate the ^{14}C in the molecules. Radioactivity in the carboxyl group was determined by decarboxylation and counting the resulting CO_2; in the aliphatic chian, by oxidation of anacardol from which three homologous fatty acids are obtained; and in ring C-6, chain C-1 and chain C-2 by decarboxylation of these fatty acids where the carboxyl groups represent these respective C atoms. Distribution of ^{14}C in the anacardic acids indicates that synthesis of the chain and of the ring are separate processes. According to its radioactivity, ring C-6 originates from the chain. A polyketide mechanism appears likely for the ring synthesis, although considerable randomization of 1-^{14}C and of 2-^{14}C from the acetates had occurred in the plants.

INTRODUCTION

Anacardic acids are salicylic acids substituted in position 6 with a saturated or unsaturated n-C_{13}, n-C_{15}, or n-C_{17} chain (1-4). (Abbreviations, such as An-13:0, are used to specify n-tridecyl or other alkyl chains of the 6-alkylsalicylic (anacardic) acids, applying the abbreviated nomenclature common for fatty acids.) A prototype for such structures, 6-methylsalicylic acid, occurs in some microorganisms and in barley (5,6). Anacardic acids so far have been encountered only in a few higher plants, namely in Anacardiaceae and in *Ginkgo biloba*. For the biosynthesis of these acids, one may anticipate that the aliphatic chains are furnished by fatty acids (7), but the ring may be formed by a polyketide or a shikimic acid pathway. Both are known for biosynthesis of phenolic compounds that are not of terpenoid type (8,9). A polyketide mechanism has been suggested, without experimental evidence, for synthesis of the ring of anacardic acids (8). However, this may be questioned in view of the occurrence of shikimic acid, particularly in *Ginkgo biloba* (10,11).

The purpose of the work reported here was to investigate if the ring is formed in direct conjunction with fatty acid synthesis or independent of that process and to investigate if one of the above pathways leading to aromatic compounds may apply to the synthesis of the salicylic moiety in anacardic acids.

MATERIALS AND METHODS

The preparation of labeled anacardic acids by infusion of [1-^{14}C] or [2-^{14}C] acetate into young plants of *Ginkgo biloba* has been reported in detail (12). The products used here are described in Table I.

Gas chromatography (GLC) of fully methylated aliquots of mixed anacardic acids showed that contamination with fatty acid methyl esters did not exceed 0.01% in composition and 0.2% in radioactivity. Chemical composition of anacardic acids in samples 1 and 2 was similar: 3.6%, An-13:0; 56.3%, An-15:1; and 40%, An-17:1. These acids contained 6.6%, 70.0%, and 20.5% (sample 1) and 5.1%, 65.8%, and 27.4% (sample 2) of the total activities.

Double bonds in the aliphatic chains of anacardic acids, their methylated derivatives, or of anacardols were hydrogenated in ethyl acetate by treatment with H_2 at atmospheric pressure for 1 hr over 10% palladium on $CaCO_3$ (K&K Laboratories, Plainview, N.Y.). From sample 3, An-13:0, An-15:0, and An-17:0 were separated as methyl esters by liquid-liquid chromatography in acetonitrile over silicone oil as stationary phase (7). The methyl esters were converted to acids. Samples of 100 mg containing at least 100,000 cpm were sufficient to carry out the whole sequence of reactions.

Chemical Degradations

The scheme of reactions is given in Figure 1, and the procedures are described in that sequence.

Decarboxylation of anacardic acids: Samples of 30-60 mg were mixed with 15-30 mg Cu powder (Schaar & Co., Chicago, Ill.) and placed into a two vessel apparatus similar to that described for wet combustion of ^{14}C labeled materials and absorption of $^{14}CO_2$ (13). The vessels, 25 ml volume each, for decarboxylation and absorption were equipped with magnetic stirrers. The apparatus was evacuated repeatedly and flushed with N_2, and the pressure was adjusted to 140 mm Hg before closing the system. Decarboxylation was complete after 1 hr at 200 C, but an additional hr was given for absorption in a measured amount of aqueous

TABLE I

Labeled Anacardic Acids from *Ginkgo biloba*

Sample	Precursor infused	Time (days)	Anacardic acids[a]	
			Percent ^{14}C incorporated	dpm/mmole
1	[1-^{14}C]acetate	1.5	0.22	3.4×10^6
2	[2-^{14}C]acetate	7	0.85	9.8×10^6
3	[1-^{14}C]acetate	9	0.51	31.5×10^6

[a]Biological dilution factors were between 1.4×10^3 and 5.2×10^4.

$Ba(OH)_2$ or in 0.5 ml 1M Hyamine 10-X in methanol (Packard Instruments, LaGrange, Ill.).

In decarboxylation of nonlabeled anacardic acids, CO_2 was measured by titrating the excess of $Ba(OH)_2$. The average yield from 7 samples was 95.2%, with a maximum deviation of 1.7%. Radioactive measurements (discussed below) were referred to this yield to calculate ^{14}C in the anacardic acid carboxyl group.

The products of the decarboxylation reaction were taken up in $CHCl_3$ and filtered from the catalyst. Pure anacardol was obtained, after removal of the solvent, in a yield of 60% by distillation at 180 C/50μ Hg (14). The distillation residue contained some anacardyl anacardate which was not used for further degradation. Isolation by thin layer chromatography, IR spectrum, saponification to anacardol and anacardic acids, R_f values, fluorescence, and oxidative coupling with 3-methyl-2-benzothiazolinone hydrazone (15) proved the identity of the constituents and of the original ester as alkylphenyl anacardate.

Oxidation of anacardol: A procedure similar to that described for oxidation of polyhydroxyfatty acids was used (16). Between 30-60 mg anacardol was dissolved in 1 ml purified glacial acetic acid. Ca. 130 mg CrO_3, wetted with a drop of water and then dissolved in 3 ml acetic acid, was added. After stirring for 16 hr at 25 C, excess CrO_3 was reduced by addition of aqueous $NaHSO_3$. Fatty acids were extracted with diethyl ether, esterified with CH_2N_2 (17), and the methyl esters were purified by TLC on Silica Gel H (E. Merck A.G., Darmstadt, Germany), using hexane:diethyl ether:acetic acid, 80:20:1, v/v. They were analyzed, preparatively separated by GLC, and saponified.

Decarboxylation of fatty acids: The reaction was carried out with hydrazoic acid in 100% H_2SO_4, as described previously (18). The average yield of CO_2, as determined with nonradioactive fatty acids, was 92%, and this was taken as basis for calculating percent molar activity in the carboxyl group.

GLC of methyl anacardate methyl ethers and of fatty acid methyl esters and determinations of radioactivity were carried out, as previously described (12,18).

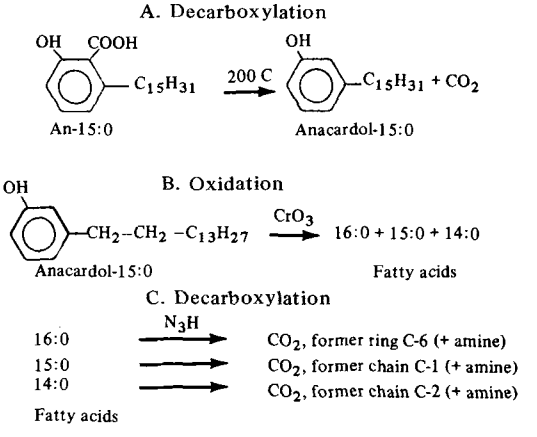


FIG. 1. Scheme for degradation of anacardic acids (example, An-15:0). A = decarboxylation, B = oxidation, and C = decarboxylation.

RESULTS AND DISCUSSION

Distribution of ^{14}C in the three samples of anacardic acids is shown in Table II, together with values calculated for even labeling of alternate carbon atoms. In all samples, activities are higher in the ring and carboxyl group and are lower in the chain than the calculated values. The difference in labeling indicates that at least two different systems are involved in the synthesis of anacardic acids, one of them forming the chain and one forming the ring.

More detailed results were obtained from the individual acids, An-15:1 and An-17:1 (sample 3), where degradations were carried through step C. As shown in Figure 1, three fatty acids were obtained by oxidation of anacardol-15:0. Their relative amounts were 16:0, 60%; 15:0, 30%; and 14:0, 10%, while shorter chain fatty acids did not arise. Anacardol-17:0 yielded 18:0, 17:0, and 16:0 acids in the same proportions. By decarboxylation of the individual fatty acids, activities of ring C-6, chain C-1 and

TABLE II

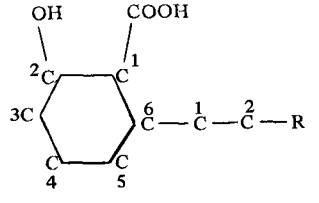
Distribution of ^{14}C in Anacardic Acids from Specifically Labeled Acetates

Part of molecule	Percent molar activity[a]					
	Sample 1	Sample 2	Sample 3			
			An-15:1		An-17:1	
	Percent ^{14}C/moiety	Percent ^{14}C/moiety	Percent ^{14}C/moiety	Percent ^{14}C/C	Percent ^{14}C/Moiety	Percent ^{14}C/C
Total	100	100	100	(9.1)	100	(8.3)
Anacardol	88.7(91.2)	96.4(100)	85.6(90.9)		87.2(91.7)	
Chain[b]	60.6(70.1)	49.4 (71)	48.7(69.1)	(6.4)	53.0(71.7)	(6.2)
Ring[b]	28.1(21.1)	47.0 (29)	36.9(21.8)	(15.4)	34.2(20.0)	(14.2)
Carboxyl	11.3 (8.8)	3.6 (0)		14.4		12.8
Ring C-6[c]				5.0		4.8
Chain C-1[c]				1.5		1.0
Chain C-2[c]				7.0		5.0

[a]Values in parentheses are theoretical for even labeling of alternate C atoms.

[b]60% of ring C-6 is counted with the fatty acids resulting from oxidation (see text).

[c]



chain C-2 in the original anacardic acids were determined.

The differences in chain C-1 and C-2 indicate an alternating pattern of labeling as expected for fatty acid synthesis from [1-^{14}C] acetate. Ring C-6 may originate with synthesis of the chain or of the ring. The radioactivities found in this carbon atom, 5.0 and 4.8% ^{14}C, are more consistent with those calculated from ^{14}C in the chains than in the rings (Table II). It seems likely that, in anacardic acids, the carboxyl of the precursor fatty acid becomes a member of the ring as it is the case with the carboxyl of acetic acid in the biosynthesis of 6-methylsalicylic acid (6,19). Fatty acyl would be the primer compound instead of acetyl and would introduce the odd numbered alkyl chain via a polyketide type synthesis.

The assumption of 2 carbon elongations of a fatty acid, followed by cyclization, finds support from radioactive data on the anacardic carboxyl group. Radioactivity in it is much higher from [1-^{14}C] than from [2-^{14}C] acetate (Table II), and the levels in samples 1 and 3 are compatible with radioactivities calculated for alternate labeling of the ring.

Chain C-1 of sample 3 and the carboxyl group of sample 2 should not be radioactive when the synthesis exclusively uses acetate units as they have been supplied. These C atoms contain some radioactivity, and our results cannot be taken as final proof for a polyketide synthesis. However, reactions of acetate which are not obligatory for the synthesis of anacardic acids may cause some randomization during experiments which lasted for 9 days. In more recent experiments (to be published), selected tissue from ginkgo synthesized anacardic acids much more efficiently than the whole plant and allowed shorter experimental periods. Radioactivity was incorporated from acetate and malonate with yields from 6-12%. Neither shikimic acid nor phenylalanine was incorporated into anacardic acids, which is an indication against the shikimic acid pathway (20). According to present data, it appears that the salicylic moiety of anacardic acid is synthesized from acetate via malonate.

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SHORT COMMUNICATION

Synthesis of Spin-Labeled Neutral Lipids: Nitroxyl Derivatives of Triglycerides and Sterol Esters

ABSTRACT

Methods for the preparation of useful spin-labeled neutral lipids are described. A spin-labeled triglyceride has been prepared by acylation of 1,3-distearoylglycerol with stearic acid anhydride bearing the 4′,4′-dimethyloxazolidine-N-oxyl ring at carbon-12. The same fatty acid anhydride has been used to acylate the 3-hydroxy group of cholesterol to obtain a cholesteryl ester with the nitroxyl function in the fatty acyl chain. The 4′,4′-dimethyloxazolidinyl-1-oxyl derivative of 5α-androstan-3-one-17β-ol has been esterified with stearic acid anhydride to obtain a steroid ester with the paramagnetic center in the steroid nucleus.

INTRODUCTION

The application of the spin-label method (1,2) to the structure and function of membranes (3-5) has depended heavily upon the design and synthesis of lipid molecules which could be introduced into the system of interest. Some of the earliest lipids prepared for this purpose included nitroxyl derivatives of the cholestane (6) and androstane (7) steroid nuclei. The synthesis of these derivatives was made possible by the development of a method for attaching the 4′,4′-dimethyloxazolidinyl-1-oxyl (doxyl) moiety to a ketonic carbon atom (6). An important feature of such derivatives is that their oxazolidine rings possess no motion independent of the carbon skeleton to which they are attached. Consequently, these rings have a fixed geometry, and the orientation of the 2 $p\pi$ orbital (which contains the free electron) relative to the host molecule is well defined. Oxazolidinyl derivatives of numerous keto fatty acids have been synthesized, and these derivatives have been incorporated subsequently into phosphatidylcholine (8) and phosphatidylethanolamine (9). Most of the spin-labeled lipids used to date have been relatively polar. Our need for chemically well defined neutral lipids of the triglyceride and sterol ester classes for the study of the more nonpolar regions of plasma lipoproteins (10) necessitated the preparation of these materials which we now report.

EXPERIMENTAL PROCEDURES

Spin-labeled fatty acids were obtained by hydrolysis of the corresponding esters in dioxane/aqueous sodium hydroxide and converted to their anhydrides by dehydration with dicyclohexylcarbodiimide in carbon tetrachloride (8). Spin-labeled derivatives of fatty acid methyl esters were prepared by m-chloroperbenzoic acid oxidation of the corresponding oxazolidine (6). Starting ketoesters were synthesized by standard methods (8). Spin-labeled androstol was prepared as described by Hubbell and McConnell (7).

The spin-labeled triglyceride (I) (Fig. 1) was prepared by the following three step synthesis. Stearic acid (28.4 g, 0.1 mole) and dihydroxyacetone (4.5 g, 0.05 mole) were dissolved in 150 ml dry, freshly distilled pyridine. To this solution was added dicyclohexylcarbodiimide (20.6 g, 0.1 mole) dissolved in 50 ml freshly distilled chloroform. Dicyclohexylurea precipitated within sec of mixing. After 48 hr, the precipitate was removed by filtration. The filtrate was subjected to rotary evaporation to remove chloroform, then shell frozen, and lyophilized to remove pyridine. The resulting solid mass was broken up, dissolved in hot acetone, and allowed to crystallize at room temperature. The colorless, crystalline 1,3-distearoxyacetone (19.8 g, 74%) had an R_f on thin layer chromatography (TLC) (Eastman chromagram sheets) of 0.90 in chloroform and 0.51 in benzene-chloroform (3:1), melted at 84-86 C (lit [11], 87.0-87.5 C), and gave an IR spectrum (KBr

wafer) exhibiting carbonyl absorption at 1730 cm^{-1}. In 20 ml dry tetrahydrofuran was dissolved 1.25 g (2 mmoles) 1,3 distearoxyacetone. Occasionally, it was necessary to heat the mixture gently to obtain complete solution. To this stirred solution at room temperature was added 2.5 ml (2.5 mmoles) diborane in tetrahydrofuran (1.0 molar in BH_3, Alfa Inorganics, Beverly, Mass.). The reaction was stirred for 18 hr after which time excess diborane was destroyed by adding 3 ml water. Tetrahydrofuran was removed by rotary evaporation leaving a white residue in water. Acetone (20 ml) was added, the residue was dissolved by heating, then set aside for crystallization at 20 C. The product (1,3-distearoylglycerol, 0.98 g, 79%) melted at 76-78 C (lit [12] 79.5 C) migrated as a single spot on TLC with an R_f of 0.75 in chloroform and 0.38 in hexane-ethyl acetate (6:1). No indication of the presence of 1,2-distearoylglycerol was observed with the latter solvent system which resolves the 1,3- and 1,2-isomers (13). The IR spectrum (KBr wafer) exhibited absorption bands at 3400 cm^{-1} (OH) and 1735 cm^{-1} (C=O). An alternative route to symmetrical 1,3-diglycerides has been published (13). This material (225 mg, 0.36 mmoles) was acylated with 12-doxyl stearic (m = 5, n = 10) anhydride (225 mg, 0.33 mmoles) in the presence of sodium oxide by a method similar to that of Robles and Van den Berg (14). (The procedure described by Robles and Van den Berg [14] calls for a three- to four-fold excess of anhydride. In the present work, equimolar anhydride and alcohol were used which, though giving lower yields, results in better overall incorporation of the spin-labeled fatty acyl chains present.) The crude product was purified partially by chromatography on a column of silica gel (Brinkmann Instruments, Westbury, N.Y.) equilibrated and eluted with methylene chloride. Purification to homogeneity was accomplished by prepatative TLC on plates of silica gel containing a fluorescent binder. The developing solvent was diethyl ether-hexane (3:7). The desired material was located by visualization with UV light. The appropriate band was scraped from the plate, transferred to a small column, and the desired material eluted from the absorbent with chloroform. The yellow product (5,10-I, 49 mg, 15%) migrated on TLC with an R_f of 0.39 in ether-hexane (3:7). Its IR spectrum (film on sodium chloride plates) exhibited strong absorption at 1735 cm^{-1} (C=O), but none at 3400 cm^{-1} (no OH). It became semicrystalline below ~10 C. Anal. calculated: $C_{61}H_{116}NO_8$: C, 73.88; H, 11.79; N, 1.41; 0, 12.91, Found: C, 73.84; H, 11.36; N, 1.75.

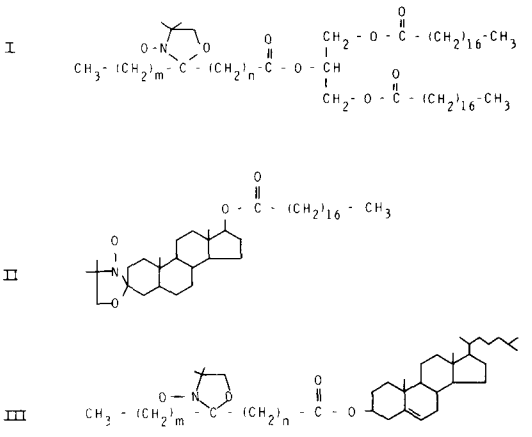

FIG. 1. Chemical structures of spin-labeled neutral lipids: 4',4'-dimethyloxazolidinyl-N-oxyl derivatives of (I) tristearin, (II) androstan-17-yl stearate, and (III) cholesteryl stearate.

The stearic acid ester of spin-labeled androstol (II) was prepared by acylation of spin-labeled androstol with stearic anhydride in anhydrous pyridine. The alcohol (95 mg, 0.25 mmole) and anhydride (275 mg, 0.50 mmole) were transferred to a 25 ml round bottom flask. Upon addition of dry pyridine (4 ml), the reaction mixture was stirred at room temperature and monitored by TLC. After 72 hr, the rate of ester formation appeared minimal. Pyridine was removed by rotary evaporation and the residue was dissolved in toluene and applied to a column of SilicAR CC-4 (Mallinckrodt, St. Louis, Mo.) equilibrated with toluene. Excess anhydride was eluted with toluene. The desired ester was eluted with methylene chloride while the alcohol remained adsorbed. The IR spectrum (KBr water) of the light yellow solid exhibited a strong band at 1727 cm^{-1}, migrated on TLC with an R_f of 0.82 in chloroform, melted at 122-124 C, and was obtained in a yield of 87 mg (54%). Anal. calculated: $C_{41}H_{72}NO_4$: C, 76.57; H, 11.29; N, 2.18; 0, 9.95, Found: C, 76.64; H, 11.01; N, 2.15.

A spin-labeled fatty acid cholesteryl ester (III; m = 5, n = 10) was synthesized by acylation of cholesterol with the fatty acid anhydride in pyridine. The anhydride (1.55 g, 2.06 mmoles) and cholesterol (1 g, 2.59 mmoles) were dissolved in 5 ml dry pyridine and stirred at room temperature. The slow formation of the desired ester was monitored for 7 days after which time solvent was removed by rotary evaporation and the residue was dried in vacuo overnight. The solid orange mass was dissolved in hexane and applied to a 2.5 x 30 cm column of silica gel (Brinkmann Instruments) equilibrated with hexane. The desired ester was

eluted with hexane:ether (95:5). TLC gave one spot with R_f = 0.58 (hexane:ether, 7:3). The viscous golden liquid exhibited an IR absorption band at 1740 cm^{-1} and was obtained in a yield of 0.87 g (36.5%). Anal. calculated: $C_{39}H_{86}NO_4$: C, 78.13; H, 11.51; N, 1.86; 0, 8.50, Found: C, 78.32; H, 11.62; N, 1.63.

JOEL D. MORRISETT
Department of Medicine
The Methodist Hospital
Baylor College of Medicine
Houston, Texas 77025

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LETTER TO THE EDITOR

Convenient Method for Concentration of Esters Prior to Gas Liquid Chromatographic Analysis

Sir: In the preparation of lipid samples for gas liquid chromatographic analysis, a recurring problem is the extraction and concentration of esters from the esterification mixture prior to injection. Often the amount of reagent is limited so as to permit injection of the reaction mixture directly; however, when working with unknown quantities of lipid extracts, this can lead to samples too dilute to analyze properly or with overload of the reagents with lipid.

For years we have used a convenient procedure which is applicable to a wide variety of esterification methods and sample concentrations. When water soluble reagents, for example 2N sodium methoxide or HCl in methanol (S.W. Christopherson, and R.L. Glass, *J. Dairy Sci.* 52:1289 [1969]), are used, the amount of reagent does not have to be limited. The sample is routinely evaporated under reduced pressure prior to the addition of reagents. When the reaction is complete, the mixture is transferred to a Babcock milk fat test bottle with a Pasteur pipette. The reaction vessel then is washed several times with small portions of hexane which are also transferred to the Babcock bottle. The bottle is then half filled with water, shaken vigorously, filled with water well into the narrow neck, and then centrifuged for 10 min. The esters are now either injected immediately or the hexane layer is removed with a Pasteur pipette for storage or for evaporation under a stream of nitrogen prior to injection. Babcock test bottles are available at low cost from most laboratory supply firms.

ROBERT E. PITAS
ROBERT G. JENSEN
Department of Nutritional Sciences
The University of Connecticut
Storrs, Connecticut 06268

[Received May 13, 1974]

Effects of Dietary Cholesterol upon Bile Acid Metabolism in Guinea Pig

HELEN I ^NSMA[1] and **ROSEMARIE OSTWALD**, Department of Nutritonal Sciences, University of California, Berkeley, California 94720

ABSTRACT

Cholesterol fed guinea pigs develop a hemolytic anemia accompanied by high cholesterol concentrations in the liver, plasma, and red cells. We have studied the bile acid metabolism of guinea pigs fed a diet with or without cholesterol in a search for the factor(s) which prevent adequate control of their body cholesterol pool and, therefore, its pathological consequences. The results show that in the cholesterol fed guinea pig the synthesis (and excretion) of bile acids was at least three times greater than in controls. This is the result of a doubling of the fractional turnover rate and a smaller increase in the pool size. The major increase of the bile acid pool was in the liver. The main bile acid in gall bladder bile and small intestines was chenodeoxycholic acid, with smaller amounts of 7-ketolithocholic and ursodeoxycholic acids. In the caecum, large intestines, and feces, the major bile acid was lithocholic acid.

INTRODUCTION

Dietary cholesterol induces a hemolytic anemia in guinea pigs. This is accompanied by a large increase in their body cholesterol pool, histopathological changes in liver and other organs, and changes in the morphology of red cells (1-3).

Conversion to bile acids is a major pathway for the elimination of cholesterol and is, therefore, one mechanism for the control of the body pool of cholesterol.

We are presenting results of a study designed to assess the extent to which bile acid metabolism contributes to the pathological expansion of the cholesterol pool in cholesterol fed guinea pigs. Guinea pigs were fed a diet with or without cholesterol (chol guinea pigs and control guinea pigs, respectively), and the kinetic parameters of bile acid metabolism were measured.

[1]Present address: Biological Sciences Department, University of California, Santa Barbara, Calif. 93106.

METHODS

Animals and Diets

Young male albino guinea pigs weighing 200-250 g (Simonsen, Gilroy, Calif.) were fed a semisynthetic diet with or without the addition of 1% cholesterol (4). Both diets contained 10% cottonseed oil, 30% casein, 36% carbohydrate (cornstarch, cerelose, and sucrose), cellulose as bulk and the necessary minerals and vitamins (4).

Bile Acid Turnover

When the red blood cell count of the chol guinea pigs had dropped below 3.0 x 10^6 cells/mm^3 (after 10-12 weeks on the diet), ^{14}C-chenodeoxycholic acid (CDC) was administered to the animals from both groups as follows: [24-^{14}C]-chenodeoxycholic acid (ICN, 35.8 mci/mmole) in ethanol was diluted fourfold with isotonic saline. Each guinea pig was injected intraperitoneally with ca. 0.5 ml (5 μci) solution. The actual volume injected was not measured accurately and varied from 0.4-0.5 ml.

Screens were placed under the cages of the injected animals to catch the feces, which were collected every 24 hr, lyophylized, and frozen. Urine was collected daily from some of the animals kept in metabolic cages.

From 5-9 days after injection, the animals were fasted for 24 hr and anesthetized with sodium pentobarbital (Diabutal, Diamond Laboratories, Des Moines, Iowa). Blood was taken by open-chest heart puncture. The contents of the gall bladder, small intestine, caecum, and large intestine were removed. These tissues were washed with 0.1% NH_4OH in 95% ethanol, and the washes were combined with the respective tissue contents. Livers were weighed, lyophylized, and frozen.

Extractions of Bile Acids

Feces: Samples (0.5 g) of lyophylized and powdered feces were suspended in 10 ml 1.25N NaOH in large stainless steel culture tube caps and hydrolyzed for 3 hr at 15 psi. The hydrolysates were transferred quantitatively to screw-cap tubes and extracted 3 times with 2-3 volumes of redistilled petroleum ether. The aqueous phase was acidified to pH 1-2 with 6N

HCl and extracted 3 times with 2-3 volumes of diethyl ether (5). The combined ether extracts were adjusted to 100 ml, and aliquots were taken for analysis of bile acids by gas chromatography and for scintillation counting.

Small intestine and gall bladder contents: These were evaporated to dryness, and the residue was dissolved in ca. 10 ml 1.25N aqueous NaOH and transferred to stainless steel caps. Samples were hydrolyzed and extracted with petroleum ether and diethyl ether as for feces.

Caecum and large intestine contents: Contents were suspended in 100-150 ml 95% ethanol with 0.1% NH_4OH, refluxed on a steam table for 45 min and filtered (6). The residues were reextracted with 100-150 ml solvent. Combined filtrates were concentrated in a rotary evaporator and transferred quantitatively to 100 ml volumetric flasks with absolute ethanol washes.

Aliquots of the ethanol extracts were evaporated to dryness, hydrolyzed, and extracted with petroleum ether and diethyl ether as for feces to obtain unconjugated bile acids.

Samples of lyophylized livers and 3 ml aliquots of plasmas and red cells were extracted with ethanol in a similar manner.

Determination of Radioactivity of Bile Acids

Aliquots of ether extracts (feces, small intestines, and gall bladder contents) were evaporated to dryness in scintillation vials, and 10 ml 0.5% diphenyloxazole (PPO) in toluene was added.

Aliquots of ethanol extracts (caecum and large intestine contents) were evaporated to 3-4 ml in scintillation vials on a steam table, and the color was reduced with several drops of 30% hydrogen peroxide. Aquasol (10 ml) (New England Nuclear, Boston, Mass.) was added. Aliquots (1 ml) from 24 hr urine collections were counted in 10 ml Aquasol.

Radioactivity was measured in a Packard model 2002 scintillation counter.

Counts/min were corrected to dpm after the addition of 1 ml ^{14}C-toluene as an internal standard.

Identification and Quantitation of Bile Acids

Gas liquid chromatography (GLC): Lithocholic acid and chenodeoxycholic acid standards (Calbiochem., San Diego, Calif.) and aliquots of the ether extracts of hydrolyzed samples were methylated and oxidized for gas chromatographic analysis. By this procedure, lithocholic acid was converted to methyl 3-ketocholanate; chenodeoxycholic acid, ursodeoxycholic acid, and 7-ketolithocholic acid were all converted to methyl 3,7-diketocholanate.

Methylation was carried out with diazomethane freshly prepared from Diazald (Aldrich Chemical Co., Milwaukee, Wisc.). Samples then were oxidized with dichromate and extracted into ethyl acetate (7).

Measured volumes of samples and standards were injected on column. All samples were analyzed in duplicate. Peak areas were calculated as (retention time) x (peak ht). The amount of each bile acid derivative was determined from standard curves which were run with each set of samples.

Thin layer chromatography (TLC): Bile acids in the hydrolysates of bile and plasma extracts were identified by thin layer chromatography on Silica Gel G in the following solvent systems: Isooctane-ethyl acetate-acetic acid (5:5:1); benzene-dioxane-acetic acid (70:20:2); and isooctane-ethyl acetate-acetic acid (5:25:0.2) (8). Developed plates were sprayed with 10% phosphomolybdic acid in 95% ethanol and heated at 110 C for 10-15 min.

Determination of Expired ^{14}C-CO_2

Guinea pigs were placed in 3 liter metabolic chambers connected to vials containing a CO_2-absorbent (ethanolamine-methyl cellosolve 2:1, 6 ml) (9). A slight vacuum was applied to the vials. Animals were left in the metabolic chambers for 1-1/2 to 4-1/2 hr, and the vials were changed every 1-1/2 hrs. Aliquots (3 ml) of the CO_2 containing solution were counted in 1 ml methyl cellosolve and 10 ml scintillation solution (5.0 g/liter PPO in toluene-methyl cellosolve 2:1 (9).

RESULTS

^{14}C-Excretion

The amount of ^{14}C in expired CO_2 was measured in 1 control and 3 chol guinea pigs at 25 or 75 hr after injection of ^{14}C-CDC. The amount (dpm/day) of ^{14}C-CO_2 expired was compared with the fecal excretion of ^{14}C for the same day; the expired radioactivity was 0.4-2.9% of the excreted radioactivity, which ranged from 5.3×10^5-1.8×10^6 dpm/day. This is comparable to Beher's, et al., (10) results with rat, mouse, hamster, and gerbil.

Radioactivity in the urine was determined daily for 2 chol guinea pigs, and the dpm/day excreted in the urine was compared with the fecal excretion of ^{14}C for each day. The urinary excretion was $8.8 \times 10^4 \pm 7.5 \times 10^4$ dpm/day which is 9.9 ± 3.8% of the fecal excretion of $1.0 \times 10^6 \pm 8.4 \times 10^5$ dpm/day.

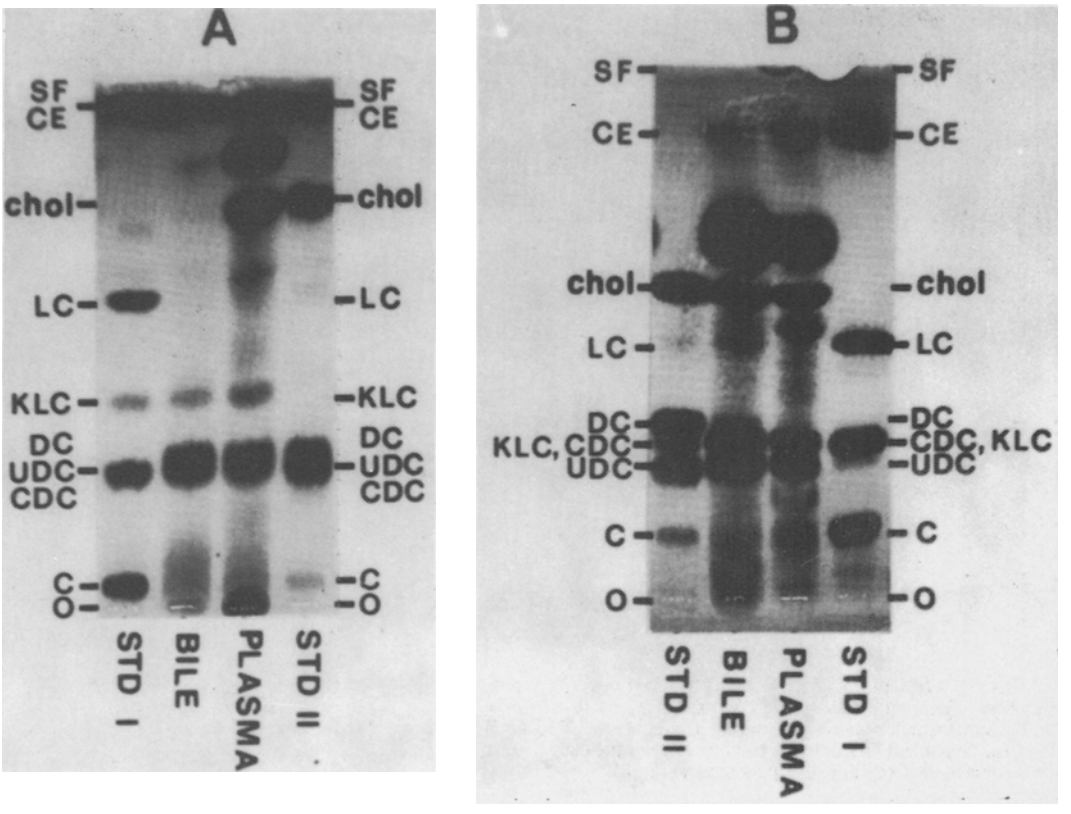

FIG. 1. Thin layer chromatography (TLC) of guinea pig bile acids. Standard mixture I: Cholic acid (C), chenodeoxycholic acid (CDC), 7-ketolithocholic acid (KLC), lithocholic acid (LC), and cholesterol ester (CE). Standard mixture II: Ursodeoxycholic acid (UDC), deoxycholic acid (DC), and unesterified cholesterol (chol). O = origin and SF = solvent front. A. Plasma: hydrolysate from a chol guinea pig. Bile: hydrolysate of taurine-conjugated bile acids (isolated by TLC) from bile of a control guinea pig. Solvent system: benzene-dioxane-acetic acid (75:20:2). B. Plasma: hydrolysate from a control guinea pig. Bile: hydrolysate of total bile acids from a control guinea pig. Solvent system: isooctane-isopropyl ether-acetic acid (2:1:1).

This value is also similar to Beher's results with the rat, mouse, hamster, and gerbil (10). Thus, ca. 90% of the excreted ^{14}C was in the feces. ($4.1 \times 10^6 \pm 8.1 \times 10^5$ and $5.8 \times 10^6 \pm 1.9 \times 10^6$ dpm total excretion by 4 control and 6 chol guinea pigs, respectively.)

Ca. 40-80% of the injected ^{14}C was recovered in the analyzed tissues (liver, plasma, gall bladder contents, large and small intestines, and caecum) and feces. Radioactivity in other tissues and carcass was not measured. Calculated recoveries are approximate values based upon a total of 5 μci ^{14}C; the actual amount injected was not precisely measured and varied from 4-5 μci. Of the total radioactivity recovered, 65-95% were in the feces.

Identification of Guinea Pig Bile Acids

Using both GLC and TLC, we have found no cholic acid in the bile of either control or chol guinea pigs. The main bile acid was chenodeoxycholic acid (CDC, 3α, 7α-dihydroxycholanic acid). Additionally, the bile contained significant amounts of 7-ketolithocholic (KLC, 7-keto, 3α-hydroxycholanic acid) and ursodeoxycholic acid (UDC, 3α, 7β-dihydroxycholanic acid). There was little or no lithocholic acid (LC, 3α-hydroxycholanic acid) in the bile of control or chol guinea pigs, (Figs. 1 and 2A). Both taurine and glycine conjugated bile acids were present. We have shown previously that the ratio of tauro-CDC/glyco-CDC in bile of control guinea pigs is 6 (11).

In contrast to the bile, the caecum contents, large intestine contents, and feces contained little or no CDC, KLC, or UDC (Fig. 2B). Both GLC and TLC indicated a large amount of lithocholic acid, presumably formed from CDC, KLC, and UDC by bacterial degradation.

These results show that only CDC and its metabolites are present in the guinea pig. Therefore, the metabolism of the total bile acid

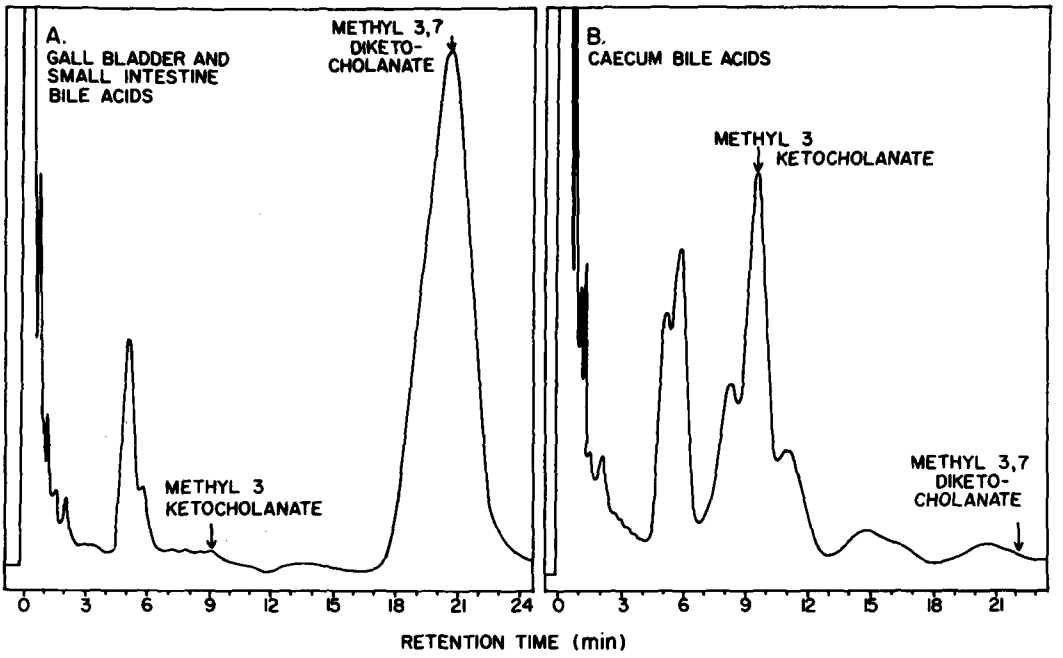

FIG. 2. Gas liquid chromatographic separation of guinea pig bile acids. Column: 3 ft x 1/8 in. inside diameter. Silanized glass packed with 2% w/w OV-17 on Chromosorb W A/W DMCS 100-120 mesh. (Varian Aerograph.) Carrier gas: N_2. Column temperature: 236 C. Wilkens Aerograph model 200 with flame ionization detector was used. Samples are from chol guinea pigs; control guinea pigs gave similar results. Peaks at 5 min have been identified tentatively as neutral sterols.

pool can be determined by administration of ^{14}C-CDC under the assumption that there is a rapid conversion of CDC to the equilibrium mixture of CDC, UDC, and KLC.

Bile Acid Metabolism

Steady state bile acid turnover follows first order kinetics, as shown by Lindstedt and Norman (12). Therefore, the semilog plot of the fraction of radioactivity remaining in the body vs time is linear and has a slope proportional to the fractional turnover. Figure 3 gives data for 6 guinea pigs; for all 11 guinea pigs studied, the graphs were linear, with correlation coefficients (r) greater than 0.98. The fractional turnover, k, was much greater for chol guinea pigs (0.42 ± 0.07 da^{-1}) than for controls (0.18 ± 0.04 da^{-1}) (Table I).

Bile acid pool sizes were calculated in two ways, both based upon the specific activity of bile acids in the bile and small intestine contents (Table I). For control guinea pigs, the pool size, derived from the sum of the tissue radioactivities, was essentially the same as that calculated from the bile acid specific activity extrapolated to zero time. For chol guinea pigs, there were large individual variations in the pool sizes derived by either method, leading to a large standard deviation. The apparent difference of these means was not statistically significant. This variability may be due to the high fractional turnover of chol guinea pig bile acids (42% of the pool/day). The rate of bile acid synthesis (and therefore of excretion) was 3-6 times larger in chol guinea pigs than in controls (Table I).

Figure 4 shows the percentage of the bile acid pool in each tissue. The major difference between control and chol guinea pigs was in the percentage of bile acids in the liver. Despite the increase in size (Table I) the chol liver had a much higher bile acid concentration (0.39 ± 0.30 mg bile acid/g liver) than the control liver (0.07 ± 0.024 mg bile acid/g liver).

DISCUSSION

We have found that guinea pig bile contains little or no cholic acid; this agrees with previous reports (13,14). Only chenodeoxycholic acid and its metabolites (7-ketolithocholic acid and ursodeoxycholic acid) were present. Assuming that CDC equilibrates rapidly with these metabolites, we have used ^{14}C-CDC to determine the metabolism of total guinea pig bile acids.

Our results show that guinea pigs can in-

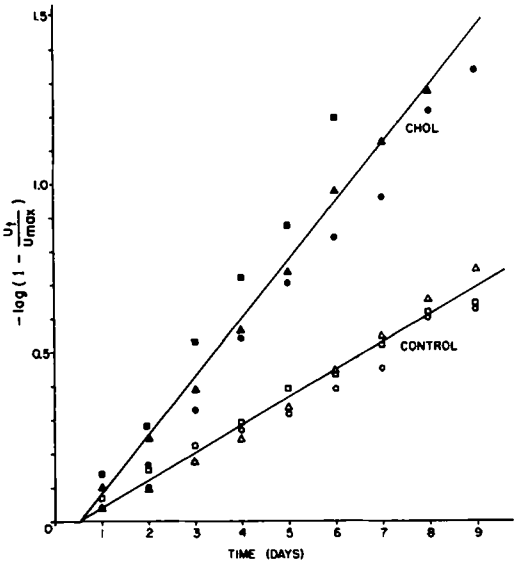

FIG. 3. Semilogarithmic plot of elimination of chenodeoxycholic acid from guinea pig bile acid pools. U_t = cumulative fecal radioactivity to time t; u max = total radioactivity recovered in feces and body tissues. Chol = cholesterol. Data for three control (open symbols) and three chol (closed symbols) guinea pigs are shown. Linear regression equations and correlation coefficients (r) are as follows: (control) O: Y = 0.075 x − 0.048 (r = 0.994), Δ: Y = 0.091 x − 0.092 (r = 0.995); □Y = 0.074 x − 0.004 (r = 0.997); (chol) ●: Y = 0.168 x − 0.148 (r = 0.997), ▲Y = 0.198 x − 0.185 (r = 0.982), ■Y = 0.183 x − 0.042 (r = 0.996).

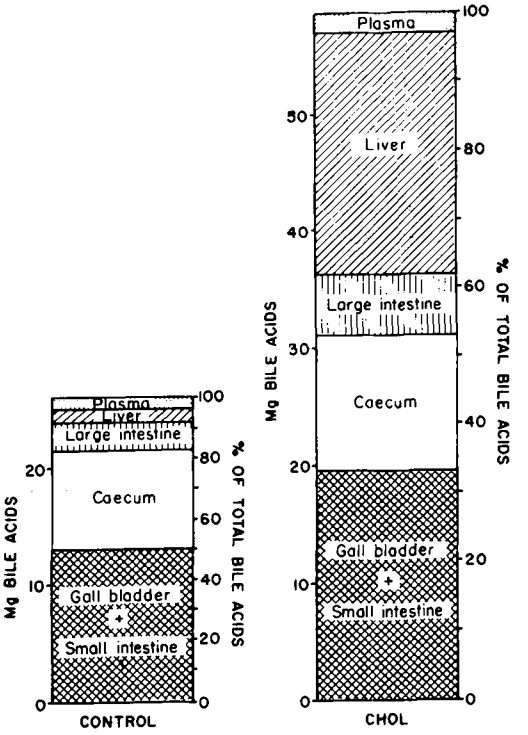

FIG. 4. Distribution of the guinea pig bile acid pool. Data were calculated from bile acid radioactivity in each tissue and from specific activity of bile acids in gall bladder and small intestine. Standard deviations of means (not shown) ranged from 25-67% of the means.

crease the conversion of cholesterol to bile acids in response to dietary cholesterol. Chol guinea pigs excreted at least 3 times as much bile acid daily as did control guinea pigs (Table I). The food intake of both groups was ca. 20 g/day (= 200 mg cholesterol/day for chol guinea pigs) (15). From this, it can be calculated that chol guinea pigs synthesized and excreted ca. 0.1 mg bile acid/mg cholesterol ingested. We have not measured the fractional absorption of cholesterol by the intestine, so this value represents the minimum efficiency of the conversion of cholesterol to bile acids.

Ginter (16) reported that cholesterol accumulation was induced in guinea pigs by chronic ascorbic acid deficiency and that the rate of conversion of cholesterol to bile acids was decreased significantly. His value for this conversion in normal guinea pigs was higher than our value for bile acid synthesis in controls (12 mg/24 hr/500 g body wt vs 3 mg). Differences in diet and experimental design may account for this discrepancy. While we have calculated bile acid synthesis from fractional turnover and pool size of [^{14}C]-CDC, Ginter calculated it from [^{14}C]-CO_2 produced from [26-^{14}C]-cholesterol and from the specific activity of liver cholesterol.

Dietary Cholesterol and Bile Acid Metabolism

Studies in rats, hamsters, and squirrel monkeys have shown that resistance to dietary cholesterol in terms of pool expansion and pathological effects is related to the ability to increase bile acid excretion.

Rats, for example, are relatively resistant to 1% dietary cholesterol. They accumulate less liver cholesterol than do guinea pigs and all the extra cholesterol is esterified (17). Much of the excess absorbed cholesterol is converted to bile acids (bile acid synthesis was 1.4 mg/day/100 g body wt, compared to 0.7 mg/day/100 g body wt in controls), while the bile acid pool size remains unchanged (18).

Hamsters, on the other hand, are even more sensitive to dietary cholesterol than guinea pigs; their liver cholesterol increased 40-fold after 3 weeks on a 1% cholesterol diet (19), but the net bile acid synthesis and excretion did not increase (18). Both the bile acid half life and the pool size were ca. tripled, which resulted in an unchanged rate of bile acid synthesis (18).

TABLE I

Guinea Pig Bile Acid Metabolism[a]

Group	Control guinea pigs (5)[b]	Chol guinea pigs (6)[b]	t-Test chol vs control
Body wt (g)	663 ±125	443 ±77	$P < 0.005$
Liver wt (g, wet)	20.8 ±1.8	55.2 ±3.5	$P \ll 0.001$
Liver cholesterol (mg/g wet wet)	4.2 ±1.7	33.5 ±9.3	$P < 0.002$
Bile acids:			
Half-life ($t_{1/2}$)[c] (da)	4.0 ±1.0	1.7 ±0.1	$P < 0.001$
Fractional turnover (k)[c] (da^{-1})	0.18 ±0.04	0.42 ±0.07	$P \ll 0.001$
Pool size (mg)			
Measured[d]	26±4	60±19	$P < 0.01$
Calculated[e]	23±7	36±10	NS[f]
Synthesis rate[g] (mg/day) calculated from			
Measured pool size	4.6±0.8	23.4±8.3	$P < 0.001$
Calculated pool size	3.8±0.7	15.2±4.1	$P < 0.001$

[a]Data are expressed as mean ± standard deviation.

[b]The number of animals in each group is given in parentheses, except for liver cholesterol: control, n = 4; chol, n = 3.

[c]Half-life ($t_{1/2}$) is (log 2)/m, and fractional turnover (k) is 2.303 m, where m is the slope of the linear regression line (Fig. 3).

[d]Measured pool size = mg BA in (GB + SI) $\times \frac{\text{dpm in all tissues}}{\text{cpm in GB + SI}}$ BA = bile acid; GB = gall bladder, and SI = small intestine. All tissues = GB + SI + caecum + large intestine + liver + plasma.

[e]Pool sizes were calculated by extrapolating the bile acid specific activity to time zero: Pool size = $\frac{\text{dpm } (t_o)}{\text{dpm/mg bile acid } (t_o).}$

[f]NS = not significant.

[g]Synthesis or excretion rate = fractional turnover (da^{-1}) x pool size (mg).

Lofland (20) has studied squirrel monkeys of two phenotypes: cholesterol-sensitive hyperresponders and cholesterol-resistant hyporesponders. The hyperresponders became hypercholesterolemic with cholesterol feeding (0.5 mg/kcal) and did not increase their bile acid excretion. The hyporesponders showed an increase in bile acid excretion soon after cholesterol feeding was begun. Synthesis and absorption of cholesterol and excretion of neutal sterols were the same in both groups and did not change with cholesterol feeding.

The accumulation of nondietary cholesterol also is accompanied in some cases by a decrease in bile acid excretion. For example, ethanol feeding induces cholesterol accumulation in the liver and plasma of rats, and the rate of excretion of bile acids is lower than normal (17).

We have shown that the guinea pig cannot prevent an expansion of the cholesterol pool in response to 1% dietary cholesterol in spite of a significant increase in bile acid synthesis. This is in contrast to some other animal species, where cholesterol pool expansion is inversely proportional to bile acid excretion. The following factors may contribute to this cholesterol accumulation in the guinea pig: continued endogenous synthesis of cholesterol, continued high rate of intestinal absorption, or failure of neutral sterol excretion to increase. Furthermore, the excess absorbed cholesterol might not be in te right location or in the right chemical form, or both, for conversion to bile acids. For example, chol guinea pigs have high levels of unesterified cholesterol in the liver and plasma (2); cholesterol in this form is incorporated more readily into cell membranes and less readily into plasma lipoproteins, which are the normal vehicle for the transport and metabolism of cholesterol.

Some red cell abnormalities and hemolytic

anemias perhaps are caused by changes in the concentration and composition of plasma bile acids. For example, Cooper, et al., (21) has shown that the ratio of CDC to cholic acid is abnormally high in cirrhotic patients with spurred red cells and normal in patients with target cells. He also has shown that the spurring of monkey red cells is caused by lithocholic acid, a CDC metabolite (22).

We have studied the spurring of red cells and the production of abnormal lipoprotein species in chol guinea pigs and have found striking similarities with those of patients with cirrhosis and other liver diseases (23,24). These patients also resemble chol guinea pigs in their abnormally high plasma unesterified cholesterol levels (21,25). Cholesterol fed guinea pigs may, therefore, be a useful model system for studying the relationship between bile acids, hemolytic anemia, and red cell morphology changes.

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Enzymic Synthesis of Ethanolamine Plasmalogens through Ethanolaminephosphotransferase Activity in Neurons and Glial Cells of Rabbit in Vitro[1]

L. BINAGLIA, R. ROBERTI, G. GORACCI, E. FRANCESCANGELI, and G. PORCELLATI,
Department of Biochemistry, The Medical School, University of Perugia, 06100 Perugia, Italy

ABSTRACT

The de novo synthesis of ethanolamine plasmalogen in isolated neuronal and glial cells from adult rabbit brain cortex was investigated in vitro, using labeled cytidine-5′-diphosphate ethanolamine as lipid precursor. The neuronal cell enriched fraction was found to possess a twofold ethanolaminephosphotransferase activity (EC 2.7.8.1), as compared to the glial fraction. The neuronal/glial ratio was similar both in the absence and in the presence of saturating alkenylacyl glycerol. Under the most favorable conditions, rates of 31 nmoles and 16 nmoles ethanolamine plasmalogen/mg protein/30 min were obtained for neurons and glia, respectively. Several kinetic properties of the phosphotransferase were found to be similar both in neurons and glia, e.g., K_m of cytidine-5′-diphosphate ethanolamine, pH optimum, need for divalent cations; the K_m value for alkenylacyl glycerol was twofold higher in glia (4 mM) than in neurons (2 mM). The neuronal/glial ratio for the phosphatidylethanolamine synthesizing activity was 2, 4.5, and 6 on using diacyl glycerols prepared from ox heart, ox brain, and soybean, respectively. It is concluded that the cytidine-dependent system for ethanolamine plasmalogen and phosphatidylethanolamine synthesis is concentrated prevalently in the neuronal cells, as compared to glia.

INTRODUCTION

Neuronal and glial cells have been studied recently in vitro in a variety of biochemical reactions (1), including protein synthesis (2). No reports have as yet appeared, however, on the ability of these cells to perform phospholipid biosynthesis, except for some studies on the base-exchange reaction (3-5) and on the synthesis of 1,2-diacyl-sn-glycero-3-phosphorylcholine (diacyl-GPC) and 1,2-diacyl-sn-glycero-3-phosphorylethanolamine (diacyl-GPE) (1).

Ethanolamine plasmalogens (alkenyl-acyl-GPE), on the other hand, account for a noticeable amount of the lipid bound ethanolamine in the mammalian brain, and their concentration changes, unlike those of the diacyl-GPEs, are known to be linked to developmental phenomena in the brain (6,7). This assumption might be reflected in possible differential activities toward ethanolamine plasmalogen precursors of different cell types during development. A study of the enzymic properties of the de novo mechanism for ethanolamine plasmalogen synthesis, both in neuronal and glial cell fractions seems, therefore, of value to understand its physiological function during development and its relationship to the membrane phospholipids.

The biosynthesis of plasmalogens in mammalian systems most probably involves the introduction of long chain alcohols into 1-O-alkyl glycerolipids and the oxidation of 1-O-alkyl-2-acyl-sn-GPEs or GPCs to the corresponding 1-alkenyl-2-acyl-derivatives (8,9). Previous work from various laboratories has, however, demonstrated (10-13) that the formation of ethanolamine plasmalogens in nervous tissue also may take place from cytidine-5′-diphosphate ethanolamine (CDPE) and added 1-alkenyl-2-acyl-glycerols (alkenylacyl glycerols). As a first step of our research we have, therefore, investigated the synthesis of ethanolamine plasmalogens from CDPE and exogenously added alkenylacyl glycerols in neurons and glia from rabbit brain. Preliminary reports of this work have been presented (14,15).

EXPERIMENTAL PROCEDURES

Preparation of Neuronal and Glial Cell Fractions

The neuronal and glial fractions were prepared from white rabbits (1.5-1.8 Kg body wt), according to Goracci, et al., (5) diluted with large amounts of 0.32 M sucrose-salt solutions (16) and pelleted by centrifugation for 15 min at 2000 g. The cell enriched pellets were homogenized in 0.32 M sucrose-10 mM-Tris-HCl, pH 7.4, by the use of glass Teflon homogenizers at 1000 rev/min with 5 or 6

[1]Presented in part at the IXth International Congress of Biochemistry, Stokholm, July 1973 and at the XIXth National Meeting of Biochemistry, Trieste, October 1973.

up-and-down strokes. The purity of the neuronal and glial suspensions was assessed before final homogenization by light microscopy, electron microscopy (16-19), and marker enzyme tests (3,5).

Chemicals and Labeled Substrates

Materials: Materials were obtained as described previously (1).

Labeled substrates: Most of the labeled substrates were obtained, or synthesized and purified, as reported previously (1). Uniformly labeled ^{14}C-diacyl-GPE, obtained from Applied Sciences Laboratory, State College, Pa., was purified by chromatography on a short diethylaminoethyl (DEAE) cellulose column before using as a reference standard. The lysoderivatives of the ^{14}C-labeled diacyl-GPE were obtained according to previous procedures (11).

Diacyl glycerols and alkenylacyl glycerols: The 1,2-diacyl-sn-glycerol (diacyl glycerol) from soybean lecithin was prepared, purified, and emulsified as reported elsewhere (1).

The diacyl glycerol from choline lipids of ox brain was prepared by the action of phospholipase c according to McMurray (10) and Kiyasu and Kennedy (20), essentially as described elsewhere (12). The separation of the diacyl glycerol from the alkenylacyl glycerol, which was discarded, was achieved by silicic acid chromatography in a benzene-chloroform gradient (20). The purity of the diacyl glycerol was checked every time by evaporating under nitrogen small aliquots of each chromatographic fraction at low temperature, treating them for 5-10 min with HCl fumes into small tubes, and dissolving the residue freed from the HCl in a small volume of diethyl ether which was spotted successively on Silicagel (G) plates. Chromatography was carried out by using petroleum ether (40-60 C)-diethyl ether (2:1, by volume) as the solvent. Those fractions which yielded after this treatment chromatographic spots not identical to those of authentic synthetic diacyl glycerols (mixed standards of DL-1,2-diolein, D-1,2-dimyristin, D-1,2-dipalmitin, and D-1,2-distearin) were discarded. The pure diacyl glycerol containing fractions were pooled, evaporated under a stream of nitrogen, and emulsified as above.

Diacyl glycerol from mixed choline phospholipids (1,2-diacyl-sn-GPC, alkenylacyl-sn-GPC, and sphingomyelin) of ox heart was prepared as described above. Thin layer chromatography (TLC) on Silicagel (G), as described in the previous section, was used to check the purity of the diglyceride.

This last procedure also yielded alkenylacyl glycerols (plasmalogenic diglycerides). The purity of this diglyceride was checked by removing from each fraction two small aliquots, which were (A) spotted on Silicagel (G) layers and (B) treated with HCl fumes as above before spotting; only those fractions which showed a complete variation of R_f of the spot upon acid treatment were pooled. TLC was carried as above.

Diacyl glycerol and alkenylacyl glycerol were stored if necessary for a few days at -20 C in diethyl ether. The sonicated dispersions were prepared just before use, as described above. This was particularly important for the alkenylacyl glycerol, whose activity was considerably lost after rather short time intervals from preparation ($t_{1/2}$ of ca. 20 days in the average of two experiments).

Analytical Methods

Protein was determined according to Lowry, et al. (21), with crystalline bovine serum albumin as a standard (1). Phospholipid P was determined in the lipid extract according to Ernster, et al. (22).

The diacyl glycerol concentration was estimated as described elsewhere (1). The alkenylacyl glycerol concentration was determined after treatment of the purified product with HCl and successive TLC using petroleum ether (40-60 C)-diethyl ether (2:1, by volume) as the solvent. The produced monoacyl glycerol (R_f = O), well separated from free aldehyde (front of the solvent), was eluted repeatedly from the layer with diethyl ether-methanol (80:20, by volume) and the glycerol content estimated as above. This procedure was adopted to overcome the interferences in the direct spectrophotometric method (23) of the vinyl ether groups of the alkenylacyl glycerol. Parallel estimations by the Rapport and Norton procedure (24) gave comparable results.

Incubation

Experiments normally were carried out in the following incubation mixture (0.15 ml final volume): alkenylacyl glycerol (8 mM), dissolved in Tween-20 (0.02% final concentration) and albumin (0.01%); Tris HCl buffer, pH 8.0 (50 mM); cysteine (12.3 mM); labeled CDPE (specific activity of 0.5-1.0 nCi/nmol), at the concentration values indicated in each table or figure; neuronal protein (150-200 μg) or glial protein (400-500 μg); and $MnCl_2$ (10 mM). Components were added at +2 C at the indicated order. The final pH value, measured at the end of incubation period, was 8.0-8.1. Incubation was carried out for 30 min at 39.5 C. When indicated, diacyl glycerol was added in place of the alkenylacyl derivative. Each incu-

TABLE I

Cytidine-5'-Diphosphate Ethanolamine Incorporation into Ethanolamine Phosphoglycerides of Rat and Rabbit Liver and Brain Tissues[a]

Tissue	Activity[b]
Rat brain	3.7
Rabbit brain	1.9
Rat liver	6.8
Rabbit liver	5.6

[a]Rats were Sprague-Dawley males, 35-40 days old and rabbits were white, 4 months old. Liver and brain homogenates were prepared according to Giorgini, et al., (27) and Goracci, et al., (5) respectively. Incubation was carried out in the following medium: Tris-HCl buffer, pH 8.00 (50 mM), labeled cytidine-5'-diphosphate ethanolamine (1.34 mM; specific activity of 1 nCi/nmol), $MnCl_2$ (10 mM), ethylenediaminetetraacetic acid (2 mM), cysteine (12.3 mM), and the equivalent of ca. 20 mg whole brain or liver. The final volume was 0.15 ml, and the incubation was performed at 39.5 for 30 min. Mean values of activity of three experiments.

[b]*nmoles/mg protein/10 min.*

bation was performed in heavy-walled, round-bottomed test tubes which were stoppered and shaken at ca. 140 strokes/min in a water-bath shaker. At the end of the incubation, the mixture was treated as follows.

Isolation and Assay

Total lipid: Reaction was terminated by adding 0.2 ml 25% liver homogenate immediately followed by 2.5 ml cold chloroform-methanol (2:1, by volume). The mixture was filtered, treated according to Folch-Pi, et al., (25) and washed by the method of Folch-Pi, et al. (26). The final lipid extract was dried under nitrogen at 30 C.

Separation of phospholipid: The lipid residue was exposed to HCl fumes for 5-10 min, the excess HCl removed under a stream of nitrogen, and the content dissolved with small aliquots of cold chloroform-methanol (2:1, by volume) mixture. Fractionation of lipid classes was performed by spotting small samples of the mixture on Silicagel (G) layers (300 μm in thickness), which then were developed with chloroform-methanol-acetone-acetic acid-water (75:15:30:15:7.5, by volume) at 16-18 C. This procedure allows complete separation of diacyl-sn-GPE (R_f of ca. 0.6) and 2-monoacyl-sn-GPE (R_f of ca. 0.3), derived from plasmalogens, from other lipids, confirmed by using reference phospholipid standards which always were included on the same plate. No radioactive monoacyl-sn-GPE was seen on chromatoplate if HCl treatment was omitted. Iodine vapor, ninhydrin spray, phosphomolibdate reagent, and radiochromatography with a radiochromatoscanner were used for detection.

Analyses of phospholipids: The identified labeled lipids were scraped off from the TLC plates and counted, as reported elsewhere (1).

Calculation of results: Since following CDPE incorporation no other labeled phospholipid was found out of the separated diacyl-sn-GPE or alkenylacyl-sn-GPE, radioactivity was estimated on the isolated spot and thus calculated. The amount of the synthesized phospholipid was calculated by dividing the estimated nCi by the specific activity of the incubated precursor. The results, expressed as nmole of synthesized lipid, then were converted into nmole x mg protein^{-1} x 30 min^{-1}.

RESULTS

Experiments with Whole Brain of Rat and Rabbit

Preliminary experiments carried out with whole brain homogenates from rat and rabbit (1) already had indicated that apparently higher rates of synthesis of ethanolamine phosphoglycerides (EPG) occurred in the rat brain than in that of rabbit, when these homogenates were incubated with labeled CDPE under comparable conditions. Data of subsequent experiments, reported in Table I, show that similar differences apparently were detected also between the liver homogenates of the two animal species. The incubation of rabbit brain homogenates with radioactive CDPE in the absence of diacyl and alkenylacyl glycerols labeled both phosphatidylethanolamine and ethanolamine plasmalogen. Higher rate of synthesis of diacyl-GPE occurred in these conditions as compared to ethanolamine plasmalogen (ratio of ca. 2).

Incorporation of CDPE into Lipid of Neuronal and Glial Cells

Experiments were performed to determine the capacity of neuronal and glial cell preparations to synthesize EPG from CDPE in the absence of lipid acceptors and to ensure that the increased incorporation of the labeled nucleotide in the presence of diacyl or alkenylacyl glycerol was due to an increased labeling of the correspondent ethanolamine phospholipid. Table II shows that, in the absence of any lipid acceptor, the neuronal cell fraction possessed a higher rate of EPG synthesis, if compared to glia. In terms of specific activity, the neurons displayed a threefold increase of activity for both phosphatidylethanolamine and ethanolamine plasmalogen synthesis; furthermore, both neurons and glial cells displayed a higher synthesis of diacyl-GPE compared to that of alkenylacyl-GPE (ratio of 1.5-1.8). The addi-

TABLE II

Incorporation of Cytidine-5'-Diphosphate Ethanolamine into Phosphatidylethanolamine (Diacyl-GPE) and Ethanolamine Plasmalogen (Alkenylacyl-GPE) of Dispersions of Neuronal and Glial Cells from Rabbit Brain in Absence and Presence of Added Diacyl Glycerol or Alkenylacyl Glycerol[a]

Synthesized lipid	Fraction	Type of added diglyceride	Activity[b]	a/b[c]
Diacyl-GPE	Neurons	—	1.36	
	Glia	—	0.43	3.1
Alkenylacyl-GPE	Neurons	—	0.76	
	Glia	—	0.28	2.7
Diacyl-GPE	Neurons	Diacyl glycerol	4.00	
	Glia	Diacyl glycerol	1.98	2.0
Alkenylacyl-GPE	Neurons	Diacyl glycerol	1.21	
	Glia	Diacyl glycerol	0.50	2.4
Diacyl-GPE	Neurons	Alkenylacyl glycerol	1.97	
	Glia	Alkenylacyl glycerol	0.68	2.9
Alkenylacyl-GPE	Neurons	Alkenylacyl glycerol	19.0	
	Glia	Alkenylacyl glycerol	5.4	3.5

[a]Neuronal or glial homogenates were incubated (ca. 0.2 mg neuronal protein and 0.45 mg glial protein) under standard conditions for 30 min at 39.5 C with 4 mM diacyl glycerol or alkenylacyl glycerol (both prepared from ox heart) and 1.2 mM cytidine-5'-diphosphate ethanolamine (specific activity of 1 nCi/nmol). Incorporation was measured as reported in the text.

[b]nmoles/mg protein/30 min.

[c]a = neurons and b = glia.

tion of 4 mM diacyl glycerol prepared from ox heart caused a 5-fold and a 3-fold stimulation of CDPE incorporation into alkali-labile EPG in glia and neurons, respectively, whereas a correspondent 20-fold and 25-fold stimulation of ethanolamine plasmalogen synthesis was obtained on adding a similar concentration of alkenylacyl glycerol prepared from the same source. On adding diacyl glycerol or alkenylacyl glycerol, the neuronal fraction displayed a noticeably higher activity, compared to glia (2-fold and 3.5-fold increases for diacyl-GPE and alkenylacyl-GPE, respectively). The increased uptake of radioactive CDPE into the two distinct phospholipid moieties following diglyceride addition was not completely specific, because a small but consistent stimulation of alkenylacyl-GPE synthesis was obtained on adding diacyl glycerol, and a smaller one viceversa (Table II).

It must be mentioned, in connection with Table II, that similar incubation conditions always have been used during these experiments, including the source of the diglycerides. This was particularly important, since it was found that the neuronal/glial ratio for incorporation of CDPE into diacyl-GPE varies considerably on changing the type of the diacyl glycerol used. Table III shows in this connection that neuronal/glial ratios ranging from 2-6 occurred throughout the experiments carried out with different diacyl glycerols.

Characteristics of Lipid Labeling

Incubation of CDPE with either neurons or glia produced only radioactive EPG. The radioactivity of EPG was found entirely in the base moiety of the phospholipid at any time of incubation, as revealed by hydrolytic procedures (11). By determining the labeling of EPG in both neurons and glia following the chromatography of the intact lipid classes ("Experimental Procedures") or hydrolytic procedures (11), similar values were obtained. Moreover, no appreciable incorporation of CDPE was observed into mild alkali-and mild acid-stable fraction of neuronal and glial EPG (1-alkyl-2-acyl-sn-GPE). In all the experiments reported in

TABLE III

Incorporation of Cytidine-5'-Diphosphate Ethanolamine into Phosphatidylethanolamine of Neurons and Glia in Presence of Different Diacyl Glycerols[a]

	Activity[b]		
Diacyl glycerol[c]	Glia	Neurons	a/b[d]
From ox heart	1.9	4.0	2
From ox brain	0.9	4.2	4.7
From soybean	3.7	22.0	6
D-1,2-distearin	2.5	6.5	2.6
DL-1,2-diolein[e]	5.1	13.5	2.6
—	0.43	1.36	3.1

[a]Experiments were performed with 4 mM diacyl glycerols, as reported in Table II.

[b]nmoles/mg protein/30 min.

[c]See the text for the preparation of the listed liglycerides.

[d]a = neurons and b = glia.

[e]2 mM concentration.

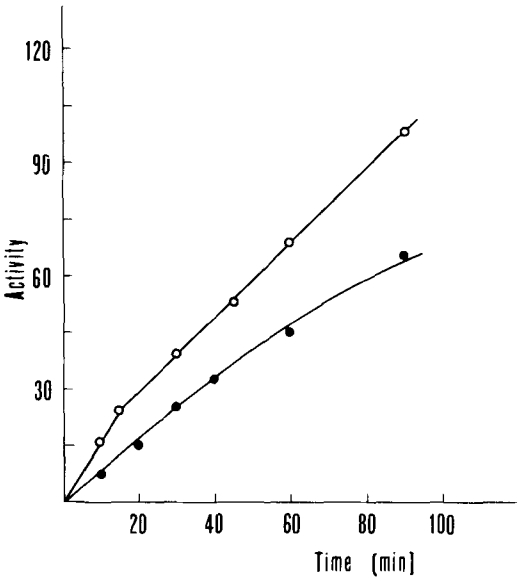

FIG. 1. The effect of the time of incubation upon the synthesis of ethanolamine plasmalogen by neuronal and glial cell homogenates of rabbit brain. The incubation system contained (in a final volume of 0.15 ml) 50 mM Tris-HCl buffer (pH 8.0), labeled 1.10 mM cytidine-5'-diphosphate ethanolamine (CDPE) (specific activity of 1 nCi/nmol), 8 mM alkenylacyl glycerol, 0.025% Tween-20, 0.012% of bovine serum albumin, 12.3 mM cysteine, 10 mM $MnCl_2$, and neuronal (90 μg) or glial (110 μg) protein. Temperature 39.5 C. The time of incubation was varied as shown. Activity reported as nmoles/mg protein. o–o–o–o–o, incubation of CDPE with neurons; •–•–•–•–•, incubation of CDPE with glia.

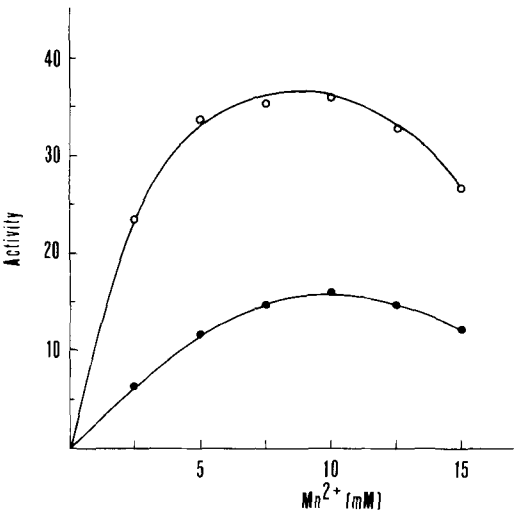

FIG. 2. The effect of Mn^{2+} ions upon the ethanolaminephosphotransferase activity (EC 2.7.8.1) of neuronal and glial fractions of rabbit brain towards 9 mM alkenylacyl glycerol. Incubation for 30 min was carried out as reported in Figure 1. Cytidine-5'-diphosphate ethanolamine (CDPE) concentration was 0.78 mM. Activity reported as nmoles/mg protein/30 min. o–o–o–o–o–, incubation of CDPE with neurons (115 μg protein); •–•–•–•–•–, incubation of CDPE with glia (190 μg protein).

this work, the radioactivity of the isolated EPG will be referred to diacyl GPE or to alkenylacyl-GPE.

Properties of Ethanolamine-Phosphotransferase Activity (EC 2.7.8.1) of Glia and Neurons

Activity and pH: The optimum of pH of the CDPE-1,2-diglyceride ethanolaminephosphotransferase activity (EC 2.7.8.1) was found to be around 8.0 in both fractions, either when synthesis of diacyl-GPE or of alkenylacyl-GPE was considered, with a successive decrease and a further increase in activity above pH 8.4-8.5. Higher phosphotransferase activity was found above these values but with more scattering of data. This effect probably is due to the instability of the enzymic system at these pH values (1,12). Owing to these circumstances a suboptimal pH of 8.0 has been used throughout our experiments. At this pH value, neuronal/glial activity ratio of ca. 2 was found either for diacyl-GPE or alkenylacyl-GPE synthesis, when ox heart diglyceride preparations were used.

Effect of time of incubation and enzyme concentration: The rate of synthesis of ethanolamine plasmalogen in neurons was proportional to time only during the first 15 min of incubation at a concentration of ca. 650 μg protein/ml, whereas the activity in glia retained linearity up to 40 min of incubation (Fig. 1). Somewhat similar findings were observed for diacyl-GPE synthesis (1). During the incubation period of 30 min, formation of products (diacyl-GPE or alkenylacyl-GPE) from CDPE was proportional to the amount of neuronal or glial protein over the range from 0.5-2.0 mg/ml.

Divalent cations: No activity was detected in the absence of Mn^{2+} or Mg^{2+}, thus confirming previous work on the synthesis of ethanolamine plasmalogens by whole brain particulate fractions (11-13). Both Mg^{2+} and Mn^{2+}-ions stimulated CDPE incorporation into glial and neuronal alkenylacyl-GPE, but low concentrations of Mn^{2+} were more effective than low concentrations of Mg^{2+} in the synthesis of this lipid, as first observed by Ansell and Metcalfe (12). The optimal Mn^{2+}-ion concentration was found ca. 10 mM (Fig. 2), the same being true also for diacyl-GPE synthesis, both in neurons and glia (1). At the optimal Mn^{2+}-ion concentration and 9 mM diglyceride the neuronal/glial ratio for ethanolamine plasmalogen synthesis was 36/16 nmoles/mg protein/30 min, while that for diacyl-GPE was ca. 12/6. An optimal

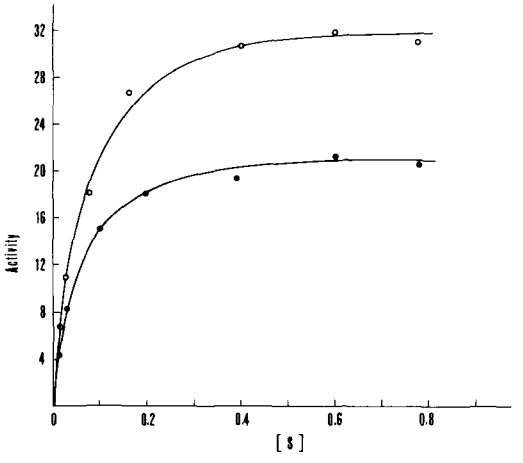

FIG. 3. The effect of cytidine-5'-diphosphate ethanolamine (CDPE) concentration upon the synthesis of ethanolamine plasmalogen by neurons (o–o–o–o–o) and glia (●–●–●–●–●–●) from rabbit brain. Each tube contained the equivalent of 160 μg neuronal protein or 325 μg glial protein incubated in the experimental conditions reported in Figure 1. The concentration of CDPE (1.22 nCi/nmol) was varied as shown. Incubation was carried out at 39.5 C for 20 min. The activity is expressed as nmoles/mg protein/30 min. [S] = CDPE, mM.

concentration of 10 mM $MnCl_2$ was used in all the experiments reported in this work; in addition Mn^{2+} ions were found more suitable than Mg^{2+} in maintaining stable diglyceride dispersions.

K_m for CDPE: The K_m of CDPE in glia and neurons was determined in the presence of saturating alkenylacyl glycerol by adding, under standard conditions, a constant amount of the radioactive substrate and a varying amount of the cold nucleotide. The results shown in Figure 3 indicate that, in both neurons and glia, the enzyme activity:CDPE concentration relationship appears to follow an almost typical Michaelis-Menten equation. The system was apparently saturated by ca. 0.6-0.8 mM substrate in both cell population. A Lineweaver-Burke double reciprocal plot indicated a K_m value of CDPE of 6.7 x 10^{-5}M in both cells. Similar results have been obtained when diacyl glycerol replaced alkenylacyl glycerol (1). Saturating concentrations of CDPE were used throughout all the experimental work of this study.

It is worth mentioning that, in whole rabbit brain microsomes incubated with alkenylacyl glycerol, the K_m for CDPE appeared to be 8.7 x 10^{-5}M; this value is sensibly lower than that reported previously for rat (12) and chick (11) brain microsomes incubated with similar lipid acceptor.

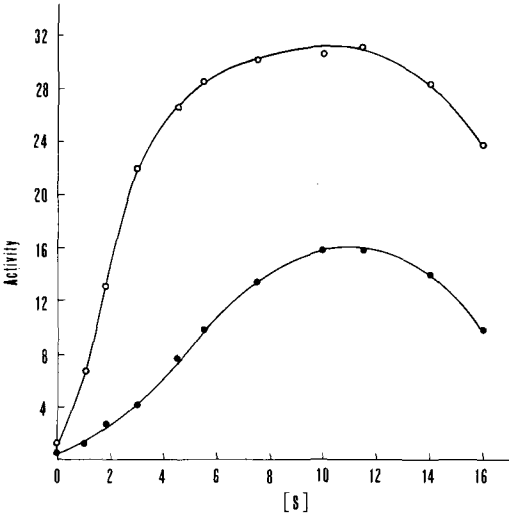

FIG. 4. The effect of the concentration of alkenylacyl glycerol upon the velocity of the ethanolamine-phosphotransferase reaction in the presence of excess cytidine-5'-diphosphate ethanolamine (CDPE). The incubation medium contained in a final volume of 0.15 ml the same components reported in Figure 1, incubated with labeled 1.2 mM CDPE (1 nCi/nmol). Neuronal and glial protein was 820 μg/ml and 1.4 mg/ml, respectively. Alkenylacyl glycerol from ox heart was added, as indicated. The amounts of Tween-20 and serum albumin always were kept constant to obtain final concentration values of 0.025% and 0.012%, respectively. Incubation was at 39.5 C for 20 min. The activity is expressed as nmoles/mg protein/30 min. [S] = alkenylacyl glycerol, mM. o–o–o–o–, neurons; –●–●–●–, glia.

K_m for alkenylacyl glycerol: Figure 4 represents the plots of reaction velocity against alkenylacyl concentration for neurons and glia. On increasing the concentration of alkenylacyl glycerol in the incubation system, an increased synthesis of ethanolamine plasmalogen from CDPE occurred after an initial inert phase both in neurons and glia, maximum reaction rates being obtained at ca. 12 mM concentration in both cell populations. A significant decrease in velocity appeared with increase in the alkenylacyl concentration for both neurons and glia. This inhibition was not due to the increase of serum albumin or Tween-20 in the system, because these components were kept constant during all these experiments. Inhibition by excess alkenylacyl glycerol on CDPE incorporation into ethanolamine plasmalogen also has been reported for rat brain microsomes (12).

Inspection of Figure 4 indicates that it would be impossible to calculate either maximal velocities or the K_m from linear transformations of results. However, it can be noticed that in the reaction rates for the synthesis of

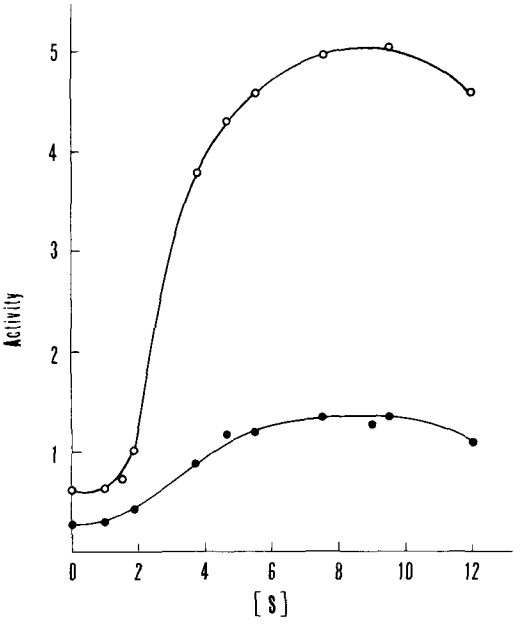

FIG. 5. The effect of the concentration of diacyl glycerol (prepared from ox brain) upon the rate of synthesis of phosphatidylethanolamine in the presence of excess cytidine-5'-diphosphate ethanolamine. The experimental conditions were similar to those reported in Figure 4, except that increasing amounts of diacyl glycerol replaced alkenylacyl glycerol. Neuronal and glial protein was 1.0 mg/ml and 1.9 mg/ml, respectively. [S] = diacyl glycerol, mM. o—o—o—o—o—, neurons; —●—●—●—●—, glia.

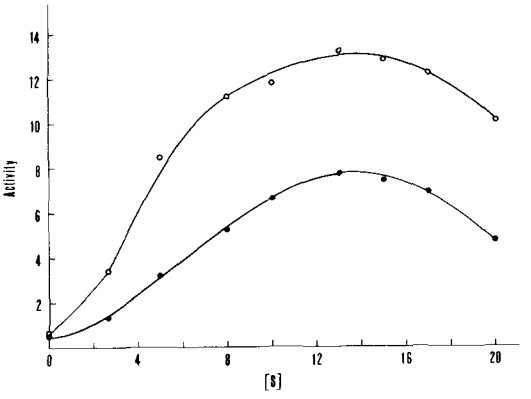

FIG. 6. The effect of the concentration of diacyl glycerol (prepared from ox heart) on the rate of synthesis of phosphatidylethanolamine in the presence of excess cytidine-5'-diphosphate ethanolamine. The experimental conditions were similar to those reported in Figure 4, except that increasing amounts of diacyl glycerol replaced alkenylacyl glycerol. Neuronal and glial protein was 730 μg/ml and 1.4 mg/ml, respectively. [S] = diacyl glycerol, mM. o—o—o—o—, neurons; —●—●—●—, glia.

alkenylacyl-GPE in neurons, half of the maximal values were reached at ca. 2 mM alkenylacyl glycerol with corresponding V_{max} values of ca. 31 nmoles/mg protein/30 min. Similarly, it can be calculated by inspecting Figure 1 that in the reaction velocity for alkenylacyl-GPE synthesis in glia, half of the maximal levels were reached at 4-5 mM alkenylacyl glycerol, with corresponding V_{max} values of ca. 16 nmol/mg protein/30 min. The apparent K_m values of alkenylacylglycerol in neurons are rather close to those of diacyl glycerol prepared from soybean lecithin (1) and to those calculated in whole brain microsomes for both types of diglycerides (11,12). Saturating concentrations of alkenylacyl glycerol have been used throughout this work.

The effect of increasing substrate concentration on the phosphotransferase activity also has been studied for diacyl glycerol prepared from bovine heart or ox brain lecithins, and the results compared with those obtained with the use of diacyl glycerol prepared from soybean lecithins (1). Figures 5 and 6 show that maximum reaction rates for diacyl-GPE synthesis are reached in both glia and neurons at saturating concentrations of 9-10 mM and 13-14 mM, when diacyl glycerols prepared from ox brain and ox heart are used, respectively. A noticeable decrease in velocity appeared with increase in the diacyl glycerol concentrations in both cases. These values of apparent saturation of the phosphotransferase activity by diacyl glycerol contrast with those obtained with the use of diacyl glycerol prepared from soybean lecithin (1), which were around 5-6 mM, and indicate that the diglyceride composition may have some importance in the kinetic parameters of the phosphotransferase reaction. The neuronal vs glial ratios of enzymic activity are, however, not different from those reported in Table III, when the incorporation of CDPE is examined under conditions of saturating diacyl glycerol concentrations. Table IV shows in this connection that increasing ratios of 1.7, 4.3, and 5.8 were obtained in these conditions, and this result is not different from the correspondent values of 2.0, 4.7, and 6.0, obtained in nonsaturating conditions of substrate concentration (Table III).

DISCUSSION

The involvement of cytidine nucleotides in the synthesis of ethanolamine plasmalogens through the participation of exogenously added alkenylacyl glycerol, first reported in brain tissue by McMurray (10) and successively examined in brain subfractions (11,12), has been clearly demonstrated in the present work in

separated neuronal and glial populations from rabbit cerebral cortex. A substantial stimulation of the ethanolaminephosphotransferase activity (20- to 25-fold stimulation) was observed with both cell populations in the presence of excess alkenylacyl glycerol prepared from ox heart.

The biosynthesis of plasmalogens in nervous tissue is known to proceed probably through the introduction of long chain alcohols into 1-O- alkyl glycerolipids and the oxidation of 1-O-alkyl-2-acyl-sn-GPE to the corresponding 1-alkenyl-2-acyl-derivative (13, 28-30). It is also possible, however, that diacyl-GPE, alkenyl-acyl-GPE and alkylacyl-GPE are synthesized independently but by analogous Kennedy's pathways with a supply of appropriate glyceride acceptors (31). This assumption would imply the presence of the correspondent neutral diglyceride in nervous tissue, a finding which apparently seems to apply only to diacyl glycerol (32) and probably to alkylacyl glycerol (33) but not to alkenylacyl glycerol (8). The significance of the findings reported in the present study must, therefore, await further investigation regarding chemical and metabolic evidence for the existence of alkenylacyl glycerols in nervous tissue, though some positive evidence already has been reported (34).

Without exogenously added alkenylacyl glycerol, both the neuronal and glial fractions displayed a higher synthesis of diacyl-GPE as compared to alkenylacyl-GPE (Table II), these findings being similar to those obtained with microsomes and whole brain homogenates (11). It must be added that previous reports have indicated that, in the absence of lipid acceptor, only trace amounts of plasmalogen material were obtained in both fractions from CDPC (1), whereas values of 0.76 and 0.28 nmoles/mg protein/30 min are reported in the present work for the ethanolamine plasmalogen synthesized from CDPE in similar conditions. Corresponding values for diacyl-GPE (Table II) and diacyl-GPC (1) synthesized in the absence of added acceptors were 1.36 and 0.43, and 1.50 and 0.40, for neurons and glia respectively.

Glial cells are less efficient than neurons in operating in vitro the de novo synthesis of ethanolamine plasmalogen in the absence of lipid acceptor (neuronal/glial ratio of ca. 3), a situation which can be compared with the results obtained for diacyl-GPE (Table II) and diacyl-GPC (1) synthesis. On adding suitable amounts of alkenylacyl glycerol, a 20- to 25-fold stimulation was found for ethanolamine plasmalogen; here again values of neuronal/glial ratio of ca. 3 were obtained. It is worth mentioning in this connection that smaller stimulation of diacyl-GPE synthesis was obtained on adding comparable amounts of diacyl glycerol from the same source (ox heart) than that of ethanolamine plasmalogen from the alkenylacyl derivative (Table II). The finding of a less efficient activity of glial cells in synthesizing ethanolamine plasmalogen as compared to neurons is not due, in our opinion, to different rates of penetration of precursors to the sites of synthesis, as explained with previously reported results (1). Although reference points for metabolic differences between glia and neurons are difficult to establish (35) and a number of glial and neuronal cells in our dispersions have not been calculated, our data of enzymic activity, based upon unit protein, seem to be valuable (35) for the evaluation of differences between glia and neurons. It is worth mentioning in this connection that the glial/neuronal ratio of phospholipid content, related to wt of neuronal and glial preparation, is ca. 1.6 (36), which is the same value of that related to protein content (37). This presumably indicates that neuronal and glial homogenates contain similar amounts of protein on a wt base, and this is particularly important, since the method which has been used in the present work has been reported to give ca. the same yield of neuronal and glial material on a wt base (16,17).

The increased uptake of labeled CDPE into diacyl-GPE and alkenylacyl-GPE following the addition of the correspondent diglyceride was not completely specific, as described in Table II. The stimulation that given amounts of diacyl glycerols exerted on the alkenylacyl-GPE synthesis was always higher than that displayed by alkenylacyl glycerol for the production of

TABLE IV

Synthesis of Phosphatidylethanolamine from Cytidine-5'-Diphosphate Ethanolamine in Neurons and Glia in the Presence of Saturating Diacyl Glycerol Concentrations[a]

Diacyl glycerol[c]	Activity[b] Glia	Neurons	a/b[d]
From ox heart	7.8	13.1	1.7
From ox brain	1.2	5.1	4.3
From soybean	3.9	22.7	5.8

[a]Experiments were performed under standard conditions for 30 min at 39.5 C. CDPE concentration was 1.2 mM. Concentrations of diacyl glycerols from ox heart, ox brain, and soybean lecithins were 13 mM 9 mM, and 5 mM, respectively.

[b]nmoles/mg protein/30 min.

[c]See the text for the preparation of the listed diglycerides.

[d]a = neurons and b = glia.

diacyl-GPE. The described phenomenon currently is being investigated in this laboratory.

The characteristics of ethanolamine plasmalogen synthesis in neurons do not appear to differ significantly from those of glia. Apart from the described quantitative differences in the phosphotransferase reaction, several kinetic properties of the enzymic system, e.g., K_m of CDPE, pH optimum, need for divalent cations, were found to be similar in both cell populations. The difference in apparent values of K_m for alkenylacyl glycerol between glia and neurons does not seem to be significantly important. It is worth mentioning that values of pH optimum and Mn^{2+} concentration similar to those described in this work were obtained for diacyl-GPC and diacyl-GPE synthesis in both neurons and glia (1), either in the absence or in the presence of lipid acceptors, and this applies also to the synthesis of diacyl-GPE in brain microsomes (12).

CDPE in neuronal and glial cells has a similar K_m value (6.7×10^{-5}M) in the presence of excess alkenylacyl glycerol, which is strikingly similar to that found in both cell populations for diacyl-GPE synthesis (5.5×10^{-5}M) and which is noticeably lower than that of rat and chick brain microsomes (11,12); a value of 8.7×10^{-5}M has been reported, however, for rabbit brain microsomes in the present study. The K_m of CDPE is, therefore, similar in the presence of diacyl- and alkenylacyl glycerols, thus indicating probably, together with other suggestions (11), that the transfer of CDPE to different lipid acceptors is catalyzed by the same enzyme protein with a different degree of specificity toward the two diglycerides (11). In addition, the K_m of CDPE was found to be similar in the presence of diacyl glycerols prepared from different sources. These findings would indicate that the diglyceride composition does not modify the K_m for the nucleotide moiety. Whether the finding of the low K_m value of CDPE in both neurons and glia as compared to that of CDPC (1) has some connection with active synthesis of ethanolamine phosphoglycerides in both compartments is not known, and it will be interesting to examine in future studies the K_m value for this nucleotide in glia and neurons and the activity of ethanolamine phosphotransferase during brain development and myelination.

The value of apparent K_m calculated in neurons for alkenylacyl glycerol is close to that found for whole brain microsomes (11,12) and to the K_m value of diacyl glycerol in neurons for diacyl-GPE synthesis (1). Under optimal saturating concentrations of alkenylacyl- and diacyl-glycerols from ox heart, a neuronal/glial ratio of ca. 2 was found in both cases, with higher V_{max} values for alkenylacyl-GPE synthesis (31 and 16 nmoles/mg protein/30 min, for neurons and glia respectively) than for diacyl-GPE (13.1 and 7.8, respectively). These last values may indicate a greater efficiency of both neurons and glia for alkenylacyl-GPE synthesis as compared to that for the diacyl derivative. It is worth mentioning that a similar neuronal/glial ratio of enzymic activity of 2 was found for both ethanolamine plasmalogen and phosphatidylethanolamine synthesis on using diacyl glycerols from the same source (ox heart). On the other hand, neuronal/glial ratios ranging from 2-6 were determined in the present study for diacyl-GPE synthesis on using diacyl glycerols prepared from different sources, added either or not at saturating levels (Tables III and IV). The lowest ratio was found for the diglycerides prepared from ox heart, whereas the highest was presented by the lipid acceptors prepared from soybean lecithin, thus confirming previous observations (1).

The finding that similar neuronal/glial ratios were found, either in the presence or in the absence of saturating levels of diglycerides, would indicate that the different levels of incorporation of CDPE into diacyl-GPE between neurons and glia are really due to different efficiency of the two cell populations toward the enzymic reaction, pointing probably to more specific interactions of neuronal or glial membranes toward given structures of the lipid acceptors. In addition, diacyl glycerol composition seems to have some importance in defining kinetic parameters as shown by the fact that the higher the substrate concentration to obtain V_{max} values the lower the neuronal/glial ratio of activity (Figs. 5 and 6 and Table IV).

It is tempting to speculate that, on using an alkenylacyl glycerol prepared from ox brain ethanolamine phosphoglyceride (which apparently appears to be a more physiological diglyceride for lipid synthesis in brain), a neuronal/glial ratio similar to that found for diacyl-GPE synthesis (Table III and IV) would be obtained, in analogy with the value obtained on using lipid acceptors prepared from ox heart. If this were the case, then probably the participation of the neuronal cells in the de novo synthesis of ethanolamine plasmalogen would be even higher compared to glia.

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Acyl-CoA Acyl-sn Glycerol-3 Phosphorylcholine Acyl Transferase of Bovine Mammary Tissue

J.E. KINSELLA and **J.P. INFANTE**, College of Agriculture and Life Sciences, Department of Food Science, Cornell University, Ithaca, N.Y. 14850

ABSTRACT

Microsomal material from lactating bovine mammary tissue possess very active acyl-CoA:1-acyl-sn-glycerol-3-phosphorylcholine acyl transferase (EC2.3.1.-1). Oleyl-CoA was the preferred substrate and rates of 6.3, 6.4, 1.8, 21.0, and 0.1 nmoles acylated/min/mg were obtained for myristyl-, palmityl-, stearyl-, oleyl- and linoleyl-CoA, respectively, when 1-oleyl-sn-glycerol-3-phosphorycholine was used compared to 5.2, 4.1, 1.0, 3.5, and 0.2 nmoles/min/mg with 1-palmityl-sn-glycerol-3-phosphorylcholine as acyl acceptor. Apparent Km values of 4.5 and 5.2 μM for oleyl-CoA and 6.5 and 4.6 μM for palmityl-CoA were obtained with 1-oleyl- and 1-palmityl-sn-glycerol-3-phosphorylcholine, respectively. The Km values of 1-oleyl-sn-glycerol-3-phosphorylcholine were 20 and 50 μM using oleyl-CoA and palmityl-CoA as acyl donors. The possible involvement of this enzyme in membrane dynamics during lipid secretion is discussed.

INTRODUCTION

In previous studies of milk glycerolipid synthesis, a preferential synthesis of phosphatidylcholine (Pc) was observed consistently both in vivo and in vitro (1-3). This has been ascribed to an active role of Pc in the assembly, intracellular mobility, and extrusion of milk fat droplets in mammary tissue by a mechanism, conceivably including a lyso-Pc/Pc acylation, deacylation cycle (1). Evidence for such a mechanism has been adduced for the analogous process of chylomicron secretion (4-7), where the Golgi membranes have been implicated (5). To examine this, the activity of acyl-CoA 1-acyl-sn-glycerolphosphorylcholine (AGPC) acyl transferase was assayed in lactating bovine mammary tissue.

MATERIALS AND METHODS

Mammary tissue from lactating bovine was fractionated, as described (8). Microsomes were lyophilized and stored in small vials at -30 C.

Bovine serum albumin (BSA) and 5-5′ dithiobis-(2-nitrobenzoic acid) (DTNB), were purchased from Sigma Co. (St. Louis, Mo.). The acyl-CoA species were obtained from P-L Biochemical (Milwaukee, Wisc.) and AGPC was procures from Applied Science (State College, Pa.). All other chemicals were reagent grade.

Microsomes were dissolved in Tris-HCl buffer (65 mM, pH 7.4) with gentle sonication (model 8845-3, Cole Palmer Instrument Co., Chicago, Ill.). Protein was quantified by the method of Lowry, et al. (9). Acyl-CoA: AGPC acyl transferase activity was measured using the spectrophotometric method of Rodgers (10) employing a Perkin Elmer model 356 UV-visible recording spectrophotometer. Assays contained acyl-CoA (2-25 μM); AGPC (5-80 μM); DTNB (1 mM); Tris-HCl (65 mM, pH 7.4), and 0.1 mg protein in a total volume of 1 ml. Control cuvettes lacked the AGPC. The reactions, at 31 C, were initiated by the addition of acyl-CoA and monitored continuously by the interaction of released CoA with DTNB (10).

RESULTS

Acylation rates were linear under conditions described earlier (11), i.e. 0.1 mg microsomal protein for 10 min at 31 C. Unlike the acyl-CoA L-α-glycerolphosphate acyl transferase, this reaction was not influenced by Mg^{++} at concentrations up to 1 mM. Optimum pH was 7.4.

With the exception of oleyl-CoA comparable rates of acylation of both oleyl-glycerolphosphorycholine (GPC) and palmityl-GPC were obtained (Table I). This is consistent with earlier reports (12,13). Oleyl-GPC was used as substrate in subsequent experiments.

Using low concentrations (ca. critical micelle concentration [CMC]) of both substrates, it was apparent that oleyl-CoA was the preferred substrate for this enzyme (Table I). The inclusion of BSA (3 mg/ml) under these conditions reduced rates by almost 50% but the substrate preferences were consistent. This effect probably was caused by binding of both substrates by the BSA and subsequent reduction of their free concentration in the assay media.

Increasing the concentrations of substrates, in the absence of BSA, caused a gradual drop in acylation rates, presumably because of the disruptive effect of these amphipathic sub-

TABLE I

Relative Rates of Acylation of 1-Oleyl-sn-Glycerol-3-Phosphorylcholine by Acyl-CoA Species Using Bovine Mammary Microsomes[a]

Acyl-CoA (concentration)	1-oleyl-sn-glycerol-3-phosphorylcholine 60 μM (nmoles/min/mg protein)	
10 μM	A[b]	B
Myristyl-CoA	4.9	2.2
Palmityl-CoA	3.0	1.8
Stearyl-CoA	1.0	0.8
Oleyl-CoA	14.0	8.0
Linoleyl-CoA	0.1	0.1
25 μM	300 μM	
Myristyl-CoA	2.3	6.3
Palmityl-CoA	2.3	6.4
Stearyl-CoA	0.1	1.8
Oleyl-CoA	9.1	21.0
Linoleyl-CoA	0.1	0.1

[a]Assays contained 5-5'-dithiobis-(2-nitrobenzoic acid) (1 mM), acyl-CoA, 1-oleyl-sn-glycerol-3-phosphorylcholine, microsomal protein (0.1 mg) in 1 ml Tris-HCl (65 mM, pH 7.4). Bovine serum albumin (3 mg) was included in B but omitted from A.

[b]Comparable rates for 1-palmityl-sn-glycerol-3-phosphorylcholine (60 μM) in absence of bovine serum albumin were 5.2, 4.0, 1.0, 3.5, and 0.2 for the acyl-CoA species listed in the order above.

strates on the microsomal lipoprotein (enzyme) complex. BSA relieved this inhibition, and the maximum rates obtained (Table I) indicate that the preference for oleyl-CoA was more pronounced. The low rates observed with stearyl-CoA and particularly linoleyl-CoA is particularly noteworthy, because these acids may constitute 5 and 14% of the fatty acids on position sn-2 of phosphatidylcholine of bovine mammary microsomes (14).

Lineweaver-Burk plots (Fig. 1) revealed apparent Km values of 4.5 and 20 μM for oleyl-CoA and oleyl-GPC, respectively. Corresponding Km values of 6.5 and 50 μM were obtained when palmityl-CoA was the acyl donor. With palmityl-GPC as acyl acceptor, Km values of 5.2 and 4.6 μM for oleyl- and palmityl-CoA were obtained, and the Km for the palmityl GPC was around 50 μM with both acyl groups.

The inclusion of BSA in incubation medium increased the apparent Km values twofold and reduced Vmax by half for each substrate (Table II).

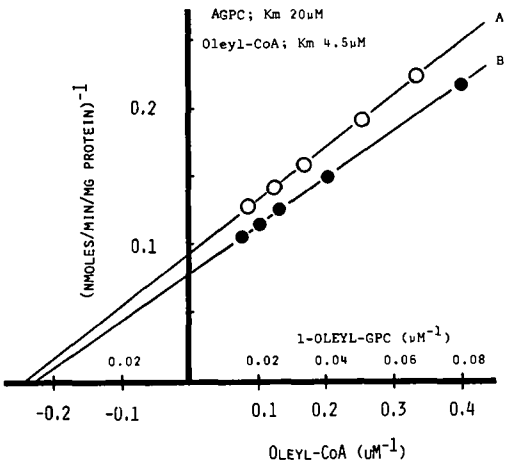

FIG. 1. Lineweaver-Burk plot showing effect of substrate concentration upon rate of acylation of 1-oleyl-sn-glycerolphosphorylcholine by bovine mammary microsomes. Assays as described in text; oleyl-CoA, 25 μM, in A; 1-oleyl-glycerolphosphorylcholine, 60 μM, in B. AGPC = 1-acyl-sn-glycerolphosphorylcholine.

DISCUSSION

These data confirm earlier evidence for the presence of acyl-CoA AGPC acyl transferase in bovine mammary tissue (1-3), and the apparent marked preference of this enzyme for oleyl-CoA is consistent with the rapid untake and incorporation of oleic acid by mammary cells in vitro (1,2). They also indicate that this enzyme can use both exogenous and endogenous oleyl-CoA and that it may be contiguous with the stearyl-CoA desaturase of bovine mammary microsomes (8), since earlier studies of bovine mammary stearyl-CoA desaturase revealed a

TABLE II

Influence of Bovine Serum Albumin on Apparent Km and Maximum Velocity of Acyl-CoA:1-Oleyl-sn-Glycerolphosphorylcholine (GPC) Acyl Transferase from Bovine Mammary Microsomes

Kinetic properties	With bovine serum albumin (3 mg/ml)		Without bovine serum albumin	
	Oleyl-CoA	1-Oleyl GPC	Oleyl-CoA	1-Oleyl GPC
Km (μM)	10.0	55.0	4.5	20.0
Vmax (nmole/min^{-1}. mg^{-1})	6.0	5.3	12.6	10.5

marked incorporation of endogenous oleic acid into microsomal phosphatidylcholine. The presence of AGPC acyl transferase in mammary tissue means that labeled fatty acids are limited for quantifying the de novo synthesis of mammary of milk phospholipids, particularly Pc.

The relative rates obtained with the various fatty acids were quite similar to those obtained using rat liver microsomes, with the exception of linoleyl-CoA which was the optimum substrate for the enzyme from rat liver (13,15). The low utilization of linoleyl-CoA was puzzling, since the linoleic acid in mammary and milk Pc occurs almost exclusively on position sn-2. However, the present acylation rates were not proportional to the concentration of the individual fatty acids on the secondary position of microsomal Pc, where C14, C16, C18, C18:1, and C18:2 are 7, 34, 5, 33, and 14%, respectively (14).

The maximum AGPC acylation rates were higher than those obtained with acyl-CoA:L-αGP-acyl transferase (11) though somewhat less than the respective rates obtained when 1-acyl-sn glycerol-3-phosphate (AGP) was the acyl acceptor, i.e. 2.2, 9.7, 1.4, 2.1, and 0.6 with L-αGP; and 13, 31, 8.7, 11.4, and 2 nmoles acylated/min/mg protein with AGP for myristyl-, palmityl-, stearyl-, oleyl-, and linoleyl-CoA, respectively (J.E. Kinsella and M. Gross, unpublished data). AGPC is apparently the best substrate for oleyl-CoA in lactating mammary tissue and coincidentally oleic acid, together with palmitic acid, is the principal fatty acid in position sn-2 of milk Pc.

The changes in Km and Vmax caused by the inclusion of BSA in the assay medium are consistent with its known affinity for acyl groups and lysophosphatides. Unless the BSA binding constant and the stoichiometry for substrates and products of the reaction are taken into account, the concentration of substrates in free form is unknown, and, of course, this situation is exacerbated when amphipathic, micelle forming substrates are used. Thus, kinetic constants determined in different studies are difficult to compare, unless concentrations of substrates, enzyme, and BSA are similar.

The presence of very active AGPC acyl transferase is consistent with the suggestion that Pc turnover may be an integral phenomenon associated with intracellular aggregation of triglycerides and secretion of lipid droplets in mammary tissue, as apparently occurs in the intestinal mucosa (4,5). Membrane lysis and reformation (16), together with active phospholipases and reacylating enzymes, occur in intestine during chylomicron secretion (5-7), indicating that an active lyso-Pc/Pc deacylation-reacylation cycle may be important in lipid secretion. Reversible hydrolysis and reacylation of Pc may facilitate motility and extrusion of lipids by continuously altering the physical properties and conformation of intracellular membranes (16). It is conceivable that a similar cycle occurs in lactating mammary tissue, where membrane material is extruded with fat droplets (17-19). In support of this suggestion is the marked labeling of phosphatidylcholine that occurs following the exposure of mammary tissue to endogenous and exogenous radioactive acyl groups both in vivo and in vitro (1-3, 19).

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Unusual Composition of Sterols in a Phytophagous Insect, Mexican Bean Beetle Reared on Soybean Plants

J.A. SVOBODA and M.J. THOMPSON, Insect Physiology Laboratory[1], T.C. ELDEN, Applied Plant Genetics Laboratory[1], and W.E. ROBBINS, Insect Physiology Laboratory[1], Beltsville, Maryland 20705

ABSTRACT

Three saturated sterols, cholestanol, campestanol, and stigmastanol, constituted 54, 72, and 77% of the total sterols of the egg, prepupa, and adult, respectively, of the Mexican bean beetle, *Epilachna varivestis* (Mulsant), reared on soybean plants. Lathosterol (7-cholesten-3β-ol), possibly formed from cholestanol in this insect, constituted 12, 16, and 11.8% of the total sterols isolated from egg, prepupa, and adult, respectively. None of these sterols have been isolated and identified previously as components of the sterols of a phytophagous insect reared on a natural host plant. Cholesterol, a major sterol of most plant feeding insects studied thus far, comprised less than 5% of the total sterols in any of the stages examined. The unique composition of the sterols in this insect in relation to the sterol composition of the host plant is compared to dietary sterol utilization and metabolism in other phytophagous insects.

INTRODUCTION

In a previous study with the Mexican bean beetle, *Epilachna varivestis* (Mulsant), certain azasteroids were shown to disrupt larval growth and development of this species when administered by foliar application (1). These azasteroids are known to inhibit the conversion of C_{28} and C_{29} plant sterols to cholesterol by blocking the Δ^{24}- and $\Delta^{22,24}$-sterol reductase enzyme systems and presumably disrupt other pathways of steroid metabolism, such as molting hormone biosynthesis or metabolism in several species of insects (2,3). To determine the possible effects of these azasteroids upon sterol metabolism in the Mexican bean beetle larvae, it was first necessary to isolate and identify the sterols of the normal insect and of the host plant on which it was reared. We found the major normal neutral sterols of the Mexican bean beetle to be quite different from those previously reported for other plant feeding insects. In this article, we describe the isolation and identification of the major sterols of the Mexican bean beetle and discuss the relationship of the insect sterols to those of the soybean plant on which these experimental insects were reared.

MATERIALS AND METHODS

The Mexican bean beetle prepupae used in the study were obtained from eggs produced by field collected adults fed leaves from the Clark variety soybean, *Glycine max* (L.) Merr., and held at 25 C in a growth chamber under a 12 hr photophase. Larvae were reared in 1 gal glass jars on bouquets of these soybeans leaves, grown under greenhouse conditions. Prepupae were weighed and frozen as they were produced. The adults were obtained from the same field collected insects, and the soybean leaves used for analysis were from plants of the Clark variety used in rearing the insects.

All insect material was homogenized in chloroform-methanol (2:1) in a Tenbroeck tissue grinder, and the total sterols were isolated and purified after saponification of the lipids, as previously described (4). Soybean leaf material was homogenzied in the same solvents with a Virtis homogenizer, and the sterols were isolated and purified in the same manner as the sterols of the Mexican bean beetle. The nonsaponifiable material from both the insect and soybean leaf lipids was subjected to digitonide precipitation to remove accompanying fatty alcohols that were detected by thin layer chromatography (TLC) and gas liquid chromatographic (GLC) analyses.

After GLC analysis of the purified sterols from Mexican bean beetle eggs, prepupae, and adults on two GLC systems (1.0% OV-17 and 0.75% neopentyl glycol succinate [NGS]), it was obvious that there was a considerable difference between the sterols of this insect (Table I) and those of previously examined phytophagous insects in which cholesterol was a predominant sterol. The sterols were acetylated, and aliquots of the acetates were chromatographed on 20% $AgNO_3$-impregnated Silica Gel H chromatoplates developed in benzene-n-hexane (1:1). The sterol acetates separated into two distinct spots, which were visualized by spraying with 50% H_2SO_4

[1]ARS, USDA.

TABLE I

Relative Percentages of Sterols Identified from Various Mexican Bean Beetle Stages and Soybean Leaves

Sterol	Mexican bean beetle (Relative % of total) Eggs (1)[a]	Prepupae (2)	Adults (2)	Soybean leaves (Relative% of total) (4)
Cholesterol	2.9	4.4	4.5	0.9
Cholestanol	19.0	29.0	50.7	<0.1
Lathosterol	12.0	16.0	11.8	—
Campesterol	1.1	T[b]	T	11.3
Campestanol	7.1	10.2	6.0	0.2
Δ^7-Campestenol + unknown	20.9	3.6	2.0	—
Stigmasterol	—	1.7	1.4	31.5
Stigmastanol	27.5	32.9	20.3	1.1
Sitosterol	3.2	2.2	2.3	55.0
Δ^7-Stigmastenol	6.3	T	1.0	—
Total saturated sterols	53.6	72.1	77.0	1.4

[a]Number in parentheses = number of samples quantitated to obtain these values.
[b]T = detectable trace.

TABLE II

Gas Liquid Chromatographic Data on Insect and Soybean Leaf Sterols

Compound	RRT[a] on 1.0% OV-17 Standard	Insect	Soybean leaves	RRT on 0.75% NGS Standard	Insect	Soybean leaves
Cholesterol acetate	3.41	3.42	3.44	6.63	6.57	6.72
Cholestanol acetate	3.41	3.44	3.51	6.51	6.49	6.52
Lathosterol acetate	4.00	4.02	—	8.01	8.02	—
Campesterol acetate	4.51	4.55	4.53	8.88	9.00	8.87
Campestanol acetate	4.50	4.57	4.55	8.78	8.84	9.10
Stigmasterol acetate	4.90	5.00	4.88	9.42	9.41	9.53
Δ^7-Campestenol acetate	5.20	5.10	—	10.70	10.74	—
Sitosterol acetate	5.63	5.68	5.56	11.14	10.87	11.36
Stigmastanol acetate	5.61	5.68	5.69	11.02	11.00	11.26
Δ^7-Stigmastenol acetate	6.45	6.50	—	13.50	13.42	—

[a]Retention time relative to cholestane. Column conditions as previously described (15,16).
[b]NGS = neopentyl glycol succinate.

and heating at 100 C. These spots corresponded ca. in R_f values to those of cholestanol acetate and cholesterol acetate, indicating the presence of a mixture of saturated sterols and sterols containing one double bond, with the saturated sterol acetates comprising the major fraction of this mixture.

The sterol acetates from each insect sample were chromatographed on a 3 g column of 20% $AgNO_3$-impregnated Unisil, as previously described (5). In a typical analysis of the sterol acetates, the column was eluted with 25 ml of each of the following: 10, 15, 20, and 25% benzene in n-hexane and 100% benzene. With TLC on $AgNO_3$-impregnated Silica Gel H, it was seen that most of the saturated sterol acetates eluted in the 15% benzene fraction; the 20% benzene fraction contained a mixture of stanol acetates and sterol acetates that probably contained one double bond. The bulk of the remaining sterol acetates eluted in the 25% benzene fraction and appeared to be monounsaturated sterol acetates when analyzed by $AgNO_3$-TLC. Rechromatography of the third fraction (20% benzene) provided adequate separation of the stanol acetates from the unsaturated components of the mixture. In this rechromatography, it was noted that the unsaturated components in the 20% benzene fraction had a slightly greater R_f than cholesterol acetate and that they fluoresced under UV light with a pale orange color that was quite different from the usual yellowish color produced by most monounsaturated sterol acetates. Though the major

component behaved similarly to desmosterol acetate by GLC, it eluted somewhat faster from the $AgNO_3$-Unisil column than the usual Δ^5-sterol acetates. The fact that this unknown compound was more polar than cholestanol acetate though less polar than cholesterol acetate suggested that its double bond was located elsewhere than at the Δ^5-position. A gas chromatograph-mass spectrum (GC-MS) of this sterol acetate which exhibited a strong molecular ion at m/e 428 confirmed that the double bond was not at the C-5 position, since Δ^5-sterol acetates do not give a parent ion but instead give a strong peak at M-60 under these conditions. Other major peaks at m/e 413, 368, 315, and 255 (base peak) indicated loss of methyl, acetic acid, C_8H_{17} (side chain) and C_8H_{17} + acetic acid, respectively. The strong peaks at m/e 315 and 255 also placed the double bond in the sterol nucleus. This and other observations previously mentioned suggest that the compound is lathosterol (7-cholesten-3β-ol) acetate. Comparative GLC of this sterol acetate (Table II) and the free sterol and GC-MS analyses with authentic lathosterol and its acetate identified this unknown as lathosterol. Similarly, two other Δ^7-sterols were identified as Δ^7-campestenol and Δ^7-stigmastenol. These sterols made up a significant portion (Table I) of the Mexican bean beetle egg sterols, and the Δ^7-campestenol was accompanied by an unidentified C_{29} sterol.

RESULTS AND DISCUSSION

Lathosterol comprises about 12, 16, and 12% of the sterols of the Mexican bean beetle egg, prepupa, and adult, respectively. Although lathosterol is the principal metabolite of cholestanol in two species of cockroaches (6,7) and also has been reported to be a major component of the sterols of certain other invertebrates, including some species of nematodes (8,9) and mollusks (10), this is the first report in which lathosterol is identified as one of the normal major sterol components of an insect fed on a natural host plant.

Of equal interest was the fact that the stanols, identified by comparative GLC and GC-MS as cholestanol, campestanol, and stigmastanol, made up ca. 54, 72, and 77% of the total sterols from the egg, prepupa, and adult, respectively, of the Mexican bean beetle (Table I). We know of no other report of the normal occurrence of such high levels of saturated sterols in insect tissues. Cholestanol and stigmastanol were the major stanols, with cholestanol accounting for over 50% of the total sterols in the adult insect.

The Δ^5-sterols cholesterol, campesterol, stigmasterol, and sitosterol, from the insect also were identified positively by the same methods, as were lathosterol and the saturated sterols. Of particular interest was the small amount of cholesterol found in the insect sterols (egg, 2.9%; prepupa, 4.4%; and adult, 4.5%). In other insects, both phytophagous and omnivorous, in which the sterols have been analyzed by specific methods, cholesterol has often been one of the principal sterols, and, in plant feeding species, with the exception of one known case, *Drosophila pachea* Patterson and Wheeler (11), it usually has been the major sterol.

The results of analyses of the sterols from soybean leaves raised some interesting questions concerning the utilization and metabolism of dietary sterols in the Mexican bean beetle. Over 98% of the soybean plant sterols are comprised of the normal phytosterols campesterol, stigmasterol, and sitosterol, plus a detectable amount of cholesterol (<1% of total). Possibly this insect possesses a selective uptake and retention mechanism that takes up saturated sterols from the diet to the partial exclusion of unsaturated sterols similar to the selective uptake and retention of certain sterols found in the house fly, *Musca domestica* (L.) (12). Since cholestanol is only present in extremely small amounts in the insect diet (Table II), such a mechanism also would require the dealkylation of stigmastanol to cholestanol and its subsequent conversion to lathosterol. However, based upon the small percentage (<2% of total) of saturated sterols in the plant sterols and the relatively large percentage of stanols found in the different developmental stages of the Mexican bean beetle, it appears that this insect can reduce the Δ^5-bond of the plant sterols; these stanols are then dealkylated to produce cholestanol. In addition, since no Δ^7-sterols were detected in the soybean leaf sterols, the Mexican bean beetle must convert the stanols to the Δ^7-sterols, a dehydrogenation that has been observed to occur in a number of species (13). The occurrence and importance of the Δ^7-bond in insect sterols and sterol metabolism have been pointed out and emphasized by recent investigations with certain other species (14,15).

The present study of the sterols of the Mexican bean beetle again points to the diversity that may exist between insect species in relation to the utilization and metabolism of dietary sterols. Interestingly, the Mexican bean beetle is a member of the subfamily Epilachninae which contains nearly all the phytophagous species of the family Coccinellidae (the ladybugs), a family of insects that are predomi-

nantly predacious. The unusual spectrum of sterols found in this insect then may result from mechanisms and pathways of phytosterol utilization and metabolism that have secondarily evolved in a phytophagous insect that was originally zoophagous and may account for the differences found in this species as compared to other plant feeding insects. Future studies with radiolabeled sterols and inhibitors of sterol metabolism used in conjunction with semidefined diets should help us to clarify and better understand the source of the sterols and pathways of sterol metabolism that occur in this insect.

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Total Glycerophospholipid Fatty Acid and Phospholipid Class Composition of Nerve Ending and Related Fractions from Fetal and Adult Pig Cerebellum and Adult Pig Whole Brain Cortex

R.L. GEISON, Waisman Center on Mental Retardation and Human Development, Department of Pediatrics and University of Wisconsin Center for Health Sciences, **A.L. FLANGAS,** Wisconsin Regional Primate Research Center, and **S.E. KORNGUTH,** Departments of Neurology and Physiological Chemistry, University of Wisconsin Center for Health Sciences, Madison, Wisconsin 53706

ABSTRACT

Membrane fractions derived from crude mitochondrial fractions of pig cerebellums were separated on a continuous CsCl-sucrose gradient. Fetal and adult brains were used as starting material. The major differences in total glycerophospholipid fatty acid composition between fetal and adult membranes were an increase with maturation in docosahexaenoic acid and a decrease in palmitic acid which occurred in all membranes, including nerve ending and smooth membrane fractions. Phosphatidylcholine levels decreased, and ethanolamine phosphatide levels increased with maturation in all adult membrane fractions. Phosphatidylserine levels increased primarily in nonmitochondrial fractions in adult tissues. The results indicate that both hydrophobic and hydrophylic characteristics of several membrane fractions, including nerve endings, change with development. The developmental changes in pig cerebellums are similar to those reported for whole brain or brain regions from other species. Mitochondrial enriched fractions derived from adult pig whole brain cortex had significantly reduced palmitate levels and significantly elevated oleate levels compared with nerve ending and smooth membrane fractions.

INTRODUCTION

The degree of membrane lipid unsaturation is known to contribute to the fluidity of the membrane and has been shown to affect active (1) and passive (2) transport processes. It is, therefore, of interest to explore membrane polyunsaturated fatty acid content in brain where transport of ions and chemical transmitters across membranes is critical for function.

Adult brain glycerophospholipids contain significant amounts of the unsaturated fatty acids, arachidonic and docosahexaenoic acids. In the brains of a variety of mammalian species, the content of both arachidonic and docosahexaenoic acids is more constant that in liver (3). In essential fatty acid deficiency, the brain levels of arachidonic and docosahexaenoic acids associated with individual glycerophospholipids are altered to a much lesser degree than in liver, muscle, or serum (4), and the unsaturation index remains constant (5). In developing brain, polyunsaturated fatty acid composition is variable, depending upon the ratio of dietary linoleic and linolenic acids. Inversely proportional changes in the levels of 22:5 (n-6) and 22:6 (n-3) occur while the sum of these two fatty acids remains constant (4,6). Adult brain also is influenced by dietary linoleic and linolenic fatty acid content (7-8). Although the brain maintains a constancy of fatty acid polyunsaturation, the percentage of unsaturation of brain glycerophospholipid, exclusive of myelin, increased coincident with maturation (6,9-12). These changes were due in part to relative increases in glycerophospholipids enriched in unsaturated fatty acids (9,10,13) and also to increases in unsaturation within glycerophospholipid classes (10,12). Significant subcellular variations in fatty acid unsaturation of phosphatidylcholine also exists (14-16), in addition to the major variation of fatty acid composition of myelin phosphatidylcholine. Mitochondrial phosphatidylcholine polyunsaturated fatty acids (14-16) and molecular species containing polyunsaturated fatty acids (15) are elevated ca. 50% compared to synaptosomes or microsomes. Small variations in fatty acid composition of individual phospholipids of neuronal and glial cell fractions were reported (16).

The mechanisms contributing to the ability of the brain to regulate a given level of polyunsaturated fatty acids are not known. To determine whether developmental increases in polyunsaturated fatty acids were related to the maturation of specific membranes, several membrane fractions were prepared from fetal and adult pig cerebellums. It previously was shown that the fatty acid composition of gangliosides from these membranes changed with

ontogenesis (17).

METHODS

Membrane Fractionation

The preparative isolation and initial lipid analyses of the cerebellar and whole brain membrane fractions have been reported previously (17-19). The tissues were obtained through the courtesy of a local slaughter house (Oscar Mayer and Co., Madison, Wisc.). Fetal and adult tissues were obtained within 25 min of death (induced by CO_2 anoxia) of the animals or of the pregnant sows. Fetal cerebellums and adult cerebral cortices were removed and placed on ice, homogenized, and subjected to fractionation within 2 hr. Fetal cerebellums at 60-70 and 77-87 days gestational age (determined by crown rump measurements [19]) were obtained at each age from a total of 50-70 individual fetuses collected from 5-7 individual mothers.

The adult cerebral cortex fractions were obtained from four individual brains which were pooled. The adult whole brain cortex fractions were obtained from five individual brains which were pooled in each of three separate experiments. Tissues were homogenized with a Dounce homogenizer in 10 volume of 0.32 M sucrose-1 mM $MgCl_2$-0.4 mM potassium phosphate buffer, pH 7.0. The homogenate was centrifuged at 1100 x g x 20 min. The supernate was centrifuged at 11,000 x g x 40 min; the pellet was resuspended in 0.32 M sucrose-10 mM $MgCl_2$-0.4 mM phosphate buffer, pH 7.0, and placed on a discontinuous sucrose gradient (0.8, 1.0, and 1.4 M sucrose containing 10 mM $MgCl_2$). After centrifugation at 55,000 x g x 90 min the 1.0-1.4 M sucrose interface (crude synaptosomes) was removed and subjected to CsCl-sucrose zonal centrifugation (18). Fractions were collected at their isopycnic densities and characterized by electron microscopy (18,19). Attempts to characterize these fractions further by enzyme analysis were unsuccessful due to CsCl inactivation of several enzyme activities (Particularly the Na^+-K^+ dependent ATPase).

Phospholipid Isolation and Assay

Phospholipids were extracted (19) from the washed membrane fractions which had been resuspended in 0.5 ml 0.73% NaCl. Lipids were extracted with 20 volume of chloroform-methanol (2:1, v/v). This extract was filtered and partitioned with 0.2 volume of 0.73% NaCl. Lower phase lipids were concentrated under a N_2 stream and dried in a vacuum over NaOH pellets at 4 C overnight. The lipids were redissolved in chloroform and a residue removed by filtration. The phospholipids were separated on two dimensional thin layer chromatographic (TLC) plates and quantified (20). Plates (0.25 mm) were prepared with 20 g Silica Gel H (Merck, Brinkman Instruments, Westbury, N.Y.), 1.5 g magnesium acetate, and 52 ml H_2O. Lipids were located by I_2 vapors and identified by relative migration rates after development with chloroform-methanol-28% aqueous NH_3 (by volume 65-25-5) and in the second dimension with chloroform-acetone-methanol-acetic acid-H_2O (by volume 3-4-1-1-0.5). Spots were scraped from the plates and digested with 70% perchloric acid (Allied Chemical, Morristown, N.J.) using an oil bath instead of a heating block. The color development procedures were as described (20). In addition to the reported major lipid classes, lipids migrating as phosphatidic acid and lysophosphatidylethanolamine were observed. The phosphate content of these lipid classes was too low to measure accurately. In some instances, analytical variations in color development of phospholipid spots on the silica gel blanks were elevated sporadically and not included in the results.

Fatty Acid Determination

Glycerophospholipid fatty acids were prepared by reacting an aliquot of the total lipid extract with 1 ml 14% (w/v) BF_3-methanol (Applied Science Laboratories, State College, Pa.) at 60 C for 15 min in screw-cap vials with Teflon liners. After cooling, 1 ml hexane and 2-4 drops of water were added, and, after mixing and centrifuging, the hexane layer was removed and evaporated to dryness under a N_2 stream. The fatty acid methyl esters were dissolved in redistilled CS_2 and separated on a column (8 ft x 5 mm) of 15% (w/w) stabilized diethylene glycol succinate on 80-90 mesh Anakrom ABS support (Analabs, Hamden, Conn.) at 205 C. The equivalent chain lengths (ECL) of the fatty acids were determined by comparison of relative retention times with saturated standards. The ECL for 20:4 (n-6), 22:4 (n-6), 22:5 (n-6), 22:5 (n-3), and 22:6 (n-3) were 22.8, 25.0, 25.5, 26.1, and 26.7, respectively, which were 2-3% higher than respective literature values of 22.43, 24.58, 24.97, 25.38, and 26.03 (21). In addition, unidentified fatty acids with ECL of 21.3, 21.9, and 22.2 were detected in all fetal and adult cerebellar fractions at respective levels of 0.2, 0.5, and 0.3% of total fetal fatty acids and 0.2, 0.3, and 0.4% of total adult fatty acids. Two peaks with ECL of 23.9 and 24.3 also were present. Their amounts were variable and decreased with in-

TABLE I

Summary of Reported Data on Membrane Composition of Fractions Isolated from Pig Brain on CsCl-Sucrose Gradients

Tissue	Density[a]	Membrane[b] morphology	Mg/100 mg protein			Phospholipid/ cholesterol molar ratio
			Phospholipid	Cholesterol	Gangliosides	
60-70 day fetal cerebellum	1.152	Sm	104	31	4.2	1.68
	1.188	Sy	65	18	2.7	1.87
	1.198	Sy, Mito, Df	50	13	1.6	1.94
	1.205	Mito, Df	49	13	1.6	1.86
Adult (ca. 6 mo) cerebellar cortex	1.146	Sm, My	125	47	8.2	1.36
	1.159	Sm	97	33	7.7	1.48
	1.164	Sm	88	27	7.0	1.64
	1.173	Sy, Sm	70	22	5.6	1.62
	1.178	Sy	59	17	4.8	1.72
	1.208	Mito, Nf	40	9	2.2	2.20
Adult (ca. 6 mo) whole brain cortex	1.147	Sm, My	113	33	6.1	1.68
	1.157	Sm	103	31	6.5	1.66
	1.165	Sm	95	28	6.3	1.67
	1.168	Sm	84	23	4.5	1.79
	1.180	Sy	71	19	3.8	1.90
	1.211	Mito, Nf	54	10	1.8	2.72

[a]Densities of fractions collected from zonal centrifugation of crude mitochondrial fractions.

[b]Fractions were submitted to electron microscopy. Identifiable structures are listed in order of decreasing abundance in this and the following tables. My = myelin, Sm = smooth membrane, Sy = nerve endings with synapses, Mito = free mitochondria, Nf = neurofilaments, and Df = large dendritic fragments.

TABLE II

Phospholipid Class Composition of Pig Cerebellar Membrane Fractions[a]

Tissue	Density	PC	PE	PS	PI	SM
60-70 day fetal cerebellum	1.152	55.9 ± 3.3[b]	26.4 ± 1.1	8.9 ± 0.9	5.0 ± 3.8	3.9 ± 3.4
	1.188	55.2 ± 3.4	23.9 ± 1.7	9.8 ± 1.5	5.7 ± 0.5	5.3 ± 3.1
	1.198	53.4 ± 3.4	27.3 ± 3.8	7.4 ± 1.0	6.1 ± 2.2	5.7 ± 2.9
	1.205	55.5 ± 3.4	26.4 ± 1.7	7.2 ± 1.0	5.8 ± 3.4	5.1 ± 4.1
Adult (ca. 6 mo) cerebellar cortex	1.146	42.2 ± 3.4	31.1 ± 4.4	14.7 ± 1.7	4.2 ± 0.3	7.7 ± 2.1
	1.159	37.2 ± 4.1	31.3 ± 2.9	17.2 ± 3.6	4.3 ± 0.5	9.9 ± 1.7
	1.164	46.8 ± 2.6	28.8 ± 3.0	13.9 ± 1.9	3.7 ± 0.3	6.8 ± 1.9
	1.173	48.3 ± 1.6	27.6 ± 2.6	10.7 ± 1.4	7.4 ± 0.6	5.9 ± 1.5
	1.178	44.8 ± 4.1	28.3 ± 1.8	10.8 ± 3.7	6.5 ± 1.8	9.6 ± 1.7
	1.208	46.7 ± 2.7	34.7 ± 1.8	8.6 ± 1.3	5.4 ± 0.3	4.6 ± 1.6

[a]PC, PE, PS, and PI refer respectively to glycerophospholipids containing choline, ethanolamine, serine, and inositol, and SM = sphingomyelin.

[b]Percentage ± one standard deviation of each lipid class determined on four individual thin layer chromatographic runs/membrane fraction.

creasing sample size. In the samples analyzed, they represented <3% of total fatty acids. Artifacts at a similar retention time have been reported previously (10). Attempts to analyze for fatty acids of individual phospholipids after two-dimensional TLC were unsuccessful due to significant losses of polyunsaturated fatty acids with the small amount of lipid applied to the plate. Fatty acid percentages were calculated by determining the peak ht x width at half ht. Minor fatty acids were within 0.3%, and major fatty acids were within 0.8% of stated wt percentages of a standard fatty acid sample (H-104, Applied Science Laboratories, State College, Pa.). Fatty aldehydes did not interfere with the reported fatty acid separations. On our column system, fatty aldehydes or dimethyl acetals chromatograph just prior to the fatty acid methyl ester of equivalent chain length or elute at the position of a fatty aldehyde of ca. 2 carbon atoms less in chain length. The earlier eluting peak also has a greater width to ht ratio than would be expected. The recovery of the fatty aldehydes is also variable. The on column conversion of dimethyl acetals to methyl-1-alkenyl ethers with reduced recoveries has been reported previously (22).

RESULTS

Phospholipid Composition

Published data on the gross compositon of the fractions analyzed is reviewed in Table I (17-19). Phospholipid to cholesterol molar ratios in nonmitochondrial fractions were similar in fetal and adult cerebellum within analytical variance. Decreases in cholesterol content occurred in adult cerebellar and whole brain cortex mitochondrial fractions; this resulted in elevation of the phospholipid to cholesterol molar ratio. Phospholipid to cholesterol ratios and total lipid to protein ratios were similar in comparable fractions from whole brain and cerebellar cortex. Phospholipid to cholesterol ratios increase in mitochondrial fractions and decrease somewhat in nerve ending fractions during development in rat brain (23).

The membrane fractions from adult cerebellums generally contained more phosphatidylserine, sphingomyelin, and ethanolamine phosphatides and less phosphatidylcholine than fetal membranes, while phosphatidylinositol levels remained fairly constant during ontogenesis (Table II). Fractions containing mitochondria tended to have less phosphatidylserine and sphingomyelin than other fractions. These results are consistent with relatively lower levels of cholesterol, phosphatidylserine, and sphingomyelin in mitochondrial fractions from adult guinea pig brain (24).

Developmental Changes in Fatty Acid Composition

Changes in fatty acid composition of all the cerebellar membranes analyzed occurred during maturation (Tables II and IV). To evaluate these changes, an average fatty acid composition of the four smooth membrane and nerve ending fractions analyzed was determined. The adult mitochondrial fractions were not included in these calculations due to the demonstration of significant variations in composition of this fraction in adult whole brain cortex (Table V). Some myelin fragments were observed in the lightest density fraction from adult tissue. A compromising effect of myelin upon the fatty acid composition of the lightest density fractions was suggested by the elevated oleic acid levels in the samples analyzed. These fractions were, therefore, excluded from the calculations. It is assumed that the small variations in fatty acid composition between the fractions averaged at each age were the result of analytical variations. The two separate fetal preparations were similar in relative fatty acid composition.

Among the polyunsaturated fatty acids, the 22:6 (n-3) relative percentage increased from 6.8-8.8% in the two fetal preparations to 11.7 ± 1.1% in the adult preparation. The percentage of 22:5 (n-3) also increased (1.5-1.9% to 3.3 ± 0.3%), while levels of 22.4 (n-6) decreased (4.4-4.6% to 3.2 ± 0.2%). The developmental increase in total polyunsaturated fatty acids (23.2-24.0% to 29.1 ± 1.7%) was primarily due to an increase in 22:6 (n-3). The levels of 22:5 (n-3) and 18:2 (n-6) remained constant at respectively 0.4-0.6% and 0.5-0.7% of total fatty acids at both ages. Arachidonic acid levels increased slightly (8.3-8.9% to 9.8 ± 0.2%) in the adult membrane fractions. Among the saturated fatty acids, 14:0 and 16:0 percentages decreased respectively (1.8-2.4% to 0.6 ± 0.1%) and (28.8-29.3% to 23.8 ± 0.5%). Oleic acid levels increased from 21.1-21.2% to 24.2 ± 0.5%. The levels of 16:1 decreased from 3.7-5.0% to 1.2 ± 0.2%, while 18:1 levels remained fairly constant (19.2-19.7% to 20.5 ± 1.2%). The levels of a combined fraction of 20:1 and 18:3 (n-3) increased in the adult fractions (0.4% to 0.7 ± 0.1%). With the exception of 16:0 and 22:6 (n-3), the fatty acid composition of the smooth membrane and nerve ending fractions in adult cerebellar cortex was similar to equivalent fractions from whole brain cortex. More detailed studies will be required to determine the significance of the regional variation in content of these two fatty acids.

Subcellular Variation in Fatty Acid Composition

The palmitate content of mitochondria was significantly lower than in other fractions in adult tissue (Tables IV and V). Excluding the light density fractions which contained some myelin fragments, the oleic acid levels also were found to be significantly higher in adult mitochondrial fractions compared to the smooth membrane fractions. Linoleic and arachidonic acid levels were elevated somewhat, although the increase was not statistically significant. Oleate levels were elevated in the two light density fractions from adult cerebral and cerebellar cortex.

DISCUSSION

Although a considerable body of literature exists on the changes in lipid class composition in developing whole brain and myelin fractions, little data exists on the phospholipid and fatty acid composition of other cellular fractions at different developmental ages. Because of complications of myelin accumulation, the changes in membrane lipid composition in whole brain, apart from myelin, have been difficult to assess. To understand the mechanisms controlling the insertion of lipids into membranes during ontogeny, it is necessary to examine the lipid

TABLE III

Total Glycerophospholipid Fatty Acid Composition of Fetal Pig Cerebellar Membrane Fractions

	60-70 day fetal cerebellum[a]					77-87 day fetal cerebellum[a]				
Density	1.152	1.188	1.198	1.205		1.135	1.143	1.177	1.181	
Membrane morphology	Sm	Sy	Sy, Mito, Df	Mito, Df	Average[b]	Sm	Sm	Sy	Sy	Average[b]
Fatty acid										
14:0	2.3[c]	2.5	2.6	2.3	2.4 ± 0.2	1.7	2.4	1.4	1.7	1.8 ± 0.4
16:0	29.0	30.7	27.8	27.8	28.8 ± 1.4	31.1	31.2	26.1	28.9	29.3 ± 2.4
16:1	4.8	5.9	4.6	4.8	5.0 ± 0.6	3.4	4.0	3.6	3.9	3.7 ± 0.3
18:0	20.7	22.4	21.6	19.8	21.1 ± 1.1	21.1	20.1	21.8	21.6	21.2 ± 0.8
18:1	20.9	19.0	19.2	17.5	19.2 ± 1.4	19.5	18.8	20.4	20.1	19.7 ± 0.7
18:2 (n-6)	1.1	0.4	0.6	0.6	0.7 ± 0.3	0.4	0.5	0.4	0.5	0.5 ± 0.1
20:1 + 18:3 (n-3)	0.5	0.4	0.4	0.4	0.4 ± 0.1	0.5	0.4	0.4	0.4	0.4 ± 0.1
20:4 (n-6)	7.3	7.4	9.2	11.6	8.9 ± 2.0	7.6	7.9	9.2	8.5	8.3 ± 0.7
22:4 (n-6)	4.5	4.0	4.9	4.9	4.6 ± 0.4	4.3	4.1	4.8	4.3	4.4 ± 0.3
22:5 (n-6)	1.8	1.5	2.1	2.2	1.9 ± 0.3	1.4	1.4	1.6	1.4	1.5 ± 0.1
22:5 (n-3)	0.4	0.4	0.4	0.5	0.4 ± 0.5	0.7	0.6	0.6	0.6	0.6 ± 0.1
22:6 (n-3)	6.5	5.4	7.6	7.7	6.8 ± 1.1	8.3	8.7	9.7	8.3	8.8 ± 0.7
Saturated	52.0	55.6	52.0	49.9	52.4 ± 2.4	53.9	53.7	49.3	52.2	52.3 ± 2.1
Monoenes	25.7	24.9	23.8	22.3	24.2 ± 1.5	22.9	22.8	24.0	24.0	23.4 ± 0.7
Polyenes	21.6	19.1	24.8	27.5	23.2 ± 3.7	22.7	23.2	26.3	23.6	24.0 ± 1.6
(n-6) Family	14.7	13.3	16.8	19.3	16.0 ± 2.6	13.7	13.9	16.0	14.7	14.6 ± 1.0
(n-3) Family	6.9	5.8	8.0	8.2	7.2 ± 1.1	9.0	9.3	10.3	8.9	9.4 ± 0.6

[a]Sm = smooth membrane, Sy = nerve endings with synapses, Mito = free mitochondria, Df = large dendritic fragments.

[b]Average ± one standard deviation fatty acid composition of four analyzed fractions.

[c]Mass percentage of reported fatty acids.

TABLE V

Total Glycerophospholipid Fatty Acid Composition of Adult Pig Whole Brain Cortex Membrane Fractions[a]

Density	1.15 (1)[b]	1.16 (3)	1.165 (1)	1.17 (3)	1.18 (2)	1.21 (3)	
Membrane morphology	Sm, My	Sm	Sm	Sm, Sy	Sy	Mito, Nf	Average[c]
Fatty acid							
14:0	0.5[d]	0.6 ± 0.1	0.4	0.5 ± 0.0	0.6 (0.1)	0.6 ± 0.2	0.5 ± 1.0
16:0	23.9	26.5 ± 0.6 SD[e]	26.3	26.4 ± 1.0 SD	25.6 (0.4)	21.3 ± 0.8	26.2 ± 0.4
16:1	1.1	1.3 ± 0.4	1.0	1.3 ± 0.1	1.3 (0.3)	1.5 ± 0.3	1.2 ± 0.2
18:0	22.7	24.0 ± 0.8	25.0	23.0 ± 0.4	24.5 (1.2)	23.8 ± 0.3	24.1 ± 0.9
18:1	22.8	21.2 ± 0.5 SD	20.6	20.2 ± 1.3 SD	21.0 (0.6)	23.9 ± 1.1	20.8 ± 0.4
18:2 (n-6)	0.5	0.5 ± 0.1 ND[f]	0.4	0.6 ± 0.0 ND	0.6 (0.1)	0.8 ± 0.2	0.5 ± 0.1
20:1 + 18:3 (n-3)	0.7	0.6 ± 0.1	0.6	0.7 ± 0.0	0.7 (0.1)	0.7 ± 0.1	0.6 ± 0.1
20:4 (n-6)	10.2	8.9 ± 0.7 ND	8.8	9.4 ± 0.7 ND	9.2 (0.0)	11.1 ± 1.1	9.1 ± 0.3
22:4 (n-6)	4.2	4.0 ± 0.2	4.2	4.2 ± 1.1	3.9 (0.3)	3.5 ± 0.4	4.1 ± 0.2
22:5 (n-6)	2.9	3.0 ± 0.6	3.0	3.4 ± 1.2	3.5 (0.8)	3.0 ± 0.6	3.2 ± 0.3
22:6 (n-3)	10.6	9.5 ± 1.1	9.5	9.7 ± 0.7	9.1 (0.8)	9.8 ± 1.3	9.5 ± 0.3
Saturated	47.1	51.1	51.7	49.9	50.7	45.7	50.9 ± 0.8
Monoenes	23.9	22.5	21.6	21.5	22.3	25.4	22.0 ± 0.5
Polyenes	28.4	25.9	25.9	28.0	26.3	28.2	26.5 ± 1.0
(n-6) Family	17.8	16.4	16.4	18.3	17.2	18.4	17.1 ± 0.9
(n-3) Family	10.6	9.5	9.5	9.7	9.1	9.8	9.5 ± 0.3

[a]Sm = smooth membrane, My = myelin, Sy = nerve endings with synapses, Mito = mitochondria, and Nf = neurofilaments.

[b]Number of experiments in parentheses.

[c]Average of fractions 1.16, 1.165, 1.17, and 1.18.

[d]Mass percentage of reported fatty acids. Where two separate fractions were analyzed the average of the two samples is given with the difference from the experimental values bracketed. Where three samples were analyzed the mean ± one standard deviation of the values is reported.

[e]SD = significantly different ($p<0.05$) from mitochondria enriched fraction.

[f]ND = not significantly different ($p>0.1$) from mitochondria enriched fraction.

TABLE IV

Total Glycerophospholipid Fatty Acid Composition of Adult Pig Cerebellar Cortex Membrane Fractions[a]

Density	1.146	1.159	1.164	1.173	1.178	1.208	
Membrane morphology	Sm, My	Sm	Sm	Sy, Sm	Sy	Mito, Nf	Average[b]
Fatty acid							
14:0	0.8[c]	0.6	0.6	0.5	0.7	0.4	0.6 ± 0.1
16:0	24.8	24.5	23.7	23.2	23.6	20.2	23.8 ± 0.5
16:1	1.4	1.1	1.3	1.0	1.5	1.2	1.2 ± 0.2
18:0	21.9	24.0	23.5	24.3	24.8	24.4	24.2 ± 0.5
18:1	26.2	22.4	20.0	19.8	19.9	24.6	20.5 ± 1.2
18:2 (n-6)	0.7	0.6	0.5	0.5	0.7	1.0	0.6 ± 0.1
20:1 + 18:3 (n-3)	0.9	0.8	0.7	0.6	0.6	0.8	0.7 ± 0.1
20:4 (n-6)	8.5	9.6	9.9	10.0	9.6	9.9	9.8 ± 0.2
22:4 (n-6)	3.0	3.0	3.4	3.3	3.0	2.9	3.2 ± 0.2
22:5 (n-6)	2.5	3.0	3.5	3.5	3.2	2.8	3.3 ± 0.3
22:5 (n-3)	0.6	0.5	0.6	0.5	0.5	0.5	0.5 ± 0.1
22:6 (n-3)	8.7	10.1	12.4	12.6	11.9	11.3	11.7 ± 1.1
Saturated	47.5	49.1	47.8	48.0	49.1	45.0	48.5 ± 0.7
Monoenes	27.6	23.5	21.3	20.8	21.4	25.8	21.8 ± 1.2
Polyenes	24.0	26.8	30.3	30.4	28.9	28.4	29.1 ± 1.7
(n-6) Family	14.7	16.2	17.3	17.3	16.5	16.6	16.8 ± 0.6
(n-3) Family	9.3	10.6	13.0	13.1	12.4	11.8	12.3 ± 1.2

[a]Sm = smooth membrane, My = myelin, Sy = nerve endings with synapses, Mito = free mitochondria, and Nf = neurofilaments.

[b]Average ± one standard deviation fatty acid composition of fraction 1.159, 1.164, 1.173, and 1.178.

[c]Mass percentage of reported fatty acids.

composition of highly purified membranes at specific periods of maturation. Unique developmental changes in lipid composition in one membrane type would be reflected by changes in the lipid composition of the particular subcellular fraction in which it is located. Changes occurring with maturation in specific subcellular fractions also may be related to variation in time of maturation of different cell types.

Although the phospholipid and cholesterol content of nonmitochondrial membranes isolated at a given density in CsCl-sucrose were similar in fetal and adult cerebellums, the relative proportion that each phospholipid contributed was changed. Decreases in molar percentages of phosphatidylcholine and increases in serine and ethanolamine phosphatides and sphingomyelin occurred in all subcellular fractions during maturation. These have been few other studies in which developmental changes in phospholipid class composition determinations were uncomplicated by concommitant myelin accumulation. In human gray matter, the molar percentage of phosphatidylcholine decreased, the ethanolamine phosphatide and sphingomyelin levels increased, and the phosphatidylinositol levels remained fairly constant (10,13), which is consistent with the present observations on membrane fractions. In the human gray matter, hower, phosphatidylserine remained constant, while in the pig cerebellum membrane fractions the phosphatidylserine content increased. The level of phosphatidylserine in synaptic plasma membrane fractions from adult rat brain (25) is similar to the levels found in the smooth membrane and nerve ending fractions from adult cerebellum in the present study.

The levels of 22:6 (n-3) increased in adult cerebellar cortex (Table IV) in agreement with the results of a more extensive study of human gray matter where developmental increases in the 22:6 (n-3) content of all glycerophospholipids were seen (10). The levels of 22:6 (n-3) increased in rat brain prior to myelination (6). During myelination the 22:6 (n-3) level also increased (6) although myelin has a lower 22:6 (n-3) content than other membranes. This indicates that membranes distinct from myelin are accumulating 22:6 (n-3) even during myelination. Developmental decreases in 22:4 (n-6) (Tables II and IV) agree with decreases in its percentage in serine and ethanolamine phosphatides of gray matter (10) and decreases in whole rat brain prior to and even during myelination (6). In total myelin fatty acids and in myelin ethanolamine phosphatides, the levels of 22:4 (n-6) are higher than in other subcellular fractions of 21 day old rat brain (R. Geison, unpublished data). The major polyunsaturated fatty acid in ethanolamine phosphatides of adult human white matter (10) or whole brain (26) is 22:4 (n-6). Decreases in 22:4 (n-6) are, therefore, significant in nonmyelin membranes, particularly because of the increase in 22:4 (n-6) in whole brain due to myelin accumulation. Developmental increases in 18:0 (Tables III and IV) concurs with its predominant localization in serine and ethanolamine phosphatides (10), which increase in relative amount (Table II). Developmental decreases in 16:0 and 14:0 (Tables III and IV) concur with their localization in phosphatidylcholine (10) which decreases in percentage (Table II). The 16:0 content of human gray matter phosphatidylcholine decreases (10), and the 16:0 and 14:0 content of whole rat brain nonmyelin phosphatidylcholine decreased (14). The 16:0 and 14:0 content of whole rat brain total fatty acids decreased prior to myelination (6). Decreases in 16:1 (Tables III and IV) concur with its primary localization and reduction in phosphatidylcholine of human gray matter (10) and rat brain nonmyelin membranes (14). In rat brain, 16:1 levels also decreased (6).

The developmental increase in 22:5 (n-6) is of interest (Tables III and IV). This acid normally decreases in relative amount in human gray matter serine and ethanolamine phosphatides (10) and in brain from rats on a grain diet (6). If rat dams, however, are switched from a grain diet to a corn oil diet at the time of birth, the pups show a relative increase in 22:5 (n-6) (6). Increases in 22:5 (n-6) proportionate to decreases in 22:6 (n-3) occur when dietary linolenic acid is low compared to linoleic acid (4,6), as is the case in a corn oil diet. The dietary history of the adult pigs from which the tissue specimens were obtained is not known, but corn is a major local feed grain; therefore, it is possible that a relative deficiency of linolenic acid which occurred after birth may have contributed to these increases. The polyunsaturated fatty acid levels were, however, constant in three separate preparations from whole brain cortex, suggesting that genetic factors also may contribute significantly. The low 22:5 (n-6) levels in the fetus suggest that sufficient placental uptake of linolenic or its product fatty acids may have occurred. In considering the polyunsaturated content and requirement of membrane phospholipids, the total polyunsaturated fatty acid content is probably more important than the amount of a single acid, such as 22:6 (n-3).

Two changes in subcellular fatty acid composition are of interest. (A) The increase in 18:1 in the mitochondrial fractions (Tables IV

and V) may, in part, be a result of its derivation from diphosphatidyl glycerol of which 50% of the fatty acid composition in ox and mouse brain is oleic acid (27). Higher linoleic acid levels in mitochondrial fractions also may be, in part, a result of derivation from diphosphatidyl glycerol. Analysis of individual phospholipid classes in mitochondrial and synaptosomal fractions indicated that the ethanolamine phosphatide fraction (which would have been mixed with any diphosphatidyl glycerol which would have been present) was enriched in oleic and linoleic acids in the mitochondrial fraction (16). Oleic acid levels are not elevated in ethanolamine phosphatides of rat (R. Geison, unpublished) or mouse (27) brain mitochondrial fractions. (B) The decreases in palmitate in mitochondrial fractions (Tables IV and V) probably is related to specific reductions in palmitate content of mitochondrial phosphatidylcholine (15,16) compared with synaptosomes.

The reason for the increase in 18:1 in the lightest density fraction of both adult whole brain cortex and cerebellar cortex is not known (Tables IV and V). This fraction contains some myelin fragments which would be enriched in 18:1; but other myelin related lipids (22:4 [n-6], 20:1) are not elevated nor are polyunsaturated fatty acids markedly decreased.

Previous studies indicated significant increases in higher mol wt phosphatidylcholine molecular species fractions containing polyunsaturated fatty acids (15) and in total polyunsaturated fatty acids (14,16) in brain mitochondrial fractions compared to synaptosomal or microsomal fractions. Ethanolamine phosphatides, however, which have a high polyunsaturated fatty acid content, are not elevated markedly in polyunsaturated fatty acid in mitochondrial fractions (16,27, R. Geison, unpublished) compared to synaptosomal or microsomal fractions. The lack of a significant elevation in total glycerophospholipid polyunsaturated fatty acid in mitochondrial fractions in the present study is, therefore, a result of a lack of significant variation in the polyunsaturated fatty acid content of the phospholipid class which contributes the large majority of the total glycerophospholipid fatty acids that are polyunsaturated.

The greater elevation of polyunsaturated fatty acids in brain mitochondrial phosphatidylcholine but not ethanolamine phosphatides may be the result of (A) the presence of two or more distinct membrane types which vary in phosphatidylcholine fatty acid composition or (B) membrane selectivity for unsaturated species of phosphatidylcholine but not ethanolamine phosphatides. The reduced palmitate content of mitochondrial fractions also may be a result of one of these factors. In the present study, nonmitochondrial membrane structures were observed in the mitochondrial fractions. Liver mitochondrial phosphatidylcholine has a similar molecular class distribution compared with liver microsomes (28) in contrast to variations in brain.

Phosphatidylcholine exchange between rat liver microsomes and mitochondria in the presence of a pH 5.1 supernate fraction was found to be nonselective for molecular species, while in the absence of the pH 5.1 supernate fraction the arachidonate containing species was exhanged less rapidly (29). Recently a slight tendency toward a greater extent of exchange with more unsaturated species was reported for both phosphatidylcholine and phosphatidylethanolamine between isolated rat liver microsomes and mitochondria in the presence of supernatant (30).

The reasons for the subcellular variations in lipid fatty acid composition in brain will require a definition of the extent of the contribution of these several factors.

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Behavior of Biliary Phospholipids in Intestinal Lumen during Fat Digestion in Rat

G. NALBONE, D. LAIRON, H. LAFONT, N. DOMINGO, and J. HAUTON, Groupe de Recherches sur le Transport des Lipides U 130. INSERM., 13009 Marseille, France, and L. SARDA, Institut de Chimie Biologique. Faculté St-Charles, 13003 Marseille, France

ABSTRACT

The phospholipids present in the intestinal lumen of rats following ingestion of triglycerides are of biliary origin. They consist of lecithins accompanied by a small proportion of lysolecithins. Their behavior in comparison with the other lipid constituents of the intestinal content was studied by subjecting the latter to gel filtration on an agarose column in the presence of a solution of 6 mM sodium taurocholate in 0.1 M NaCl. Part of the phospholipids is present with the triglycerides and diglycerides in the emulsified phase excluded from the gel where pancreatic lipase and colipase also are found. The remainder is found in optically clear fractions containing fatty acids, monoglycerides, and bile salts. These fractions are eluted at 2.0 column volumes, while mixed fatty acids, monoglycerides, bile salts micelles emerge from the column at 2.4 column volumes in the same chromatographic conditions. This difference in behavior may be explained by the presence of biliary lecithins. This presence could have an important bearing upon the mucosal uptake of the lipolysis products of triglycerides.

INTRODUCTION

Boucrot (1) recently has shown that, unlike dietary phospholipids, bile lecithins are very stable with respect to enzymatic hydrolysis in the intestinal lumen. Their particular resistance to hydrolysis by the pancreatic juice may be explained by the existence of a lipoprotein complex formed with a polypeptide fraction of the bile (2,3). With cholesterol and part of the bile salts, this complex constitutes an association of a different type from mixed micelles prepared in vitro, the lecithins of which can be hydrolyzed by phospholipase (4,5).

Boucrot (1) demonstrated that a high proportion of the bile lecithins are absorbed intact by the cells of the intestinal mucosa and found in the portal blood. He has postulated the existence of an enterohepatic circulation of the bile lecithins.

In addition, it has been shown in vitro (6,7) that the bile phospholipids play a part in the solubilization of other lipids, such as cholesterol. It is, therefore, of interest to study their possible role in the phenonema connected with the intestinal absorption of triglycerides. For this reason, we have investigated the behavior of bile phospholipids in the lumen of the rat small intestine during the digestion of fats. The results of preliminary experiments have been published recently (8).

METHODS AND MATERIALS

Male Wistar rats (200-250 g) fed on a balanced diet are starved before the start of the experiments, each of which is carried out on a group of 4 rats. At the beginning of the experiment, each rat is given 2 ml emulsion of triolein in water by stomach intubation. The emulsion is prepared directly by sonication. Three types of experiments are performed. Rats are ingested either with 800 mg emulsified triolein and sacrificed after 60 and 75 min, or with 300 mg emulsified triolein and killed after 90 min. These conditions were selected to provide intestinal contents representative of different stages of lipolysis of ingested triglycerides. The small intestine is ligatured both at the pylorus and at the junction of the jejunum and ileum and the intestinal contents are collected on ice. No rinsing is performed. The average total volume collected is ca. 10 ml for 4 rats. The fraction is filtered through glass wool twice to remove intestinal debris. In some cases, it is subjected to ultracentrifugation at 130,000 g at 4 C (MSE Superspeed 65 centrifuge, rotor SW 40) for between 15 min and 20 hr. The infranatant is collected.

Collection of rat bile and pancreatic juice is performed by cannulation. A first catheter (type PE 10) is placed in the proximal portion of the common bile duct, and a second is placed at the distal end of the pancreatic bile duct. Under these conditions uncontamined bile and pancreatic juice are obtained. The bile and pancreatic juice are collected on ice. The bile is used within 24 hr of collection. The pancreatic juice may be stored for several days at -20 C without loss of lipase activity.

Reconstitution in vitro of the intraluminar phase is achieved by mixing rat bile and

pancreatic juice with triolein. Rat hepatic bile (1.5 ml) containing 5 mg lecithins and 11.3 mg bile salts/ml is added to 0.150 ml rat pancreatic juice containing a total number of 225 lipase units with gentle stirring for 30 min at 4 C. Triolein (200 mg) then is added, and the mixture is emulsified by mechanical stirring for 15 min at the same temperature. In some experiments, the bile is replaced by a solution of purified bile salts (Calbiochem, A grade) similar in composition to rat bile (sodium taurocholate/sodium taurodeoxycholate:90/10) and in the same concentration (11.3 mg/ml). In each case, 1 ml mixture is subjected to gel filtration.

The gel filtration is performed on agarose (Bio-Gel A-5m, 100-200 mesh) columns (85 cm x 1.5 cm). The gel is equilibrated with a solution of 6 mM sodium taurocholate in 0.1 M NaCl adjusted to pH 6.8, and the columns are eluted with the same solution. Gel filtration is carried out at 4 C at a mean flow rate of 24 ml/hr. The volume of the fractions is 2.5 ml. The procedures used to determine the breakthrough volume (V_0) and to calibrate the columns are those previously described (8).

Extraction of the phospholipids is performed by the addition of 20 volumes chloroform-methanol 2/1 (v/v) to one volume of bile, intestinal content, or fractions collected from the columns, respectively. After evaporation to dryness under nitrogen, the residue is taken up in chloroform-methanol 9/1 (v/v), and an aliquot of this solution is subjected to thin layer chromatography (TLC) on silica gel (Ready plastic sheets F1500, Schleicher and Schuell, Dassel, W. Germany. Separation of the phospholipids is effected by means of the following development phase: chloroform-methanol-20% ammonia:70/30/5 (v/v/v). The lipids are located by spraying with molybdenum blue reagent (9). The silica gel corresponding to the spots is scraped off, and the phosphorus is measured after mineralization (10). Identification of diglycerides and triglycerides is made by TLC on an other aliquot with a development phase made up as follows: pentane-ethyl ether-99% acetic acid:90/10/1 (v/v/v). Location of the glycerides is effected at 110 C after spraying with ferric chloride reagent (11). Cholesterol, fatty acids, and monoglycerides are separated from the bile salts and phospholipids on silicic acid microcolumns, using a simplified technique adapted from the method of Nakayama (12), and they are analyzed by gas liquid chromatography (GLC). Total bile salts are measured by the enzymatic technique of Domingo, et al. (13).

The lecithin containing fractions eluted from the columns are extracted with 10 volumes of ether. After evaporation of the solvent, the residue is taken up in ether-methanol (10/1), and the protein precipitate is collected by centrifugation. Amino acid analysis of the protein is determined by the use of an automatic amino acid analyzer (Jeol JLC 5AH) after hydrolysis in 6 N HCl for 4 hr at 110 C under vacuum.

Lipase activity is measured potentiometrically at pH 9.0 and at 25 C (14), using an emulsion of triolein stabilized with gum arabic in the presence of an optimum amount of bile salts and of an adequate amount of cofactor (colipase) to obtain maximum activity (15). The total lipase activity/fraction is expressed as enzyme units. One lipase unit corresponds to the liberation of one microequivalent of fatty acid/min.

TABLE I

Amount of Phospholipids in Rat Intestinal Contents after Ingestion of Triolein[a]

N°	Lecithins[b]	Lysolecithins[b]	Lecithins[c]	Lysolecithins[c]
1	0.9	0.6	61	39
2	1.6	0.9	64	36
3	2.1	0.7	75	25
4	1.2	0.2	86	14
5	2.5	0.2	94.3	5.7
6	1.9	0.3	87.5	12.5
7	1.4	0.3	84	16
8	1.3	0.2	86.8	13.2
9	1.4	0.1	97.2	2.8
10	1.3	0.2	86.2	13.8
Mean	1.55	0.37	83	17

[a]Rats having ingested 800 mg triolein are sacrificed after 1 hr.

[b]μmoles/ml intestinal content.

[c]Molar percentage.

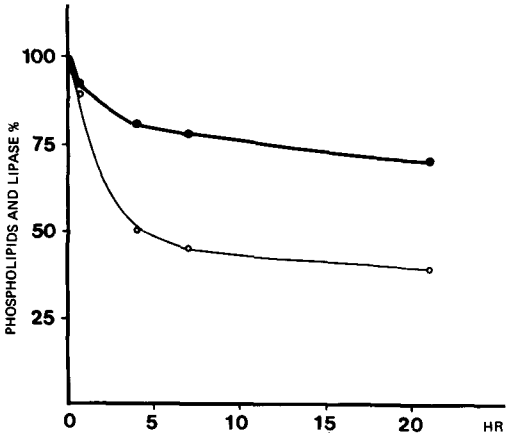

FIG. 1. Study of the phospholipids and lipase content of the infranatant during ultracentrifugation (130,000 x g) of the total intestinal contents of rats. Rats having ingested 800 mg triolein are sacrificed after 1 hr. Results are expressed as percentage of the amount present in the sample subjected to ultracentrifugation. ●—● = phospholipids and o—o = lipase.

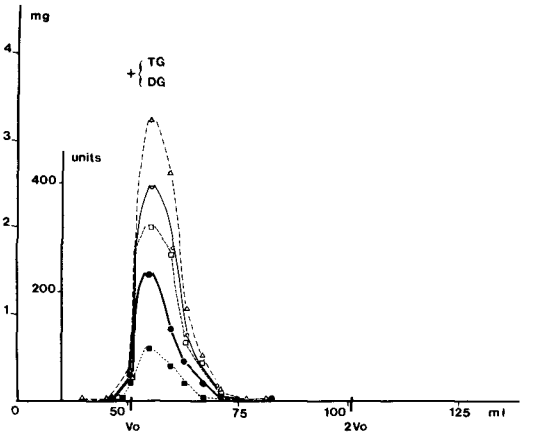

FIG. 2. Gel filtration on an agarose column of the intestinal contents collected 60 min after ingestion of 800 mg of triolein. The agarose column is equilibrated with a solution of 6 mM sodium taurocholate and 0.1 M NaCl and eluted with the same solution. Amounts of phospholipids (PL), free fatty acids (FFA), monoglycerides (MG), and bile salts (BS) are expressed in mg/fraction. Lipase activity is expressed in enzyme units/fraction. Diglycerides and triglycerides are identified by thin layer chromatography, but no quantitative determination is made. V_o represents the void volume of the column. ●—● = PL, △—△ = BS, □—□ FFA, ■—■ = MG, o—o = lipase.

RESULTS AND DISCUSSION

As shown in Table I, analysis of the phospholipids present in the intestinal content collected 1 hr after ingestion of 800 mg triglycerides reveals the presence of lecithins (83%) and of a smaller proportion of lysolecithins. It is not possible to demonstrate the presence of significant amounts of phosphatidyl serines, phosphatidyl ethanolamines, or sphingophospholipids. In other experiments, rats with a biliary fistula are given 1 ml 10 mM solution of sodium taurocholate by duodenal perfusion after ingestion of triglycerides. After 1 hr digestion, the intestinal contents are collected and analyzed. No significant amount of phospholipids are identified. This indicates that the lecithins and lysolecithins found in the previous experiments are of biliary origin. Yet, these lecithins appear to be particularly resistant to enzymatic hydrolysis in the intestinal lumen.

It generally is accepted that the lipids which penetrate into the mucosal cells are present in the micellar phase obtained by ultracentrifugation of the crude intestinal content (16). In previous experiments, we had shown that the biliary phospholipids in the micellar phase were associated with the products of lipolysis of triglycerides (8). Yet, under the conditions used for the preparation of the micellar phase, parts of the constituents are separated off in a pellet. Curves on Figure 1 indicate that, after 18 hr, centrifugation at 130,000 g, only 72% of the phospholipids and 40% of the lipase units are found in the micellar phase. Concentration gradients of the various lipids present in the micellar phase have been reported (17). The same phenomenon was found during centrifugation of synthetic mixed bile salts-fatty acids-monoglycerides micelles (18). Moreover, it is probable that part of the constituents of the emulsified phase return to the aqueous phase as a result of the changes in the properties and area of the interface during prolonged ultracentrifugation. In particular, this would explain the presence of pancreatic lipase in the micellar phase prepared by centrifugation (8).

Porter, et al., (17) proposed to prepare the micellar phase by passing the intestinal content through Millipore cellulose filters. However, it already has been observed that part of the biliary phospholipids and cholesterol are retained on the filters (19).

Feldman and Borgstrom (20) have shown that gel filtration on Sephadex in the presence of a detergent solution can be used for this type of separation. In these studies, we elected to separate the lipid constituents of the total intraluminal contents by placing the intestinal contents collected from the animal directly on a column of agarose gel equilibrated with a solution of 0.1 M NaCl containing 6 mM sodium taurocholate. When the crude intestinal contents of rats which have ingested triglycerides are placed on the agarose column, the

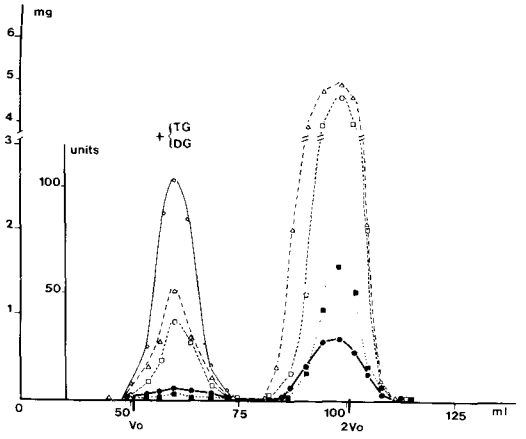

FIG. 3. Gel filtration on an agarose column of the intestinal contents collected 90 min after ingestion of 300 mg triolein. ●—● PL, △—△ BS, □—□ FFA, ■—■ = MG, and ○—○ = lipase.

constituents of the emulsified phase are excluded from the gel, while the lipid constituents eventually present in the aqueous phase are retarded.

Analysis of intraluminar contents of rats is carried out at three different stages of digestion of triglycerides. Figure 2 presents the elution diagram from an agarose column loaded with intestinal contents collected at the beginning of lipolysis. All the lecithins, cholesterol, and bile salts are eluted within the first column volume (1 V_o) in highly opalescent fractions containing the triglycerides, diglycerides and small amounts of free fatty acids, and monoglycerides. Chromatography of rat bile performed on the same column shows that lecithins, cholesterol, and bile salts are eluted with the second column volume. The opalescent fractions contain all of the lipase and of its cofactor, whereas in the same conditions the lipase of rat pancreatic juice is eluted at 2.35 V_o. Therefore, the enzyme present in the intestinal contents has a high apparent mol wt (rapid lipase) due to specific protein-lipid interaction at the interface (21-24). It is noteworthy that colipase the function of which is to give lipase its maximum activity in the presence of physiological concentration of bile salts accompanies lipase in the emulsified phase.

Analysis of the intraluminal contents at an intermediate stage of lipolysis shows that 75% of the biliary phospholipids and cholesterol are eluted with the first column volume at the same time as triglycerides, diglycerides, lipase, and colipase. These fractions contain 33% of the free fatty acids, 14% of the monoglycerides, and 26% of the bile salts eluted from the

TABLE II

Amino Acid Analysis of Polypeptide Fraction Associated with Lecithins in Rat Bile and in Rat Intestinal Contents[a]

Residues	Rat bile[b]	Rat intestinal content[b]
Alanine	12.1	11.9
Arginine	6.7	6.3
Aspartic acid	9.0	10.2
Glycine	8.4	5.5
Glutamic acid	8.7	8.3
Histidine	1.6	2.9
Isoleucine	4.6	5.3
Leucine	7.1	7.2
Lysine	6.2	7.1
Phenylalanine	3.2	4.7
Proline	4.8	4.4
Serine	10.0	10.2
Threonine	7.6	6.5
Tyrosine	2.7	2.4
Valine	7.3	7.1

[a]No correction is made for amino acid destruction. Tryptophane, cysteine, and methionine are not determined.

[b]Molar percentage.

column. They are very trubid. The remainder of the biliary phospholipids and cholesterol is eluted with the second column volume in optically clear fractions containing the major part of the free fatty acids, monoglycerides, and bile salts. Their elution volume corresponds to that of proteins having a mol wt of ca. 100,000 daltons. Figure 3 presents the diagram of the filtration of the intraluminar content at the end of lipolysis. As shown on the diagram, the first column volume contains small amounts of triglycerides and diglycerides and a small percentage of the lecithins (10%), monoglycerides, (2%), free fatty acids (14%), and bile salts (6%) eluted from the column. These fractions contain all the lipase activity and colipase. The major part of the bile lecithins, cholesterol, bile salts, and of the lipolysis products is eluted in clear fractions with the second column volume. Their chromatographic behavior is similar to that of the micellar phase of the intestinal contents prepared by centrifugation (8). Their composition (wt percentage) is: 31% fatty acids, 13% monoglycerides, 48% bile salts, 7% phospholipids, and 1% cholesterol. They contain a polypeptide which after characterization by amino acid analysis appears to be very similar in composition to that of the polypeptide fraction associated with the lecithins in rat bile (Table II). In both cases, the polypeptide represents ca. 2% of the lecithins. This polypeptide could not be identified in the nonlecithin containing fraction.

These results are confirmed by studies of the

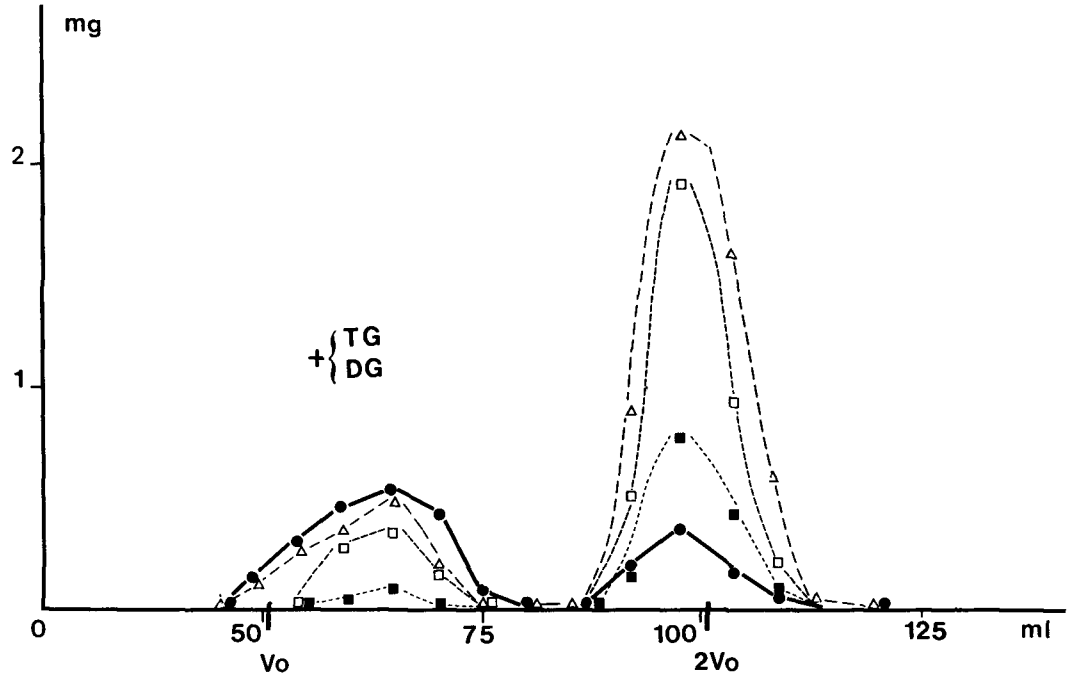

FIG. 4. Gel filtration on an agarose column of a sample obtained from an in vitro mixture of triolein, rat bile, and pancreatic juice. ●—● PL, △—△ BS, □—□ FFA, and ■—■ MG.

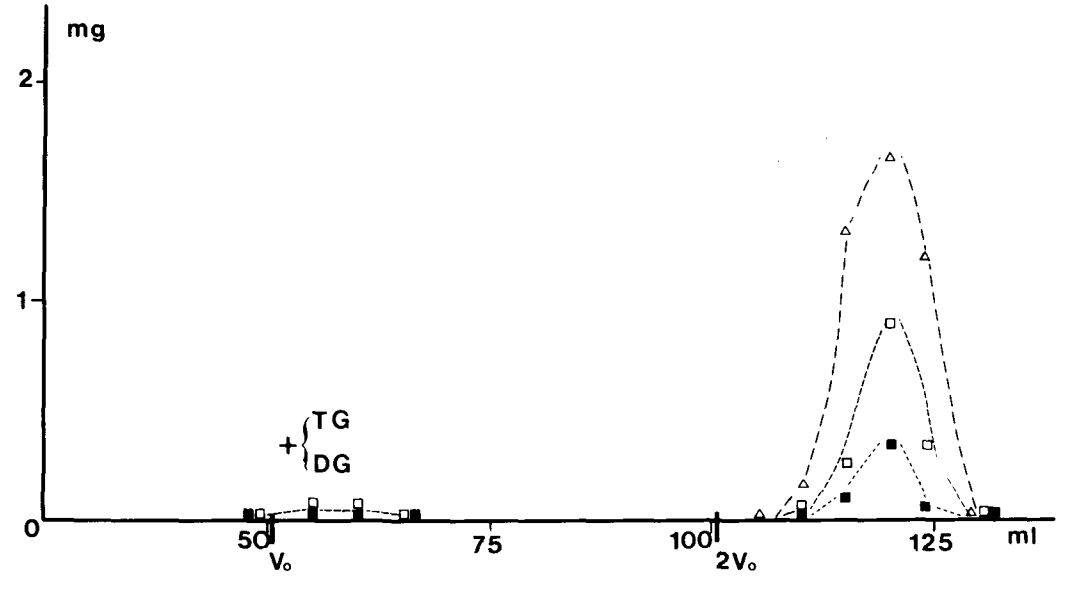

FIG. 5. Gel filtration on an agarose column of a sample obtained from an in vitro mixture of triolein, purified bile salts, and rat pancreatic juice. △—△ BS, □—□ FFA, ■—■ = MG.

chromatographic behavior of in vitro reconstituted intraluminal contents.

Figure 4 shows the diagram of the gel filtration of a mixture of rat bile and pancreatic juice with triolein. The lipids are divided into two peaks emerging in the first and the second column volumes, respectively. The first peak contains all the triglycerides and diglycerides, as well as 73% of the lecithins, 17% of the free fatty acids, 12% of the monglycerides, and 16%

of the bile salts. The greater part of the fatty acids, monoglycerides, and bile salts is eluted in the second lipid peak along with the remainder of the lecithins.

In further experiments, rat bile was replaced by a solution containing the same amount of purified bile salts. The corresponding elution diagram is presented in Figure 5. Triglycerides and diglycerides emerging with the first column volume are accompanied by a low percentage of free fatty acids and monoglycerides (1-2%). These later constituents are almost totally found in optically clear fractions eluted at 2.4 V_o, along with the bile salts loaded on the column. Their elution volume corresponds to that of a protein of a mol wt of ca. 25,000 daltons. This behavior is identical to that observed with synthetic mixed micelles fatty acids-monoglycerides-bile salts (8).

These experiments demonstrate that biliary lecithins are associated to fatty acids, monoglycerides, and bile salts in the aqueous phase. These complexes have a much higher mol wt than those formed in the absence of lecithins. Although the presence of bile lecithins does not appear to be a determinant factor with the respect to the solubilization of fatty acids and monoglycerides in bile salts, they might participate to the process of intestinal fat absorption. O'Doherty et al. (25) and Rampone (26) already have shown that the presence of luminal lecithins alter the kinetics of mucosal uptake of fatty acids and cholesterol mixed micelles. From this point of view, Higgins's (27) finding that phospholipids are involved in the uptake of chylomicrons by liver cells is noteworthy.

ACKNOWLEDGMENTS

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Studies on Phospholipids with Particular Reference to Cardiolipin of Rat Heart after Feeding Rapeseed Oil

ROLF BLOMSTRAND and **LENNART SVENSSON**, Department of Clinical Chemistry, Huddinge University Hospital Karolinska Institutet, 141 86 Huddinge, Sweden

ABSTRACT

The influence of dietary rapeseed oil on the lipid classes and fatty acid pattern of rat heart homogenate and mitochondria has been investigated after feeding a diet with 9.8% by wt erucic acid for 10 days and 1.4% and 2.6% erucic acid for 28 days. The rats treated with 9.8% erucic acid showed a significant increase in the triglycerides of the heart mitochondria. This tendency was much less pronounced in rats treated with 1.4 and 2.6% erucic acid, respectively. In all experiments, the triglycerides of the heart mitochondria showed a high content of erucic acid. The fatty acids of phosphatidylcholine, phosphatidylethanolamine, and cardiolipin were all influenced by the dietary rapeseed oil, but the erucic acid seemed to have a specific affinity to cardiolipin. Cardiolipin of rat heart mitochondria was isolated and identified with gas chromatography and mass spectrometry. The isolated cardiolipin was found to contain 12% erucic acid after feeding 9.8% erucic acid as rapeseed oil for 10 days. Similar results were obtained after feeding glyceryl trierucate for 5 days to rats. The incorporation of erucic acid into cardiolipin was followed by a corresponding decrease of linoleic acid. This observation is of great interest because the molecular structure of fatty acids in lipid molecules has a profound influence on the packing of these molecules in a bilayer. Since cardiolipin is a component of the inner membrane of mitochondria its high affinity for erucic acid might influence the normal function of the inner membrane of heart mitochondria.

INTRODUCTION

Roine, et al., (1) was the first to show that rats fed rapeseed oil for 2-3 months showed foci of histiocyte infiltration in the myocardium. Abdellatif and Vles (2) investigated the pathological effects of dietary rapeseed oil in rats. Fatty acid infiltration was found in heart, skeletal muscle, and adrenals after feeding 60 cal% of rapeseed oil for only 2 weeks. Houtsmuller, et al., (3) investigated the amount and composition of the lipid clsses in the heart of rats fed a diet containing 50 cal% rapeseed oil for periods varying from 1 day-6 weeks. A sharp increase in lipid content was observed after 3 days on the diet, which is mainly due to an increase in triglycerides. An increase in the content of free fatty acids also was observed. Of the total lipids, 27% of the fatty acids was erucic acid. Studies of heart mitochondria in vitro revealed that the rate of adenosine 5′-triphosphate (ATP) synthesis is lower after feeding a diet containing erucic acid than for a diet containing sunflower oil. The degree of inhibition was roughly proportional to the erucic acid content of the diet.

It has been suggested by several authors (4-6) that the phospholipids play a major role in the maintenance of normal function within the cell membrane. The reason for the occurrence of mixtures of different phospholipid classes and the variation in the fatty acid composition from one type of membrane to another is not yet understood.

Considerable study has been given to the role of unsaturated fatty acids in mitochondrial function (7). There is evidence that changes in the fatty acid composition are fundamental to metabolic and physical differences in mitochondria. Recently, a close correlation between the alteration of cardiolipin and mitochondria adenosine triphosphatase (ATPase) activity has been reported, indicating the existence of specific association between this enzyme and cardiolipin independently of other phospholipids (8).

Cardiolipin is a characteristic phospholipid of heart mitochondria, and cardiolipin normally contains a high concentration of linoleic acid. We have investigated the phospholipid composition and fatty acid pattern of rat heart mitochondria in a series of experiments where the erucic acid content in the diet and the feeding time were varied.

The results indicate that erucic acid is incorporated into several phospholipids of the rat heart but that erucic acid seems to have a specific affinity to be incorporated into the cardiolipin molecule of the rat heart mitochondria.

MATERIALS AND METHODS

Five groups of 10 male and 10 female 4

week old Sprague-Dawley rats in each group were fed a diet containing 40 cal% of fat. The feeding time and the erucic acid content were varied, 1 group was fed 9.8% erucic acid for 10 days and 2 groups 1.4% and 2.6% erucic acid for 28 days. The erucic acid was given as rapeseed oil. The remaining two groups were fed peanut oil instead of rapeseed oil. Further two groups of rats have been investigated. One group of 10 rats fed a basic diet consisted of pellets with an addition of 1.85 g glyceryl trierucate/day given by tube for 5 days. The other group was fed only basic diet. The purity of the glyceryl trierucate (AB Karlshams Oljefabriker, Karlshamn, Sweden) used in our experiments was investigated with gas liquid chromatography (GLC). The total fatty acids of trierucate were found to contain 92.5% erucic acid (C 22:1), 2.5% gadoleic acid (C 20:1), and 5% of other fatty acids.

Preparation of Homogenate and Isolation of Mitochondria

Rat heart homogenate was obtained by homogenization in ice-cold 0.25 M sucrose containing 1 mM neutralized ethylenediaminetetraacetic acid (EDTA). Rat heart mitochondria were isolated by ultracentrifugation in a Spinco L2-65B at 600 g for 5 min and the supernatant at 8000 g for 10 min. Mitochondria and homogenate were extracted according to Folch-Pi (9). Protein content was analyzed with micro Kjeldahl (10).

Separation of Total Lipids into Different Lipid Classes Using Silicic Acid Chromatography

Separation on silicic acid was carried out mainly as described previously (11). Ca. 10 mg total lipids dissolved in n-hexane were transferred to the column. Three fractions were eluted using the following elution mixtures: (A) pentane/benzene 85:15 (cholesterol esters are eluted); (B) chloroform (glycerides, cholesterol, and free fatty acids are eluted); and (C) methanol (phospholipids and other polar lipids are eluted).

Separation of Phospholipids by Thin Layer Chromatography (TLC)

The plates were prepared with an automatic TLC-coated (Camag, Muttenz, Switzerland). Air dried Silica Gel H (Merck, Darmstadt, Germany) plates (20 x 20 cm and 0.25 mm layer thickness) were activiated for 2 hr at 110 C before use. Ca. 7 mg extracted lipids dissolved in 100 μliter chloroform/methanol 2:1 were applied over a 5 cm line with a 100 μliter Hamilton syringe. The plates were first developed with chloroform/methanol/25% aqueous ammonia 14:6:1 to a ht of 12 cm from the application line. After air drying for 0.5 hr, the plates were developed with chloroform/methanol/acetic/water 80:13:8:0.3. The detection reagent was $(NH_4)_2SO_4$ 100 g and H_2SO_4 5 ml made up to a volume of 500 ml with water. The plates were charred for 90 min at 190 C. The relative distribution of mass among TLC-separated phospholipids was estimated by measuring the light absorption in the charred phospholipid bands in a Vitatron TLD 100 (Vitatron, Dieren, Holland).

Fatty Acid Analysis

The relative distribution of fatty acids in cholesterol esters, triglycerides and phosphatidylethanolamine, phosphatidylcholine, and cardiolipin from the TLC-separated phospholipids has been investigated. The lipids were hydrolyzed in acid methanol and the fatty acids were converted to their fatty acid methylesters with 1,2-dimethoxypropan (12). The fatty acid methylesters were separated and identified by GLC using a 4 m x 4 mm inside diameter glass column packed with 3.5% EGSS-X on acid washed and siliconized Chromosorb W (100-120 mesh). The different peak areas were calculated, and the results were expressed as area percentages. The identity of erucic acid incorporated in different lipids was confirmed by gas chromatography-mass spectrometry (GC-MS) using purchased erucic acid as reference.

Isolation and Identification of Cardiolipin

Cardiolipin was separated from the other phospholipids by TLC as described above. The following isolation and identification procedures are summarized in Figure 1. The cardiolipin band was scraped from the TLC plate and was eluted from the adsorbent by shaking vigorously with 2 x 10 ml chloroform/methanol 2:1 saturated with water followed by filtration. The filtrate was evaporated, and the lipid was dissolved in a small volume of chloroform/methanol 19:1 before transferring to a Sephadex G-25 column (13). The sample was eluted with 50 ml chloroform/methanol 19:1 saturated with water. The cardiolipin was deacylated, and the product was converted to the free acid form before silylation (14). The trimethylsilyl (TMS) deacylated cardiolipin was analyzed with a LKB 9000 GC-MS. The column used was a 0.5 m x 4 mm inside diameter glass column mainly packed with 1% SE-30 on acid-washed and siliconized Chromosorb W (80-100 mesh) and filled at the top with 10% SE-30 to a ht of 2 cm. Column temperature 180-240 C, 4 C/min. Spectra were obtained at 70 eV ionizing

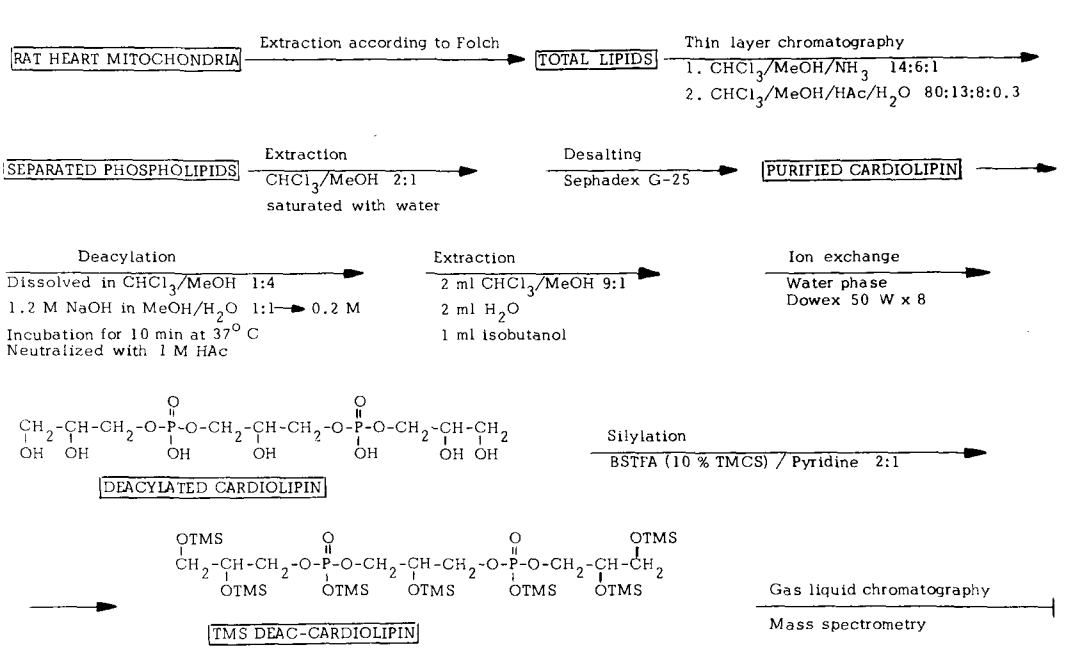

FIG. 1. Schematic outline of the isolation and identification procedures of cardiolipin.

potential, a trap current of 60 μA, accelerating voltage 3.5 kV, ion source 250 C, flash heater 250 C, scan speed 6, UV paper speed 100 mm/sec, filter 120 cps, electron multiplier sensitivity 125, and slits 0.2 and 0.3 mm. The unknown spectra were normalized and compared with a spectrum of reference cardiolipin treated in the same was as the unknown. When only small amounts of sample were available, cardiolipin was identified by mass fragmentography. Three fragments were focused on the multiple ion detector (MID) namely those having the highest mass numbers: m/e=889 (M-15), m/3=814 (M-90), and m/e=801 (M-103). They have been detected on channel 1,2, and 3, respectively with an amplification of 900 x 300 x and 90 x. Filter was 0.25 cps on each channel, electron multiplier sensitivity 110 and measuring time 20 msec. All other conditions were the same as when taking mass spectra, except electron energy which was only 20 eV when using the MID. The three ions were detected simultaneously and had equal retention time.

RESULTS

Identification of Cardiolipin

Cardiolipin of rat heart mitochondria from rats fed a diet containing 1.4 and 2.6% erucic acid for 28 days was identified by GC-MS. Only one peak was obtained at the gas chromatogram. A reasonably good agreement could be obtained when comparing the spectrum of TMS deac-cardiolipin from rat heart mitochondria with the spectrum of a standard (Fig. 2). There is, however, a slight difference between the two spectra in that they differ in base peak. The fact that cardiolipin has three asymetrical carbon atoms which give rise to different optical isomeres may be the cause of the different fragmentation. Another explanation is that there can be a small amount of contaminations in the purified sample despite the rigorous isolation and purification procedures described in Figure 1. In this case, the intense fragment at m/e=147 partly comes from the contamination.

Cardiolipin of rat heart mitochondria from rats fed a diet containing 9.8% erucic acid for 10 days was identified by mass fragmentography. Mass fragmentograms of isolated TMS deac-cardiolipin and standard are compared in Figure 3. Sample and standard have equal retention time and the ratios of the peak hts are the same.

Identification of Docosenoic Acid

The identity of docosenoic acid isolated from cardiolipin of rat heart homogenate was confirmed by GC-MS. The mass spectrum of docosenoic acid from cardiolipin and purchased erucic acid are compared in Figure 4.

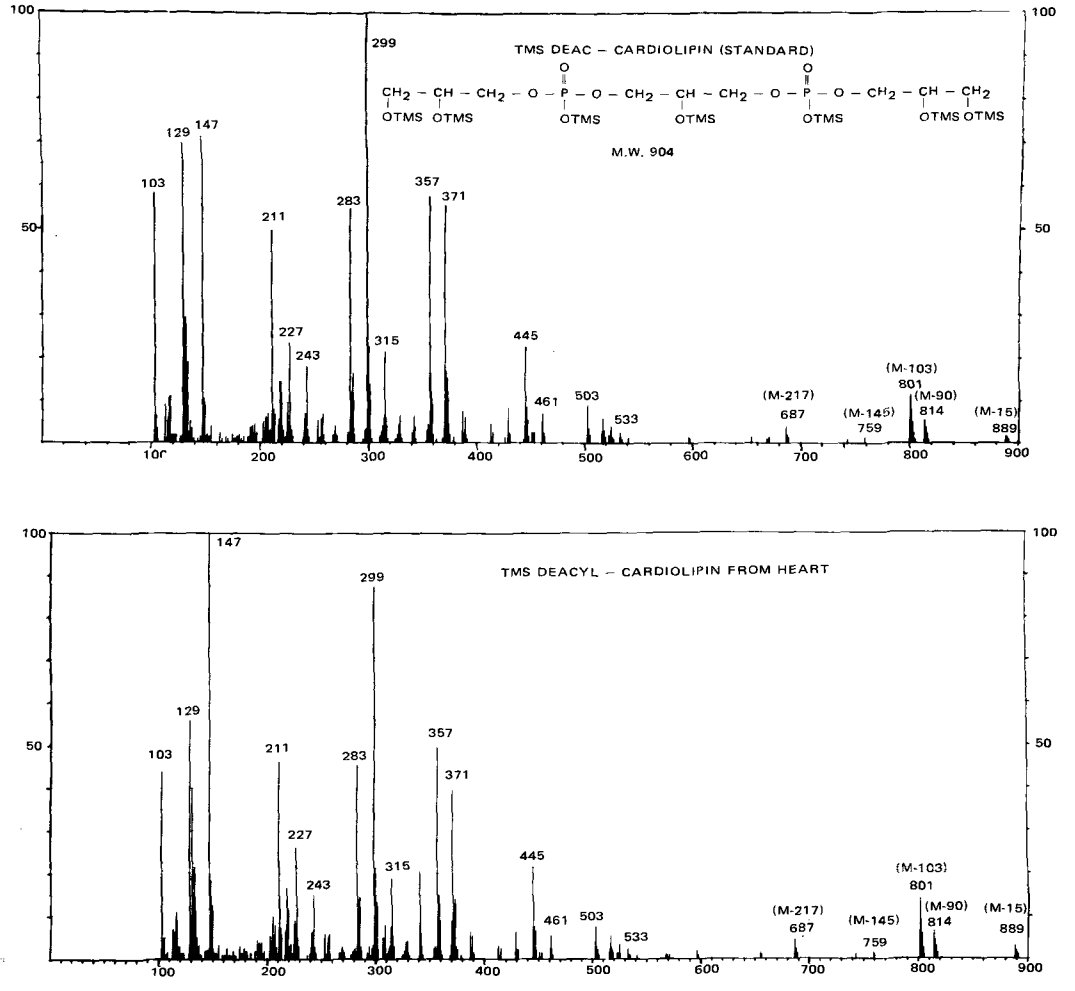

FIG. 2. Mass spectrum of trimethylsilyl (TMS) deac-cardiolipin reference (upper spectrum) and mass spectrum of TMS deac-cardiolipin of pooled rat heart mitochondria from rats fed a diet containing 2.6% erucic acid for 28 days (lower spectrum).

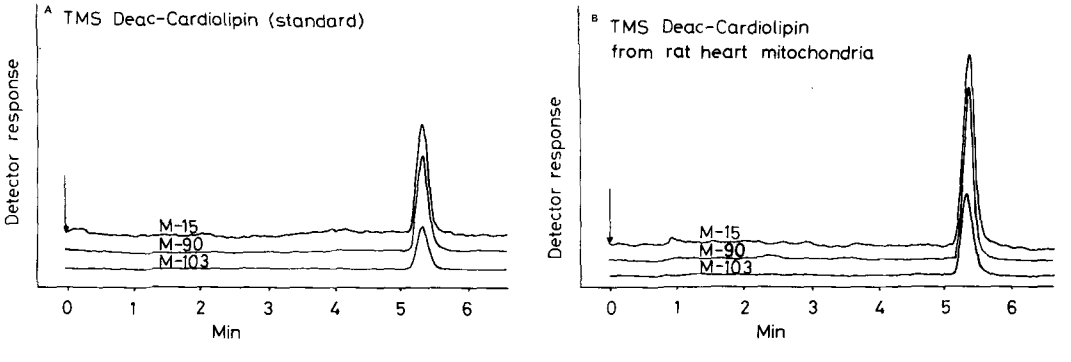

FIG. 3. (A) Mass fragmentogram of trimethylsilyl (TMS) deac-cardiolipin reference and (B) mass fragmentogram of TMS deac-cardiolipin of pooled rat heart mitochondria from rats fed a diet containing 9.8% erucic acid for 10 days.

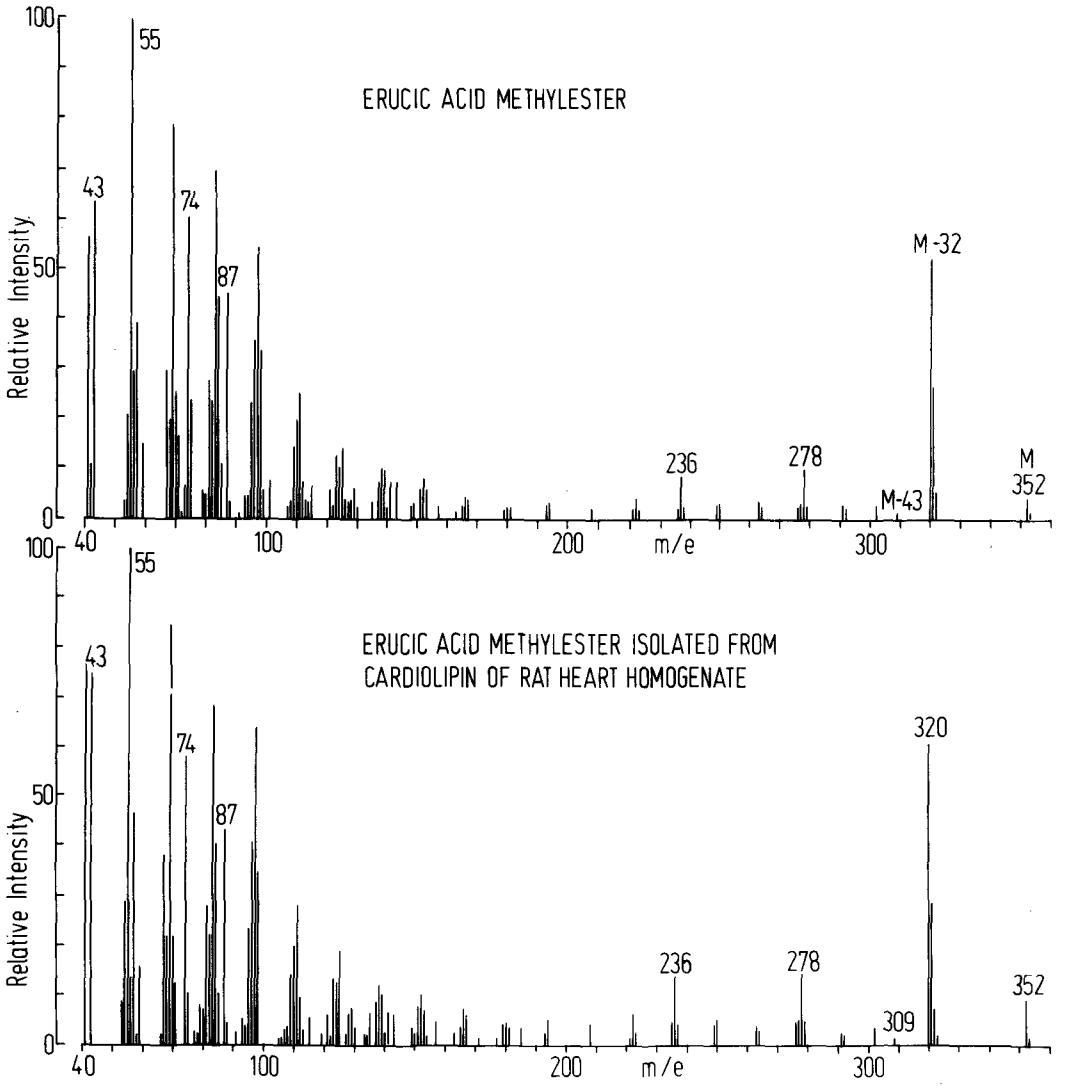

FIG. 4. Mass spectrum of erucic acid methylester reference (upper spectrum) and mass spectrum of erucic acid methyl ester isolated from cardiolipin of rat heart homogenate (lower spectrum).

Distribution of Different Lipid Classes in Homogenate and Mitochondria of Rat Heart

The distribution of cholesterol esters, triglycerides, and phospholipids in homogenate and mitochondria of rat heart are shown in Figure 5. Rats treated with 9.8% erucic acid in the diet for 10 days show a significant increase in triglycerides, while the phospholipids have a tendency to decrease. This accumulation of triglycerides in rat heart mitochondria after short term feeding of rapeseed oil confirms the results of other investigators (3,15). The tendency is much less pronounced in rats treated with 1.4 and 2.6% erucic acid for 28 days. The total phospholipids slightly increase in this experiment.

Distribution of Different Phospholipids in Homogenate and Mitochondria of Rat Heart

The influence of rapeseed oil and trierucate on the distribution of different phospholipids is shown in Figure 6. In the experiment with 9.8% erucic acid in the diet, there is a tendency to an increased concentration of phosphatidylcholine and a decreased concentration of phosphatidylethanolamine. The concentration of cardiolipin is mainly unchanged in all of the experiments. There is not any significant difference in the relative distribution of different phospholipids

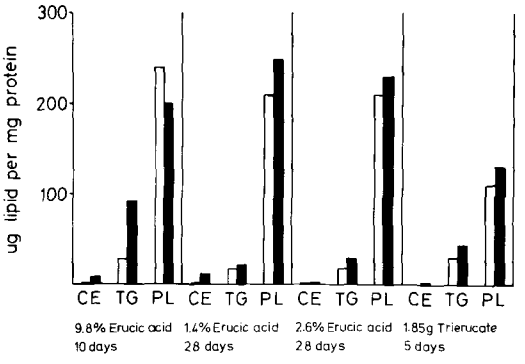

FIG. 5. The distribution of cholesterol esters (CE), triglycerides (TG), and phospholipids (PL) in pooled rat heart mitochondria from rats fed a diet of 40 cal% fat containing 9.8, 1.4, and 2.6% erucic acid given as rapeseed oil for 10, 28, and 28 days, respectively, and in pooled homogenate of rat heart from rats fed a diet consisting of pellets with an addition of 1.85 g trierucate/day for 5 days. ▭ = control ▬ = erucic acid.

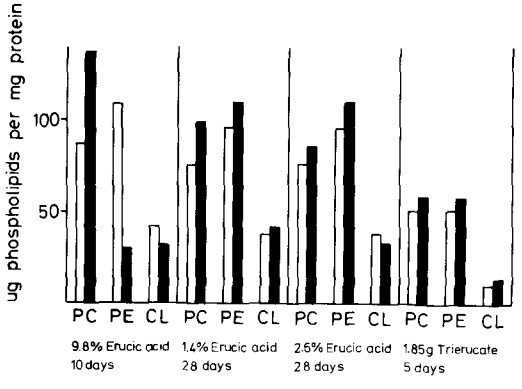

FIG. 6. The distribution of phosphatidylcholine (PC) phosphatidylethanolamine (PE), and cardiolipin (CL) in pooled rat heart mitochondria from rats fed a diet of 40 cal% fat containing 9.8, 1.4, and 2.6% erucic acid given as rapeseed oil for 10, 28, and 28 days, respectively, and in pooled homogenate of rat heart from rats fed a diet consisting of pellets with an addition of 1.85 g trierucate/day for 5 days. ▭ = control, ▬ = erucic acid.

between mitochondria and homogenate of rat heart.

Composition of Fatty Acids of Different Lipid Classes in Homogenate and Mitochondria of Rat Heart

The influence of rapeseed oil diet containing 9.8% erucic acid on the fatty acid pattern in cholesterol esters, triglycerides, and phospholipids from rat heart mitochondria is shown in Figure 7 and Table I. Erucic acid predominantly is incorporated in the heart triglycerides which contain 38% erucic acid. In the experiments with 1.4 and 2.6% erucic acid in the diet,

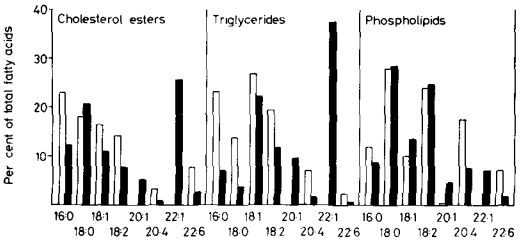

FIG. 7. Relative distribution of total fatty acids of different lipid classes in pooled rat heart mitochondria from rats fed a diet of 40 cal% containing 9.8% erucic acid given as rapeseed oil for 10 days. ▭ = control, ▬ = rapeseed oil.

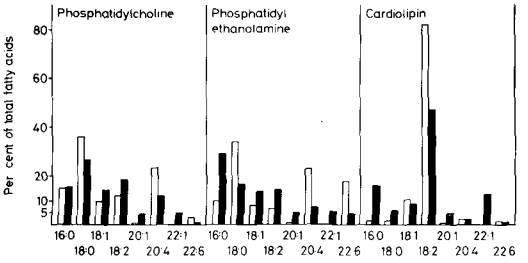

FIG. 8. Relative distribution of total fatty acids of separated phospholipids in pooled rat heart mitochondria from rats fed a diet of 40 cal% fat containing 9.8% erucic acid given as rapeseed oil for 10 days. ▭ = control, ▬ = rapeseed oil.

the erucic acid content in the triglycerides are 3.2 and 7.0%, respectively (Table II). There is also an incorporation of erucic acid in cholesterol esters and phospholipids, but to a less extent than in triglycerides. In the experiment with 9.8% erucic acid, the erucic acid content in the cholesterol esters is 26%, and there is a corresponding decrease in oleic and linoleic acid content.

Composition of Fatty Acids of Different Phospholipids in Homogenate and Mitochondria of Rat Heart

The influence of rapeseed oil diet containing 9.8% erucic acid on the fatty acid pattern in phosphatidylcholine, phosphatidylethanolamine, and cardiolipin from rat heart mitochondria is shown in Figure 8 and Table III. The fatty acids of phosphatidylcholine, phosphatidylethanolamine, and cardiolipin are all influenced by the diet. Erucic acid seems to have a specific affinity to be incorporated into cardiolipin from rat heart. The isolated cardiolipin from rat heart mitochondria is found to contain 12% erucic acid. Of great interest is that there is a decrease in the linoleic acid (C 18:2) content

TABLE I

Relative Composition of Total Fatty Acids of Different Lipid Classes in Pooled Rat Heart Mitochondria from Rats Fed Diet Containing 9.8% Erucic Acid for 10 Days

	Cholesterol esters		Triglycerides		Phospholipids	
Fatty acid	Control erucic acid 0.2%	Treated erucid acid 9.8%	Control erucic acid 0.2%	Treated erucic acid 9.8%	Control erucic acid 0.2%	Treated erucic acid 9.8%
14:0	1.6	2.4	2.2	0.4	0.1	0.3
16:0	23.0	12.1	23.3	7.0	11.9	8.7
16:1	10.3	4.6	3.1	1.4	0.2	1.0
17:0	---	2.9	1.1	0.3	0.3	0.5
18:0	18.0	20.8	13.8	3.6	28.1	28.5
18:1	16.5	11.0	26.8	22.2	10.0	13.5
18:2	14.2	7.7	19.5	11.7	23.9	24.8
18:3	---	---	---	2.8	---	0.6
20:0	1.8	1.3	---	0.5	0.3	0.4
20:1	---	5.2	---	9.5	0.3	4.6
20:2	3.6	2.9	1.1	0.6	0.3	1.1
20:4	3.3	0.7	6.9	1.6	17.5	7.4
22:1	---	25.8	---	37.8	---	6.9
22:6	7.7	2.6	2.2	0.6	7.1	1.7

TABLE II

Relative Composition of Total Fatty Acids of Different Lipid Classes in Pooled Rat Heart Mitochondria from Rats Fed Diet Containing 1.4 and 2.6% Erucic Acid for 28 Days

	Cholesterol esters			Triglycerides			Phospholipids		
	Control erucic acid	Treated erucic acid		Control erucic acid	Treated erucic acid		Control erucic acid	Treated erucic acid	
Fatty acid	0.25%	1.4%	2.6%	0.25%	1.4%	2.6%	0.25%	1.4%	2.6%
14:0	2.2	5.1	6.6	1.8	1.6	0.8	0.2	0.2	0.1
16:0	26.5	23.8	23.7	18.6	18.6	15.0	8.4	7.8	7.8
16:1	3.2	8.5	8.5	3.0	4.1	3.2	0.7	0.5	0.5
17:0	13.7	1.7	4.2	0.6	1.3	1.4	0.4	0.4	0.4
18:0	14.0	18.5	12.8	7.3	7.2	7.8	25.3	24.2	25.8
18:1	9.2	12.6	11.0	30.7	27.4	25.7	8.6	8.2	9.1
18:2	11.2	18.4	15.3	27.1	25.2	24.2	28.3	28.3	27.9
18:3	2.9	2.6	2.1	0.3	0.6	1.0	---	---	0.1
20:0	---	---	---	0.7	0.5	0.6	0.3	0.2	0.2
20:1	---	---	0.2	1.1	1.3	2.1	0.4	0.6	0.8
20:2	3.3	---	2.4	0.5	0.7	1.1	0.4	0.3	0.4
20:4	4.0	0.3	2.2	7.0	7.8	9.7	21.9	22.3	20.0
22:1	---	---	0.9	0.8	3.2	7.0	---	0.6	1.0
22:6	9.8	8.5	10.1	0.5	0.5	0.4	5.1	6.4	5.9

from 82 to 47%. The fatty acid pattern of phospholipids from rats treated with 1.85 g trierucate/day for 5 days is influenced in the same way but to a less extent (Table IV). The cardiolipin is found to contain 4.4% erucic acid, and the linoleic acid content decreases from 82 to 68%. In the long term feeding experiment (1.4 and 2.6% erucic acid given as rapeseed oil for 28 days), there can be seen a small incorporation of erucic acid into the different phospholipids and to a slightly higher degree into cardiolipin (Table V).

The incorporation of erucic acid into phosphatidylethanolamine and phosphatidylcholine causes a corresponding decrease in the arachidonic acid (C 20:4) content.

DISCUSSION

The results reported here confirm and extend earlier observations that erucic acid is predominantly incorporated in the heart triglycerides. The most interesting observation in these results is that erucic acid seems to have a specific affinity to be incorporated in to cardiolipin from rat heart mitochondria.

Since phospholipids are membrane constituents, knowledge about their distribution and metabolism may be important for a better understanding of the phenomena involved in lipidosis of heart and other diseases of the heart muscle. This information also might contribute to our knowledge of the importance of phos-

TABLE III

Relative Composition of Total Fatty Acids of Separated Phospholipids in Pooled Rat Heart Mitochondria from Rats Fed Diet Containing 9.8% Erucic Acid for 10 Days

	Phosphatidylcholine		Phosphatidylethanolamine		Cardiolipin	
Fatty acid	Control erucic acid 0.2%	Treated erucic acid 9.8%	Control erucic acid 0.2%	Treated erucic acid 9.8%	Control erucic acid 0.2%	Treated erucic acid 9.8%
14:0	0.1	0.1	0.1	1.6	0.1	0.1
16:0	14.9	15.5	9.7	28.9	1.2	15.9
16:1	0.1	0.8	0.1	2.2	0.6	0.3
18:0	36.0	26.6	33.9	16.5	1.5	5.7
18:1	9.5	14.3	8.1	13.3	10.2	8.2
18:2	12.1	18.6	6.4	14.3	81.9	47.1
18:3	---	0.6	---	0.8	0.1	1.3
20:0	0.2	0.4	0.3	---	---	---
20:1	0.3	4.5	0.3	4.8	0.4	4.7
20:2	0.1	1.1	0.1	1.0	0.9	1.2
20:4	23.7	11.7	22.9	6.9	1.8	2.0
22:1	---	4.9	---	5.3	---	12.4
22:6	3.0	0.8	18.0	4.4	1.3	1.0

TABLE IV

Relative Composition of Total Fatty Acids of Separated Phospholipids in Pooled Rat Heart Homogenate from Rats Fed Diet Containing 1.85 g Trierucate/Day Given by Tube for 5 Days

	Phosphatidylcholine		Phosphatidylethanolamine		Cardiolipin	
Fatty acid	Control	Treated erucic acid 1.85 g	Control	Treated erucic acid 1.85 g	Control	Treated erucic acid 1.85 g
14:0	0.2	0.2	0.2	0.1	0.2	1.0
16:0	17.7	14.3	9.7	7.1	1.1	6.8
16:1	0.6	0.7	0.2	0.5	0.6	1.1
17:0	0.8	0.6	0.5	0.7	0.3	1.0
18:0	23.5	25.9	28.4	26.9	2.1	4.3
18:1	10.2	10.4	6.0	7.7	7.2	9.0
18:2	15.2	15.3	7.1	5.9	81.8	67.8
18:3	0.1	0.1	---	0.1	0.2	0.7
20:1	0.3	0.7	0.2	0.7	0.2	1.6
20:2	0.3	---	0.1	0.2	0.5	0.3
20:4	20.7	21.1	22.0	20.3	2.0	0.9
22:1	0.1	1.5	---	1.1	---	4.4
22:6	10.3	9.2	25.6	28.7	3.8	1.1

TABLE V

Relative Composition of Total Fatty Acids of Separated Phospholipids in Pooled Rat Heart Mitochondria from Rats Fed Diet Containing 1.4 and 2.6% Erucic Acid for 28 Days

	Phosphatidylcholine			Phosphatidylethanolamine			Cardiolipin		
	Control erucic acid	Treated erucic acid		Control erucic acid	Treated erucic acid		Control erucic acid	Treated erucic acid	
Fatty acid	0.2%	1.4%	2.6%	0.2%	1.4%	2.6%	0.2%	1.4%	2.6%
14:0	---	---	0.2	---	0.1	0.5	0.1	0.1	0.3
16:0	10.5	13.1	10.7	7.2	10.9	7.9	1.2	0.6	1.0
16:1	0.2	---	0.3	---	0.3	0.9	0.4	0.4	0.3
17:0	---	0.3	---	0.3	0.2	---	---	---	---
18:0	34.6	29.6	31.5	37.2	32.0	30.1	4.0	1.0	1.9
18:1	7.9	9.9	9.1	9.3	11.2	10.0	8.5	6.4	6.3
18:2	12.7	16.7	17.2	8.0	14.0	9.6	79.4	87.4	85.2
18:3	---	---	0.1	---	---	---	0.1	0.2	0.3
20:0	0.3	0.1	0.3	0.4	0.2	0.3	0.1	---	---
20:1	0.4	0.4	0.9	0.5	0.8	1.0	0.6	0.6	0.9
20:2	0.2	0.4	0.3	0.2	0.6	0.5	1.0	0.7	0.7
20:4	30.9	28.1	28.4	25.7	22.8	23.4	2.4	1.1	1.3
22:1	0.2	---	0.8	0.1	0.5	1.0	0.1	0.6	1.3
22:6	2.1	1.4	0.2	11.1	6.4	14.8	2.1	0.9	0.5

pholipids in the aging process of heart cells.

The incorporation of erucic acid into the cardiolipin molecule is of particular interest because cardiolipin is synthesized by the mitochondria and may be required for the integrity of the inner membrane. Following the incorporation of erucic acid into cardiolipin, there was a corresponding decrease in the content of linoleic acid in cardiolipin.

The molecular structure of fatty acids in lipid molecules has a profound influence on the packing of these molecules in a bilayers (16). In general, the longer the fatty acid, the more tightly packed are the molecules in a monolayer and, the greater the unsaturation, the more expanded the film. Thus, at the same surface pressure, oleic acid forms a more expanded film than erucic acid does, 48 and 40 $Å^2$/molecule, respectively. Erucic acid and other long chain monounsaturated fatty acids have physical characteristics like those of saturated fatty acids. Thus, the incorporation of erucic acid into cardiolipin and the corresponding decrease in the linoleic acid might influence the physical properties of this phospholipid characteristic of the mitochondrial inner membrane.

The role of polyunsaturated fatty acids in biological membrane has been discussed widely (5, 17-19). It has been reported that in rats loss of fatty acids of the linoleate series together with the replacement of those by palmitoleate and oleate series, results in mitochondria which are more fragile during or after isolation (20,21).

Kramer (22) recently reported changes in liver phospholipid composition of rats fed rapeseed oil diets. Positional analysis of phosphatidylethanolamine and phosphatidylcholine of rat liver showed that erucic acid was incorporated preferentially in position 2 of these phospholipids which might indicate an influence of physical properties of mitochondrial membranes.

The data presently available indicate a high degree of fatty acid selectivity of heart phospholipids, particularly with regard to the synthesis of cardiolipin. There is the additional possiblity of ester interchange which, if extensive, would mask any specificity, or lack thereof, that existed during the initial synthesis of the phospholipids.

The mechanism by which this specific distribution of the erucic acid in membrane phospholipids is brought about remains to be established.

The existence of two pools of cardiolipin has been reported in rat liver mitochondria (23). One pool of cardiolipin is synthesized de novo and the other pool of cardiolipin which contains linoleic acid is synthesized by transacylation of the former. Erucic acid might be incorporated into cardiolipin in the same way as linoleic acid and qualitatively affect this pool. This is of great interest, because it has been found that cardiolipin is tightly bound to cytochrome oxidase (24), which indicates the importance of this phospholipid as a structural component of the respiratory chain.

It also has been shown that a certain proportion of unsaturated fatty acids are necessary to maintenance of mitochondrial function in a yeast mutant (25,26) unable to synthesize unsaturated fatty acids. Nearly complete loss of oxidative phosphorylation, respiratory control, and valinomycin-dependent K^+ uptake occurred when the level of cellular unsaturated fatty acids fell below a certain minimum.

Reports from different laboratories (27,28) indicate that erucic acid inhibits the oxidation of other long chain fatty acids in the mitochondria with an increased triglyceride synthesis in the heart tissue as a consequence. The specific effect of erucic acid upon the cardiolipin with a decreased linoleic acid content might have a specific inhibitory effect upon the mitochondrial fatty acid catabolism, as well as on the mitochondrial respiration and the energy supply of the heart.

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Metabolic Effects Following Medium Chain Triglycerides Load in Dogs: IV. Influence of Administration of Methylene Blue

A. BACH[1] and **P. METAIS**, Laboratoire de la Clinique Médicale A, C.H.U., Strasbourg, and **D. GUISARD** and **D. DEBRY**, Groupe de recherches de nutrition et de diététique INSERM U59, Nancy, France

ABSTRACT

The perfusion of 1 g/kg of medium chain triglycerides in 1 hr in the dog causes an increase in triglycerides, nonesterified fatty acids, and ketone bodies in the plasma. Hyperinsulinemia and hypoglycemia are observed at the same time. Simultaneous perfusion of fat and a hydrogen acceptor, such as methylene blue, does not modify the triglycerides, nonesterified fatty acids, and total ketone bodies variations. On the other hand, the dye modified the redox state of the liver, the consequence of which is an increase in glycemia, despite high concentrations of insulin.

INTRODUCTION

We have shown that a medium chain triglycerides (MCT) load causes a decrease in plasmatic glucose in man (1) as in the dog (2-6). Campbell, et al., (7,8), Jenkins (9), and Sanbar, et al., (10,11) ascribed this hypoglycemia to a decrease in hepatic glucose outflow.

Now Madison, et al., (12) has shown that an intravenous load of alcohol also produces hypoglycemia and that methylene blue, which is a powerful hydrogen acceptor, counteracts the decrease in hepatic glucose outflow.

It, therefore, appeared useful to associate a load of methylene blue to the perfusion of medium chain triglycerides during our experiments on the dog. It was logical, indeed, to consider that the rise in the redox potential observed during medium chain triglycerides load would be modified by methylene blue and that this modification would affect glucose metabolism. This hypothesis is confirmed by the results reported in the present study.

MATERIALS AND METHODS

The fat used contained 49% $C_{10:0}$, 47% $C_{8:0}$, and traces of $C_{14:0}$ and $C_{6:0}$. It was used in the form of a 20% fat emulsion in 0.9% NaCl with 1% soya lecithins and perfused at the dose of 1 g/kilo body wt at a constant rate for 1 hr.

Dogs of both sexes, weighing between 13-22 kg and fasting since the previous evening, were anesthetized as required with sodium pentobarbital.

Ten dogs were perfused with medium chain triglycerides. Eight dogs (4 of which were from the previous batch) were perfused in the same manner, but, after 30 min perfusion of fat, methylene blue (in oxidized blue form) was perfused at the rate of 10 mg/kg body wt for 1 hr.

The proper action of methylene blue was studied in 4 dogs of the preceding series, which were perfused for 1 hr with methylene blue (10mg/kg).

The experiments lasted 4-5 hr. Venous blood was taken continuously for automatic measurement of glucose, lactate, and pyruvate, and at repeated intervals for measurement of triglycerides, nonesterified fatty acids (NEFA), β-hydroxybutyrate, acetoacetate, and insulin (IRI) in the plasma. The procedure adopted and the methods of measurement have been described in a previous publication (2).

We calculated the averages ± the standard deviation of the averages of the various parameters from the results obtained. The significance of each was calculated by Student's t-test.

RESULTS

Perfusion of methylene blue produces blue staining in the plasma, which rapidly returns to its normal color when perfusion stops. We verified the fact that staining does not affect continuous measurements. In the case of measurements at repeated intervals, the extraction eliminates the dye.

Perfusion of triglycerides alone: The results obtained after the perfusion of medium chain triglycerides already have been described (2,3). Plasma triglycerides and NEFA levels rose; then a rise in ketonemia and insulinemia was observed. Plasma glucose level decreased at the beginning of the perfusion. The lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios presented a higher maximum than the original level, then decreased to a figure below this level.

Perfusion of methylene blue: Figure 1 shows the variations of all the parameters studied in the four control dogs. The variations are not

[1]Chargé de recherches INSERM.

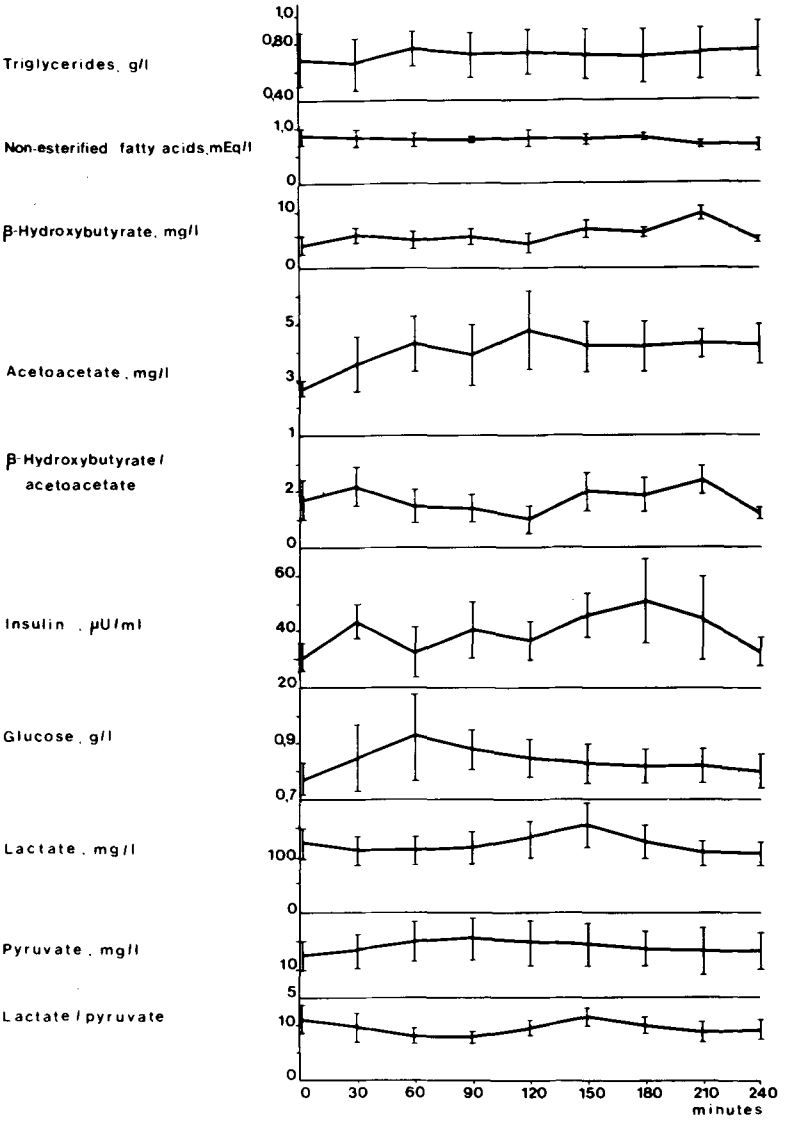

FIG. 1. Variations in plasma concentrations of the different parameters studied after perfusion of methylene blue (10 mg/kg) (4 dogs) during 1 hr in a 0.9% NaCl solution. The results are expressed in $\bar{X} \pm \frac{S}{\sqrt{n}}$.

significant. Between 30 min-4 hr, the average value of plasmatic levels is respectively: 0.72 ± 0.05 g/liter for triglycerides; 0.868 ± 0.059 mEq/liter for NEFA; 6.3 ± 0.5 mg/liter for β-hydroxybutyrate; 4.2 ± 0.3 mg/liter for acetoacetate; 42 ± 3 μU/ml for IRI; 0.86 ± 0.03 g/liter for glucose; 119 ± 9 mg/liter for lactate; and 13.9 ± 1.0 mg/liter for pyruvate. During this period, the β-hydroxybutyrate/acetoacetate ratio is 1.7 ± 0.2, and the lactate/pyruvate ratio is 9.2 ± 0.5.

Perfusion of triglycerides and methylene blue: Perfusion of fat and methylene blue causes an increase in plasma triglycerides and NEFA, which is significant in comparison with the original levels ($p<0.005$ for triglycerides, $p<0.0025$ for NEFA). The same gradient of increase is found with or without methylene blue.

The increase in plasma acetoacetate + β-hydroxybutyrate is significant ($p<0.005$), when maximum levels are compared with the original levels. This increase is the same during perfusion of fat alone or fat with methylene blue (Fig. 2).

Pyruvate concentration first presents a minimum, then a maximum, that are both sig-

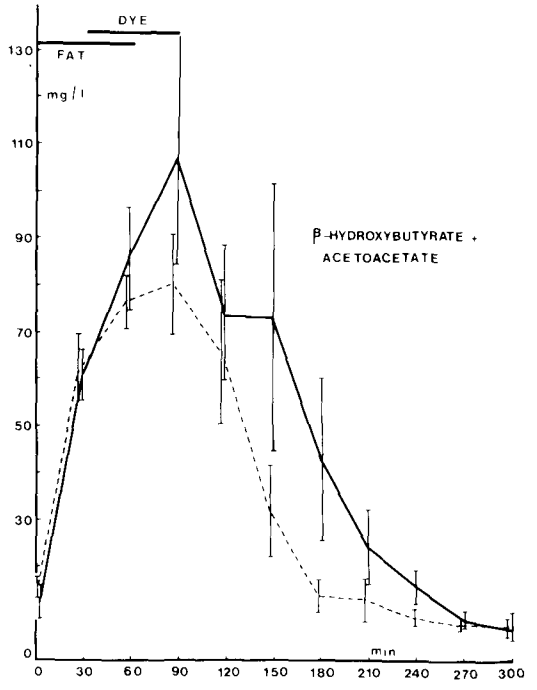

FIG. 2. Variations in plasma concentrations of total ketone bodies after perfusion for 1 hr of medium chain triglycerides (1 g/kg body wt) without (10 dogs) (dotted line) or with (8 dogs) (solid line) methylene blue (10 mg/kg body wt). The results are expressed in $\bar{X} \pm \frac{S}{\sqrt{n}}$. Fat: between 0 min-1 hr perfusion of 20% fat emulsion of medium chain triglycerides in 0.9% NaCl at the dose of 1 g/kg body wt. Dye: between 30 min and 1-1/2 hr perfusion of methylene blue at the dose of 10 mg/kg body wt.

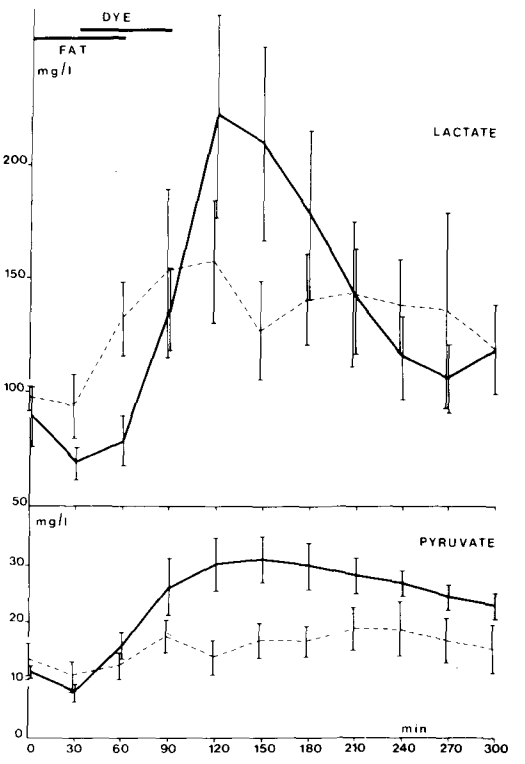

FIG. 3. Variations in plasma concentrations of lactate (above) and pyruvate (below) after perfusion of medium chain triglycerides without (dotted line) or with (solid line) methylene blue. Experimental conditions the same as those in Figure 2.

nificantly different from the original level ($p<0.005$ and $p<0.0005$, respectively). It is the same for lactate (minimum, $p<0.02$ and maximum, $p<0.005$). These two parameters undergo the same variations without methylene blue. However, plasma pyruvate is constantly higher after perfusion of the dye (Fig. 3) ($p<0.05$, 2 hr after beginning the experiment). The dispersion of the results of lactate in the presence of methylene blue is too great for significant differences to be indicated (Fig. 3).

Simultaneous perfusion of medium chain triglycerides and methylene blue causes significant hyperglycemia ($p<0.02$) which is not observed with perfusion of triglycerides alone ($p<0.005$) (Fig. 4).

Insulin increases significantly during fat and methylene blue load ($p<0.001$) (Fig. 4). Comparison of variations in insulinemia during this experiment, with variations observed during control experiment, does not show significant difference. However, in the presence of methylene blue, IRI increase appears higher.

DISCUSSION

According to the results we obtained, the presence of methylene blue does not affect variations in plasma triglycerides and NEFA observed during medium chain triglycerides load.

Variation in ketone bodies and in the β-hydroxybutyrate/acetoacetate ratio is not affected by the dye either.

The maximum of the sum of β-hydroxybutyrate + acetoacetate (Fig. 2) appears to be a little higher when the dye is present. Return to normal concentrations of ketone bodies also seems slower. However, no significant difference was observed.

In the presence of methylene blue, we find that pyruvatemia is higher, lactacidemia variable, and the lactate/pyruvate ratio (Fig. 5) lower ($p<0.001$, 3-1/2 hr after the beginning of the experiment) than during experiments carried out without the dye.

In its role of hydrogen acceptor, particularly of nicotinamide adenine dinucleotide phosphate, reduced form + hydrogen$^+$ (NADPH + H$^+$) but also of nicotinamide adenine dinucleo-

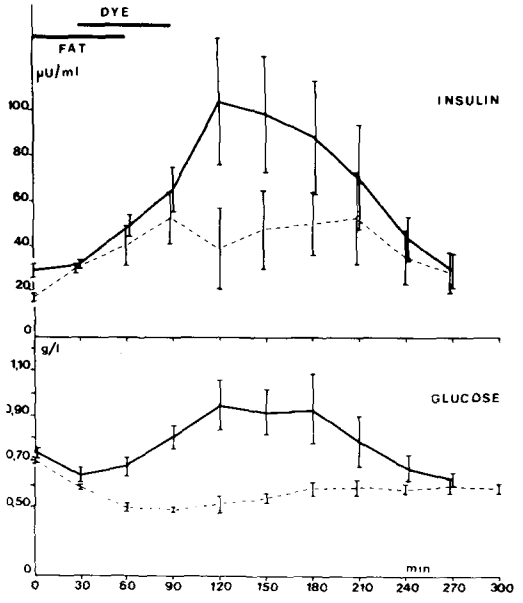

FIG. 4. Variations in plasma concentrations of insulin (above) and glucose (below) after perfusion of medium chain triglycerides without (dotted line) or with (solid line) methylene blue. Experimental conditions the same as those in Figure 2.

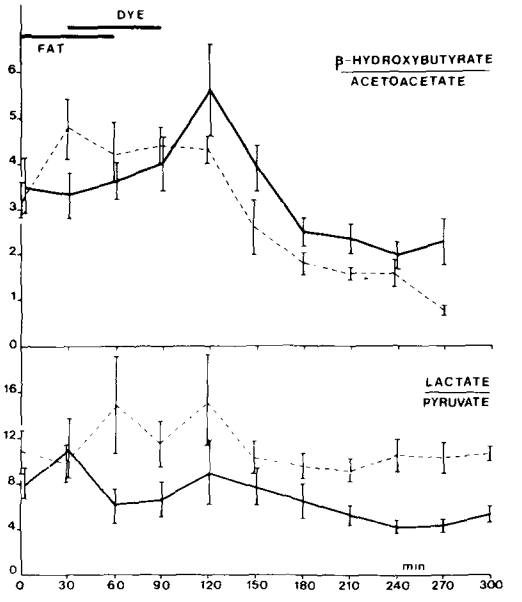

FIG. 5. Variations in plasma concentrations of β-hydroxybutyrate/acetoacetate (above) and lactate/pyruvate (below) after perfusion of medium chain triglycerides without (dotted line) or with (solid line) methylene blue. Experimental conditions the same as those in Figure 2.

tide, reduced form + hydrogen$^+$ ($NADH + H^+$) (13,14), methylene blue seems to cause shifting of lactate toward pyruvate.

The introduction of methylene blue during perfusion of fat causes an increase in plasma glucose, which is 0.65 ± 0.03 g/liter before methylene blue, and 0.95 ± 0.11 g/liter 2 hr afterwards.

The hypoglycemia we have observed after a medium chain triglycerides load might be explained by a decrease in hepatic glucose outflow. As we have seen, methylene blue alone does not significantly modify glycemia. On the contrary, its association with fat might increase hepatic glucose outflow despite considerable concomitant production of insulin. In fact, maximum increase of IRI attains 327 ± 66% during perfusion of fat and methylene blue, while it is only 255 ± 63% (the difference is not significant) without the dye.

During the very fast oxidation of medium chain fatty acids in the liver (15-17), a large quantity of acetyl-CoA is formed at the same time as numerous molecules of NAD^+ are reduced to $NADH + H^+$. The acetyls-CoA combine to give rise to the ketone bodies that stimulate the secretion of insulin (18). Part of the $NADH + H^+$ is reoxidized during the reduction of acetoacetate (massively produced during the load) to β-hydroxybutyrate and another part during the transformation of pyruvate into lactate. This results in an increase of β-hydroxybutyrate/acetoacetate and lactate/pyruvate ratios in the liver, which is reflected by the increase of these ratios in the plasma (Fig. 5, dotted lines) (2,3,19-21).

We believe that the increase of the redox state in the hepatic cell and the concomitant production of insulin (22,23) cause decrease in glucose release by the liver. The infusion of methylene blue leads to a reversal of the redox state as shown by the decrease of β-hydroxybutyrate/acetoacetate ratio between 0 min-1-1/2 hr (not significant) and especially by the decrease of the lactate/pyruvate ratio (highly significant after 3 hr). It may be concluded that there is passage of lactate to pyruvate, which increases, as shown, by Figure 3. This observation supports that of Tranquada, et al., (24) who administered methylene blue to five subjects with metabolic acidosis and excessive lactate after which they observed an increase in plasma pyruvate. In the case of perfusion of medium chain triglycerides, pyruvate may furnish oxaloacetate (since citrate-synthetase is blocked by the acetyls-CoA [25]) which is transformed into phosphoenol-pyruvate and then glucose.

ACKNOWLEDGMENTS

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Bile Acid Metabolism in Mammals: VII. Studies on Sex Differences in Deoxycholic Acid Metabolism in Isolated Perfused Rat Liver

M.M. FISHER, V.M. PRICE, R.J. MAGNUSSON, and I.M. YOUSEF[1]
Departments of Medicine and Pathology, University of Toronto,
Toronto, Ontario, Canada M5S 1A8

ABSTRACT

The hepatic metabolism of deoxycholic acid was studied using the isolated perfused rat liver technique. In 20 perfusions, 10 involving the livers of male rats and 10 involving the livers of female rats, 30 μmoles deoxycholic acid was added to the perfusion medium. In 10 perfusions, 5 male and 5 female, 1 μmole deoxycholic acid was added to the perfusion medium. In 10 of the high dose studies and in the 10 low dose studies, 1 μCi deoxycholic acid-C-24-C^{14} also was added. The deoxycholic acid was added to 100 ml perfusion medium after 2 hr of baseline perfusion, and the studies were continued another 3 hr. Biliary bile acids were analyzed by combined thin layer and gas chromatography, and the radioactivity content of the perfusion medium and liver was documented. Although there was no sex difference in total bile acid secretion in the high dose studies, there were sex differences in the bile acid secretion rate and in the quantitative secretion of individual bile acids. The biliary secretion of deoxycholic acid and cholic acid was immediate in the female studies and delayed in the male, and the amounts of cholic acid and sulfated deoxycholyl-taurine secreted were considerably greater in the male studies. In the low dose studies the isolated perfused liver of the female rat converted more deoxycholic acid to cholyl-taurine than did that of the male rat. There are sex differences in the hepatic metabolism of deoxycholic acid. In contrast to those found in the case of chenodeoxycholic acid, these sex differences are not impressive when physiological amounts of deoxycholic acid are presented to the liver.

INTRODUCTION

Deoxycholic acid (DOCA) is a secondary bile acid produced by the 7α-dehydroxylation of cholic acid (CA) in the intestines. It is, at least quantitatively, an important bile acid in the rat. Lindstedt and Samuelsson (1) have estimated that ca. 50% of the total CA pool of the rat is converted to DOCA each day. Studies with bile fistula animals have demonstrated that DOCA constitutes 15-20% of the total bile acid pool of the rat with more present in the male than in the female (2). In the rat, DOCA undergoes an efficient enterohepatic circulation, and most of that which returns to the liver is rapidly rehydroxylated to CA (3). Besides rehydroxylation at C-7, DOCA can undergo hydroxylation at C-6 with the formation of 3α,6β,12α-trihydroxycholanic acid (4). The studies reported in this article concern some of the sex differences involved in the hepatic metabolism of DOCA in the rat.

MATERIALS AND METHODS

DOCA (3α,12α-dihydroxy-5β-cholanic acid) was obtained from Sigma Chemical Co., St. Louis, Mo. No trace of contaminants was found on either thin layer (TLC) or gas chromatographic (GLC) analysis and the compound was, therefore, used without further purification. Lecithin (bovine) was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio; carbenicillin (Pyopen) from Ayerst Laboratories, New York, N.Y., and heparin sodium (U.S. Pharmacopeia) from Connaught Medical Research Laboratories, Toronto, Canada. DOCA-C-24-C^{14}, specific activity 1-5 mCi/mmole, was obtained from ICN Research Products, Irvine, Calif. An aliquot of this material was taken to dryness under nitrogen, and 4 mg nonradioactive DOCA was added. The mixture was dissolved in 2 ml methanol and the methyl esters formed as previously described (2). The methyl esters were analyzed by TLC using chloroform-acetone-methanol, 70/25/5, v/v/v. The bands were isolated and the radioactivity determined. DOCA accounted for 99.9% of the radioactivity which was recovered. On the basis of this recovery, the radioactive DOCA was used without purification.

A standard preparation of sulfated deoxycholyl-taurine (S-DOC-T) was prepared accord-

[1]A Scholar of the Canadian Hepatic Foundation.

ing to the method of Sobel and Spoerri outlined by Jenkins and Sandberg (5). Equal wt of DOC-T and dried complex of sulfur trioxide pyridine (Aldrich Chemical Co., Milwaukee, Wisc.) were refluxed for 20 min in a volume of benzene-pyridine-acetic anhydride (10/1/1, v/v/v) sufficient to afford ca. 7% solution of the pyridine sulfur trioxide. After cooling the reaction mixture, the sulfated bile acid was precipitated by adding 4 volumes of petroleum ether. The precipitate was recovered by centrifugation and then dissolved in n-butanol. The S-DOC-T was not subjected to detailed chemical analysis, but it ran as a single spot on TLC using butanol-acetic acid-water, 10/1/1, v/v/v, with an R_f of 0.14, a position distinctly more polar than that of DOC-T whose R_f is 0.44. After solvolysis, the S-DOC-T ran on TLC with an R_f of 0.44, and hydrolysis of this conjugate produced a bile acid which was identical to DOCA on analysis, using both TLC and GLC.

Perfusion Experiments

Wistar rats, (High Oak Ranch, Toronto, Canada) of both sexes, weighing ca. 250 g were used as liver and as perfusion medium donors. Prior to use, the animals were kept in rooms maintained at a constant temperature (22 C), were in darkness for 12 hr each day, and were allowed water ad lib. The studies involved two types of diet. Ten experiments involved animals which had been maintained on a standard commercial diet (Purina Rat Chow). Twenty experiments involved animals which had been maintained 1-2 weeks on a semisynthetic diet (General Biochemicals, TD-72460 Basal Diet with 27% casein and Salt Mixture USP XIV, modified Farber formulation). The animals were starved 24-26 hr before use, anesthetized with diethyl ether, and sacrificed 3-4 hr after the start of their light cycle. The perfusion technique, which involves recirculation of the perfusion medium, has been described in other communications (6,7). The perfusion medium used was whole rat blood containing heparin, 7 units/ml, carbenicillin 200 μg/ml, and added glucose 1 mg/ml. After 2 hr of perfusion 6 ml saline solution containing lecithin and bile acid was added as a single bolus to the perfusion medium which at this point measured ca. 100 ml in volume. The saline solution contained lecithin at a concentration of 7.5 mg/ml. DOCA was added in a total amount of 30 μmoles in 20 perfusions and of 1 μmole in 10 perfusions, with the perfusions at each dose level equally divided between the two sexes. In 20 of the perfusions, 10 of the high dose experiments and the 10 low dose experiments, 1 μCI DOCA-C-24-C^{14} also was added. In the radioactive studies, special procedures were invoked to ensure rapid and complete mixing of the bile acid in the perfusion medium. Immediately after the bile acid was added to the main perfusion medium reservoir, the pumping mechanism was accelerated, and the hydrostatic reservoir from which the perfusion medium flows to the liver was drained through the side arm of the portal vein cannula with care taken not to interrupt the blood flow to the liver. Preliminary studies demonstrated that these maneuvers ensure complete mixing of the bile acid in the perfusion medium within 5 min, and, after this time, the pumping mechanism was decelerated, flow through the side arm of the portal vein cannula was stopped, and the standard protocol was used. Samples of the perfusion medium were obtained from the main reservoir at frequent intervals to determine the rate at which the radioactivity disappeared from the perfusion medium. Each perfusion was continued for 3 hr following the addition of the bile acid to the perfusion medium, and bile was collected in hourly aliquots.

Bile Acid Analyses

Bile acids were extracted from the bile by the addition of 10 ml hot ethanol-methanol solution (95/5, v/v). The tubes were capped, vibrated, and then centrifuged at 3000 rpm and 4 C for 15 min. The alcoholic supernatant was decanted and the precipitate washed once with 2 ml methanol. The alcoholic extracts were combined, delipidated with petroleum ether, and then evaporated to dryness under nitrogen. The residue then was dissolved in 1 ml methanol and 0.5 ml of this used for the analysis of the conjugated bile acids and 0.5 ml for analysis of the total bile acids.

Conjugated Bile Acids

A known volume of the bile acid extract was applied to thin layer plates (20 x 20 cm) coated with Silica Gel G 0.25 mm thick. The plates were developed in a solvent containing butanol, glacial acetic acid, and water (10/1/1, v/v/v). After 4 hr, the plates were dried and the bile acids located by iodine vapor. Each spot was scraped and the bile acids extracted from the silica gel by 0.05N HCl in 75% ethanol. Recovery experiments have indicated an efficiency of greater than 95% for this procedure.

The bile acid content of that band corresponding to the S-DOC-T standard also was subjected to solvolysis. The bile acids were dissolved in 1 ml ethanol, acidified to pH 1 with 2N HCl, diluted with 9 ml acetone, and left at room temperature for 72 hr. Following evaporation to dryness, the bile acids were identified

TABLE I

Animal Body and Liver Weights (g)[a]

Group	Body wt (g)	Liver wt. (g)	Body wt / Liver wt
I			
30 μmoles deoxycholic acid laboratory chow			
Female[b]	287 ± 24	7.00 ± 0.46	41.00 ± 2.57
Male[b]	325 ± 67	8.78 ± 0.52	37.02 ± 1.52
P value	NS[c]	NS	NS
II			
30 μmoles deoxycholic acid deoxycholic acid C-24-C^{14} semisynthetic diet			
Female[b]	239 ± 11	8.22 ± 0.16	29.08 ± 3.02
Male[b]	270 ± 33	10.12 ± 0.38	26.68 ± 1.89
P value	NS	<0.001	NS
III			
1 μmole deoxycholic acid deoxycholic acid C-24-C^{14} semisynthetic diet			
Female[b]	234 ± 4	7.10 ± 0.26	32.96 ± 0.93
Male[b]	260 ± 13	9.24 ± 0.21	28.14 ± 1.29
P value	<0.001	<0.001	<0.05
P values			
Group I vs group II			
Female	NS	NS	<0.05
Male	NS	NS	<0.01
Group I vs group III			
Female	NS	NS	<0.05
Male	NS	NS	<0.01

[a]Values are $\bar{x}$ ± standard error.

[b]n = 5.

[c]NS = not significant (P>0.05).

by TLC, as previously described.

In the experiments involving radioactive bile acids, an aliquot of each extract was counted 10 min in triplicate and with an efficiency greater than 80%. A Packard Liquid Scintillation spectrometer equipped with an automatic external standard was used, and the counts were corrected for quenching.

The taurine and glycine conjugates and the solvolyzed bile acids were subjected to hydrolysis in hot NaOH (2). The unconjugated bile acids so produced were extracted with diethyl ether, taken to dryness, dissolved in methanol, and separated into trihydroxy-, dihydroxy-, and monohydroxy- bile acids by TLC system 1 of Hofmann (8). Following development in iodine vapor, the TLC plates were scraped and the bile acid content of each band eluted with methanol acidified with hydrochloric acid.

The bile acids were derivatized to their methyl esters (2) and the trimethylsilyl (TMS) esters of these prepared by incubating them in dry pyridine (0.2 ml), hexamethyldisilazane (0.5 ml), and trimethylchlorosilane (0.1 ml) at room temperature for 6 hr. The TMS-methyl esters were subjected to GLC-mass spectrometry (MS) in the laboratory of A. Kuksis.

In the appropriate experiments, the radioactivity of each band was measured, as previously described.

Total Bile Acids

The total bile acids were measured quantitatively by GLC using 5β-cholanic acid as an internal standard (2).

Radioactivity Analysis

The radioactive content was determined in duplicate samples of the perfusion medium and liver. Perfusion medium (0.1 ml) was shaken 1 hr at 55 C in 0.5 ml Protosol. Fresh 20% benzoyl peroxide in toluene (0.35 ml) then was added and shaking continued 1 hr at room temperature. Aquasol (15 ml) was added, the mixture cooled 48 hr and the samples then counted with an efficiency of 75-85%. Liver tissue, 0.5 ml of a 1/4 homogenate in 0.15M NaCl, was shaken 4 hr at 55 C in 2 ml Protosol. Fresh 20% benzoyl peroxide in toluene (1.0 ml) then was added and shaking continued 4 hr at room tem-

TABLE II

Bile Flow (μliter/g liver/hr)[a,b]

Group	Hr 0-1	1-2	2-3	3-4	4-5
I					
30 μmoles deoxycholic acid laboratory chow					
Female[c]	33 ± 4	37 ± 5	32 ± 13	20 ± 7	20 ± 5
Male[c]	45 ± 6	52 ± 4	70 ± 5	49 ± 4	39 ± 4
P value	NS[d]	NS	<0.05	<0.01	<0.05
II					
30 μmoles deoxycholic acid deoxycholic acid C-24-C^{14} semisynthetic diet					
Female[c]	39 ± 7	44 ± 4	58 ± 6	53 ± 4	42 ± 6
Male[c]	39 ± 6	52 ± 7	58 ± 5	52 ± 4	42 ± 3
P value	NS	NS	NS	NS	NS
III					
1 μmole deoxycholic acid deoxycholic acid C-24-C^{14} semisynthetic diet					
Female[c]	56 ± 12	65 ± 8	73 ± 6	61 ± 4	52 ± 5
Male[c]	52 ± 5	64 ± 4	72 ± 4	50 ± 2	38 ± 3
P value	NS	NS	NS	NS	NS

[a]Values are $\bar{x}$ ± standard error.

[b]Deoxycholic acid was added to the perfusion medium after 2 hr of perfusion.

[c]n = 5.

[d]NS = not significant (P>0.05).

perature. Aquasol (15 ml) was added, the mixture cooled 48 hr, and the samples then counted with an efficiency of 70-75%.

Statistics

Significant differences were determined by Student's t-test. No significant difference = P>0.05.

RESULTS

Because of dissatisfaction with the qualitative control of commercial laboratory chow, our laboratory converted to the semisynthetic diet mentioned under "Materials and Methods." This conversion was done with recognition of the influences of diet on bile acid metabolism (9-11). Table I, which presents the data concerning body wt and liver wt, demonstrates that those animals which had received the semisynthetic diet did present a decrease in the ratio of body wt to liver wt. Furthermore, in the studies involving the larger dose of DOCA, there was no sex difference in the volume of bile secreted by the livers of animals maintained on the semisynthetic diet, Table II. Possible influences of the semisynthetic diet on bile acid metabolism are under current study, but the studies reported in this article are concerned with sex differences in the hepatic metabolism of DOCA, and these differences have been found to be the same in the two dietary groups.

30 μmole DOCA Studies

There was no sex difference in total bile acid secretion, 78 ± 3% of the added DOCA being secreted in the bile in both the male and female studies. However substantial sex differences were documentated in the rate at which bile acids were secreted into the bile and in the quantitative secretion of individual bile acids, Table III. After the addition of 30 μmoles DOCA to the perfusion medium, the biliary secretion of DOCA and CA was immediate and largely confined to the first hr in the female studies but delayed and equally distributed over the second and third hr after the addition of the bile acid in the male studies. Furthermore, the male liver converted substantially more DOCA to CA. Of the total bile acid secretion, CA comprised 31.6% in the male studies, 15% in the female, P<0.001.

Chromatographic analysis of the biliary bile acids documented further sex differences. Figure 1 is a reproduction of a TLC of the conjugated bile acids in the bile. In studies of both sexes, cholyl taurine (C-T) and DOC-T were prominent. However, in the male studies, there also was a substantial amount of a conjugate more polar than C-T. Solvolysis, hydrolysis, and

TABLE III

Bile Acid Secretion After Addition of Deoxycholic Acid (DOCA) to Perfusion Medium (μmoles)[a,b]

Group	DOCA				Cholic acid (CA)				Total (DOCA + CA)
	2-3 hr	3-4 hr	4-5 hr	Subtotal	2-3 hr	3-4 hr	4-5 hr	Subtotal	
I									
30 μmoles DOCA									
laboratory chow									
Female[c]	16.91 ± 0.85	1.68 ± 0.14	0.13 ± 0.05	18.72 ± 0.74	2.51 ± 0.22	0.47 ± 0.04	0.08 ± 0.02	2.06 ± 0.15	20.78
Male[c]	0.41 ± 0.27	7.15 ± 0.18	7.13 ± 0.42	14.69 ± 0.24	0.12 ± 0.12	2.60 ± 0.50	3.60 ± 0.84	6.32 ± 1.05	21.01
P value	<0.001	<0.001	<0.001	<0.002	<0.001	<0.01	<0.01	<0.01	NS[d]
II									
30 μmoles DOCA									
DOCA C-24-C^{14}									
semisynthetic diet									
Female[c]	15.80 ± 0.84	1.32 ± 0.11	0.16 ± 0.05	17.28 ± 0.82	2.51 ± 0.31	0.47 ± 0.04	0.11 ± 0.07	3.09 ± 0.54	20.37
Male[c]	0.98 ± 0.43	7.51 ± 0.11	7.00 ± 0.32	15.49 ± 0.39	0.19 ± 0.11	2.52 ± 0.44	3.59 ± 0.77	6.30 ± 1.11	21.79
P value	<0.001	<0.001	<0.001	NS	<0.001	<0.01	<0.01	<0.05	NS
III									
1 μmole DOCA									
DOCA C-24-C^{14}									
Female[c]	0.15 ± 0.03	0.09 ± 0.01	trace	0.24 ± 0.04	0.25 ± 0.02	0.45 ± 0.05	trace	0.70 ± 0.09	0.94
Male[c]	0.20 ± 0.01	0.14 ± 0.01	trace	0.34 ± 0.02	0.20 ± 0.02	0.40 ± 0.05	trace	0.60 ± 0.06	0.94
P value	NS	<0.02		NS	NS	NS		NS	NS
P values*									
I vs II									
Female	NS	NS	NS	NS	NS	NS	NS	NS	NS
Male	NS	NS	NS	NS	NS	NS	NS	NS	NS

[a]Values are $\bar{x}$ ± standard error.

[b]DOCA was added to perfusion medium after 2 hr of perfusion, and bile was collected for the subsequent 3 hr.

[c]n = 5.

[d]NS – not significant (P>0.05).

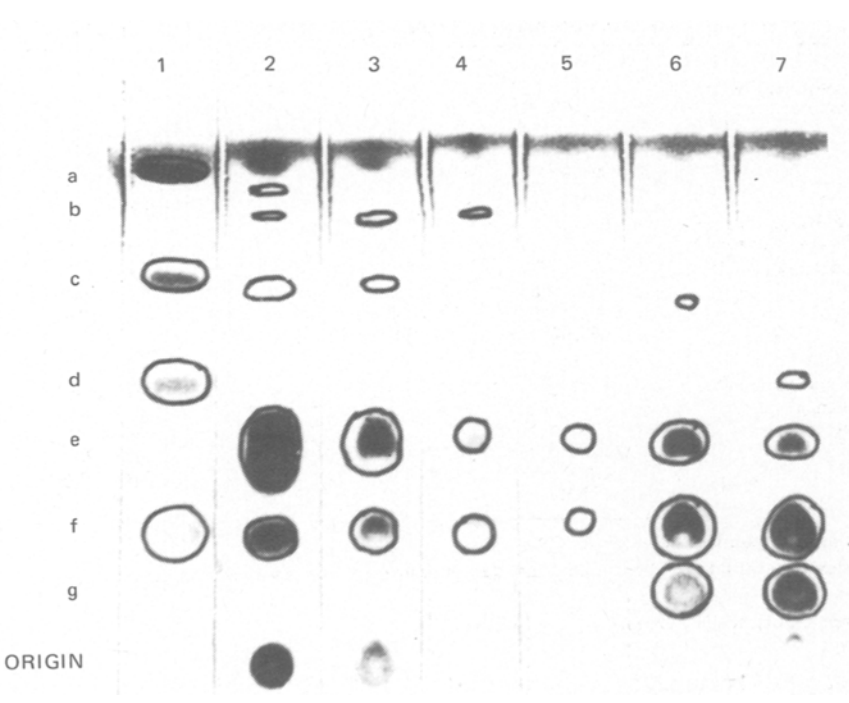

FIG. 1. Thin layer chromatogram demonstrating the presence of a conjugated bile acid more polar than cholyl-taurine in the bile produced by the isolated perfused liver of the male rat during the second and third hr after addition of 30 μmoles deoxycholic acid (DOCA) to the perfusion medium. 1. Standard mixture (a = free bile acid, b = unknown, c = chenodeoxycholyl-glycine, d = cholyl-glycine, e = deoxycholyl-taurine, f = cholyl-taurine, and g = unknown. 2-4. Female experiment (laboratory chow), hr 1-3 after addition of 30 μmoles DOCA. 5-7. Male experiment (laboratory chow), hr 1-3 after addition of 30 μmoles DOCA.

chromatography with an appropriate standard indicated that this conjugate was sulfated DOC-T (S-DOC-T). In the studies involving animals maintained on laboratory chow, 27.7% of the total biliary bile acid secretion in the male experiments was S-DOC-T (Table IV). None of this conjugate was found in the bile in the female studies. The experiments involving animals maintained on the semisynthetic diet and the use of radioactivity, rather than mass data, did demonstrate the biliary secretion of S-DOC-T by the isolated perfused liver of female rats, but once again it was much less than that found in the male studies (Table IV). Of the total radioactivity added in these experiments 79 ± 3% was recovered in the bile in the male studies, 78 ± 5% in the female.

In 2 perfusion experiments involving male animals and the addition of 30 μmoles DOCA to the perfusion medium, 375 μCi $Na_2S^{35}O_4$ (New England Nuclear, Boston, Mass.; specific activity 660 mCi/mmole) were added along with the DOCA. Only 1.02% of the radioactivity was secreted in the bile during the 3 hr following its addition to the perfusion medium. However, 30% of this was in the bile acid fraction, and 83% of this was in the conjugate identified as S-DOC-T (Table V).

Sex differences in the biliary bile acid secretion rate in these studies raised the possibility of sex differences in the hepatic uptake of DOCA from the perfusion medium. Frequent sampling permitted analysis of the rate of disappearance of the DOCA-C-24-C^{14} from the perfusion medium. At the end of 3 hr of perfusion, 2% of the added radioactivity remained in the perfusion medium, ca. 20% remained in the liver, and no significant sex differences were involved. Furthermore, there was no sex difference in the slopes of the plasma disappearance curves (Fig. 2). The perfusion medium radioactivity rose initially to above 100% because of incomplete mixing of the bile acid in the perfusion medium but began to fall within 5 min. The initial fall was linear, but the shoulder in

TABLE IV

Biliary Bile Acid Conjugates (Percent)[a,b]

Group	Sulfated deoxycholyl-taurine	Cholyl-taurine	Deoxycholyl-taurine	Cholyl-glycine	Deoxycholyl-glycine
I					
30 μmoles deoxycholic acid laboratory chow					
Female[c]		12.8 ± 0.7	79.2 ± 1.2	2.8 ± 0.9	5.0 ± 0.4
Male[c]	27.7 ± 1	29.5 ± 0.3	39.0 ± 0.5	1.5 ± 0.2	2.2 ± 0.6
P value	<0.001	<0.001	<0.001	NS[d]	<0.01
II					
30 μmoles deoxycholic acid deoxycholic acid C-24-C^{14} semisynthetic diet					
Female[c]	4.0 ± 0.6	13.0 ± 1.0	75.0 ± 1.0	2.0 ± 0.2	6.0 ± 1.0
Male[c]	18.0 ± 1.0	30.0 ± 2.0	47.0 ± 2.0	2.0 ± 0.2	3.0 ± 0.4
P value	<0.001	<0.001	<0.001	NS	<0.05
III					
1 μmole deoxycholic acid deoxycholic acid C-24-C^{14} semisynthetic diet					
Female[c]	11.0 ± 3.0	64.0 ± 2.0	19.0 ± 2.0	4.0 ± 0.4	2.0 ± 0.4
Male[c]	7.0 ± 0.5	55.0 ± 0.71	30.0 ± 0.2	5.0 ± 0.8	2.0 ± 0.2
P value	NS	<0.01	<0.002	NS	NS

[a]Values are $\bar{x}$ ± standard error.

[b]The values in group I are based upon mass data, in groups II and III upon radioactive data.

[c]n = 5.

[d]NS = not significant (P>0.05).

TABLE V

Biliary Bile Acid Radioactivity in $Na_2S^{35}O_4$ Perfusions[a]

Bile acid	Expt. 457	Expt. 460
Cholyl-taurine	7	7
Deoxycholyl-taurine	9	10
Sulfated-deoxycholyl-taurine	84	82
Lipids	-	1

[a]375 μCi $Na_2S^{35}O_4$ (specific activity: 660 mCi/mmole) were added to the perfusion medium along with 30 μmoles deoxycholic acid. The bile collected for the next 3 hr contained 1.02% of the added radioactivity, and 30% of this was in the bile acid fraction. Figures represent percent radioactivity in bile acid fraction.

the disappearance curve suggests that there was some retardation in hepatic uptake or possibly even reflux of radioactivity back into the perfusion medium between 10-20 min after the addition of DOCA to the perfusion medium. During the first hr after its addition to the perfusion medium, ca. 80% of the DOCA-C-24-C^{14} was taken up by the liver.

1 μmole DOCA Studies

Sex differences in the hepatic metabolism of DOCA were much less impressive in the studies involving 1 μmole DOCA. On the basis of both mass and radioactivity, 92-94% of the added

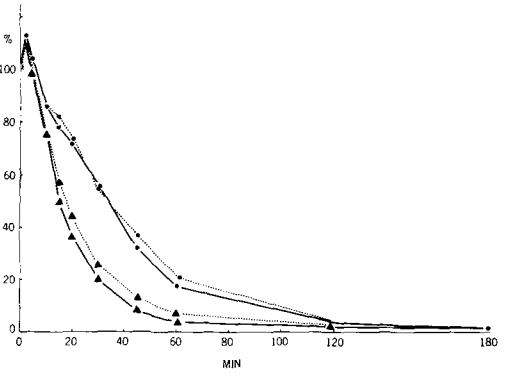

FIG. 2. Deoxycholic acid (DOCA) C-24-C^{14} was added to the perfusion medium along with nonradioactive DOCA after 2 hr of baseline perfusion. Perfusion medium radioactivity was monitored over the course of the subsequent 3 hr. n = 5 for each sex and each dose. ● = 30 μmole DOCA, ▲ = 1 μmole DOCA, ····· = male, and —— = female.

DOCA was secreted in the bile in both the male and the female studies, and no sex differences in bile acid secretion rates were involved. In both the male and female studies, bile acid secretion was distributed equally over the course of the first 2 hr following addition of the DOCA. The male liver did not secrete more S-DOC-T and actually converted somewhat less DOCA to CA than did that of the female

($P<0.05$). In terms of hepatic uptake of the DOCA-C-24-C^{14}, it was found that ca. 93% of the radioactivity was secreted in the bile, 2% remained in the perfusion medium, and ca. 5% remained in the liver with no sex differences involved. In these studies, there was no sex difference in the slopes of the plasma disappearance curves (Fig. 2). It is to be noted that, in contrast to the high dose studies, the disappearance curves in the 1 μmole studies were linear for the first 30 min and did not present evidence of retarded uptake of radioactivity between 10-20 min. Furthermore, the rate of disappearance was more rapid than in the 30 μmole studies. It is of interest that the percentage conversion of DOCA to CA was greater than in the 30 μmole studies, ca. 74% in the female studies, 64% in the male.

DISCUSSION

Increasing attention is being paid to the metabolism of DOCA, because the strong detergent properties of the bile acid imply a considerable potential for cellular toxicity (12-14). The bile acid pool of the animals used in these studies contains ca. 5 μmoles DOCA (2). The 1 μmole studies involved a perfusion medium concentration of ca. 10 nmoles/ml and were undoubtedly physiological from the point of view of the amount of bile acid involved. In these studies there were no sex differences in the hepatic uptake of DOCA, the biliary secretion rate of DOCA, or in the amount of S-DOC-T which was secreted. However, the isolated perfused liver of the female rat did convert slightly more DOCA to CA.

The 30 μmole DOCA studies involved the addition to the perfusion medium of an amount of DOCA ca. equal to the total bile acid pool of the animal, ca. 6 times the total DOCA pool and at a perfusion medium concentration of ca. 300 nmoles/ml. Under these conditions, substantial sex differences in the hepatic metabolism of DOCA were documented. Although there were no sex differences in the hepatic uptake of DOCA or in the total bile acid secretion, the isolated perfused liver of the female rat secreted the bile acids without delay and secreted less CA and less S-DOC-T. It seems unlikely that the delay in bile acid secretion in the male studies can be explained merely on the basis of the time required for 7α-hydroxylation of DOCA and sulfation of the DOC-T, because the biliary secretion of conjugated DOCA itself was delayed. It is possible, however, that the conjugation mechanism, in general, is less efficient in the male liver and that the relative delay in this process provides time for the sulfation and 7α-hydroxylation steps.

Although no evidence for the conversion of DOCA to 3α,6β,12α-trihydroxy-5β-cholanic acid (4) was found in these studies, the biliary secretion of S-DOC-T is of some interest. Palmer (15-17) has discussed some of the properties of the sulfated bile acids. In view of the fact that they appear to be associated with less toxicity than their nonsulfated analogues, we can speculate that the liver of the male rat, able to synthesize S-DOC-T and able to convert DOCA to CA more efficiently than that of the female, is more able to tolerate large doses of DOCA, a bile acid with strong detergent properties.

These studies have demonstrated that impressive sex differences in the hepatic metabolism of DOCA are not apparent when physiological amounts of DOCA are presented to the liver. This is not so in the case of chenodeoxycholic acid (18). However, when larger than physiological amounts of DOCA are presented to the liver, sex differences in the hepatic metabolism of the bile acid become obvious. The biological significance of these sex differences remains to be established.

ACKNOWLEDGMENT

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Altered Stearoyl-CoA Desaturase Activities in Morris Hepatomas 5123C and 7800

PULLARKAT K. RAJU[1], Department of Biochemistry and Biophysics,
Texas A&M University, College Station, Texas 77843

ABSTRACT

The nicotinamide adenine dinucleotide, reduced form, dependent microsomal stearoyl-CoA desaturase activities were determined in Morris hepatomas 5123C and 7800 and compared with those of the host livers. In hepatoma 5123C, the desaturase activity was ca. one-third that of the host liver. One week on a fat-free high-carbohydrate diet did not alter the desaturase activities of the hepatomas, whereas the host liver desaturase activities increased ca. three times. Fatty acid synthetase activity of the hepatoma 5123C was ca. one-fourth and that of hepatoma 7800 was ca. the same as that of the host liver, suggesting that the decreased desaturase activity of the hepatoma 5123C is mainly due to the decreased overall lipogenesis.

INTRODUCTION

Little information is available in the literature on the biosynthesis of unsaturated fatty acids in neoplastic tissues. Wood and Healy (1) have shown that Ehrlich ascites cells are capable of elongating palmitoyl-CoA but incapable of desaturating the stearoyl-CoA produced. On the other hand, the lipids of these cells, especially triglycerides, have an increased level of stearic acid without a corresponding decrease in the oleic acid content (2). This suggests that Ehrlich ascites cells may have an alternate route for the biosynthesis of oleic acid, other than the most prevalent aerobic desaturation route. In the present study, the microsomal stearoyl-CoA desaturase activities of two Morris hepatomas were determined to see whether low desaturase activity is a fundamental characteristic in neoplastic tissues.

MATERIALS AND METHODS

Animals, Tumors, and Diets

The tumors used in these experiments were the Morris hepatomas 5123C and 7800 and were obtained from the McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisc. Tumor cells were inoculated intramuscularly into both hind legs of inbred Buffalo strain rats (Texas Inbred Mice Co., Houston, Tex.) which were kept on a stock ration (Wayne Lab Blox, Allied Mills, Chicago, Ill.). A few animals were kept on a fat-free high-carbohydrate diet (Nutritional Biochemicals Corp., Cleveland, Ohio), for a week prior to sacrifice.

Preparation of Enzymes

The rats were killed by decapitation, bled, and their livers and tumors quickly removed. The tissues were homogenized in 2 volumes of 0.1 M phosphate buffer (pH 7.4) in a Potter Elvehjem glass homogenizer with 3 up-and-down strokes. The homogenate was centrifuged at 15,000 x g for 30 min, and the resultant supernatant was centrifuged at 104,000 x g for 60 min. The microsomal pellet was washed with phosphate buffer, resuspended in phosphate buffer at pH 7.4 at a concentration of ca. 10 mg protein/ml, and used for the assay of desaturase. The 104,000 x g supernatant was used for fatty acid synthetase activity determination.

The assay system contained 25 nmoles stearoyl-CoA (20,000 cpm), 0.5 μmoles nicotinamide adenine dinucleotide, reduced form (NADH), 100 μmoles phosphate buffer pH 7.4, ca. 1 mg enzyme, and water to bring the total volume to 0.5 ml. Incubations were carried out at 37 C for 5 min in a Dubnoff metabolic shaker and the reaction stopped by the addition of 1 ml 8% potassium hydroxide in 95% alcohol. Fatty acids were extracted after saponification and acidification. Diazomethane was used to prepare the methyl esters. The isolation of stearate and oleate and calculation of the desaturase activities were made according to the previously reported procedures from our laboratory (3). The enzyme activity is expressed as nmoles of oleate formed/min/mg protein. Since certain cancer tissues are known to utilize dihydroxyacetone phosphate preferentially to sn-glycero-3-phosphate (4), no glycerophosphate was added to the incubation system, even though it is known to stimulate the desaturase acitvity (5).

Fatty Acid Synthetase Assay

The fatty acid synthetase activity in the

[1]Present address: Department of Neurochemistry, Institute for Research in Mental Retardation, Staten Island, New York 10314.

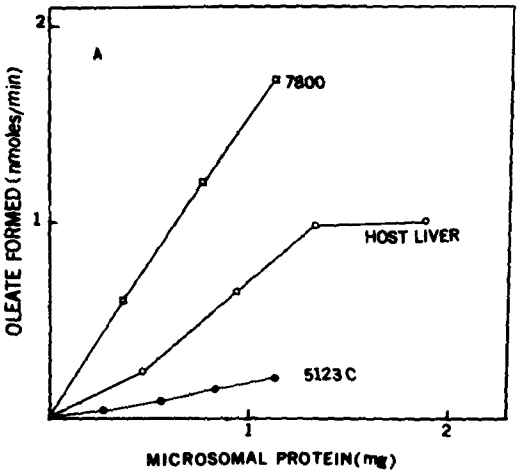

FIG. 1A. Dependency of the desaturase activities on microsomal protein concentration. Incubations were carried out for 5 min under the conditions described in the text.

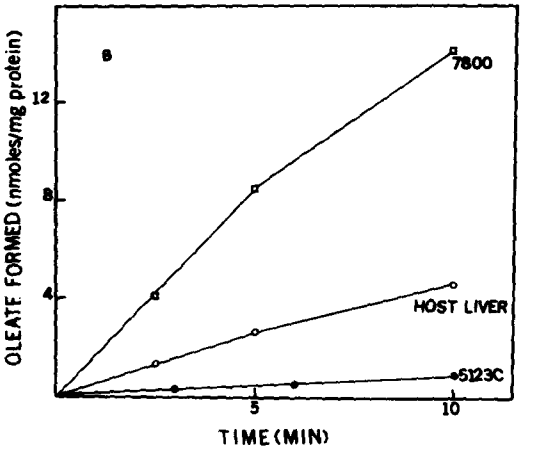

FIG. 1B. Dependency of the desaturase activity of the hepatomas and host liver microsomes on time of incubation. All animals were maintained on a stock ration.

104,000 x g supernatant was determined according to the procedure of Hsu, et al., (6) using [2-^{14}C]malonyl-CoA as the radioactive substrate. The enzyme activity is expressed as nmoles of malonyl-CoA incorporated into long chain fatty acids/min/mg protein.

Protein determinations were made according to the procedure of Lowry, et al., (7) using bovine plasma albumin as standard.

[1-^{14}C]Stearoyl-CoA and [2-^{14}C]malonyl-CoA were purchased from New England Nuclear, Boston, Mass. Unlabeled stearoyl-CoA, malonyl-CoA, acetyl-CoA, nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), and NADH were purchased from P-L Biochemicals, Milwaukee, Wisc.

RESULTS AND DISCUSSION

The stearoyl-CoA desaturase assay employed in the present study is similar to the system reported by Oshino and Sato (8). A substrate concentration of 50 μM was found to give maximum activity in the host livers and the heptomas. NADH was used as the electron donor, since it gave ca. 15% higher desaturase activities than NADPH in both host liver and hepatomas.

Figure 1A and 1B shows the dependency of the desaturase activity on the microsomal protein concentration and on the duration of incubation. Since the host livers of the rats bearing hepatomas had the same desaturase acitivity, only one value for liver is shown. The reaction

TABLE I

Stearoyl-CoA Desaturase Activities of Microsomes and Fatty Acid Synthetase Activities of High Speed Supernatant of Hepatomas and Host Livers[a]

		nmoles/min/mg protein	
		Desaturase	Synthetase
Tissue	Diet	oleate formed	malonyl-CoA incorporated
5123C Host liver	Stock ration	0.66 ± 0.08	3.94 ± 0.57
5123C Hepatoma	Stock ration	0.20 ± 0.04	0.98 ± 0.17
5123C Host liver	Fat-free	1.92 ± 0.30	
5123C Hepatoma	Fat-free	0.19 ± 0.04	
7800 Host liver	Stock ration	0.70 ± 0.07	4.81 ± 0.48
7800 Hepatoma	Stock ration	1.36 ± 0.11	3.58 ± 0.55
7800 Host liver	Fat-free	2.13 ± 0.25	
7800 Hepatoma	Fat-free	1.39 ± 0.10	

[a]Values are averages of 3-5 animals with the standard error of the mean. All determinations were made with at least three different protein concentrations, and linear portions of the curves were used to calculate the specific activities.

is linear up to ca. 5 min and a protein concentration from 0.5-ca. 1.25 mg in both the hepatomas and the host liver. Under all conditions, hepatomas 7800 had the highest, and hepatoma 5123 had the lowest stearoyl-CoA desaturase activities.

Table I shows a comparison of the stearoyl-CoA desaturase activities of the hepatomas and their respective host livers under a stock ration and a fat-free dietary condition. The desaturase activities of the host liver in rats fed the stock ration and fat-free diet are similar to that reported by Paulsrud, et al. (9). The tumor desaturase activities were unaffected by the type of diet, whereas the host livers increased ca. three times on a fat-free diet. On a stock ration, hepatoma 5123C desaturase value was ca. one-third that of the host liver, but, in hepatoma 7800, it was ca. twice that of the host liver. The lack of dietary influence on the desaturase in hepatomas agrees with earlier observations on other fatty acid synthesizing enzymes (10-12).

To test whether the altered desaturase activity represents an altered overall fatty acid synthesis, the fatty acid synthetase activities of the hepatomas and host livers of rats on the stock ration were determined (Table I). The synthetase activity is ca. the same as reported by Majerus, et al., (10) for Buffalo strain rats. In hepatomas 5123C, the synthetase activity was one-fourth that of the host liver. Thus, the low desaturase activity of the hepatomas 5123C may, for the most part, represent an overall low lipogenic activity of this neoplasm. The synthetase activity of the hepatoma 7800 is ca. the same as in the host liver. Thus, compared to normal liver cells in hepatoma 7800, desaturase and synthetase do not appear to be controlled coordinately. Chicken adipose tissue, which has extremely low activity for fatty acid synthetase, has also low stearoyl-CoA desaturase activity (13).

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Effect of Excessive Fatty Acid Ingestion upon Composition of Neutral Lipids and Phospholipids of Snail *Helix pomatia* L.

R.C.H.M. OUDEJANS and **D.J. VAN DER HORST**, Laboratory of Chemical Animal Physiology, Utrecht, The Netherlands

ABSTRACT

The effect of an excessive intake of oleic acid on the lipids of the Roman snail (*Helix pomatia* L.) was studied. The total lipid content increased by 30% which was fully attributable to a marked elevation in the neutral esters and free fatty acids, as phospholipid and free sterol contents remained unaffected. The fatty acid composition of the phospholipids, characterized by high amounts of stearic, linoleic, homolinoleic, and, particularly, arachidonic acids, appeared to be nearly insensitive to this excessive oleic acid ingestion. By contrast, the effect of oleic acid upon the depot lipids was striking: active intestinal resorption of the acid from the dietary supply was shown by the fourfold level of oleic acid in the free fatty acid fraction, whereas a fivefold level of this acid in the glyceride and sterol ester fraction was proof of a substantial esterification. These data support the view that the composition of the structural lipids is specifically species oriented, whereas both the content and the composition of the depot lipids are highly governed by dietary fat intake.

INTRODUCTION

Many factors may affect the lipid composition of animals. Sexual maturation, stress, season, and fluctuating environmental conditions, such as temperature, can cause an animal to modify its metabolism accordingly (1-8).

Although the influence of the food chain on the lipid composition of marine organisms is well documented (9-15), there is only a limited amount of information available on the response of the lipid composition of poikilothermic terrestrial animals to dietary intake variations.

In recent studies on the lipid metabolism of the pulmonate land snail, *Cepaea nemoralis*, it was shown that the fatty acid composition remained surprisingly constant throughout the year, indicating an apparent lack of seasonal variation (16). Moreover, in a preliminary experiment, no discernable alteration in the fatty acid pattern upon feeding green lettuce could be detected, suggesting the characteristic fatty acid profile of *Cepaea* to be maintained irrespective of dietary lipid ingestion (16).

However, considering the minute differences in the fatty acid compositions of the leaves of green plants (17), this independence may be based essentially upon the close similarity of plant fatty acid spectra.

From these considerations, it is pertinent to study the effect of one particular fatty acid upon the lipid composition of a land snail, and, therefore, the present communication is concerned with the influence of an excessive ingestion of oleic acid on the fatty acid profile of the more accessible Roman snail, *Helix pomatia* L. Oleic acid was selected for this purpose considering the low level of this fatty acid in green plants (1-2%) (17,16) and its prominence in the fatty acid compositions of land snails (10-20%) (18-21), pointing to an important function in their lipid metabolism which is confirmed by the active biosynthesis of oleic acid from injected 1-^{14}C-acetate (22).

Since phospholipids are strictly characteristic of cellular organization, whereas triglycerides and other neutral esters may be regarded chiefly as depot lipids primarily for energy reserve, the fatty acid patterns of these specific lipid classes were compared, rather than the fatty acid compositions of the total lipids. In addition, the reflection of the oleic acid in the free fatty acids was examined.

EXPERIMENTAL PROCEDURES

Materials

Adult snails of the species *H. pomatia* L. (Gastropoda:Pulmonata) were obtained commercially from Robert Stein Zuchtanstalt, Lauingen a/d Donau, West Germany. Upon arrival at the laboratory, the animals were housed at 15 C in terraria containing moist marly soil and preconditioned by feeding green lettuce once nightly for several weeks. They were given constant access to water.

Two groups of snails were studied under similar conditions. One group of 150 snails was fed lettuce leaves coated with pure oleic acid (Merck AG, Darmstadt, West Germany) (ca. 70 mg a leave) for 3 weeks, while a control group, consisting of an equal number of snails, was kept solely on lettuce. In both groups, daily

TABLE I

Variation in Total Lipid and Lipid Class Content of *Helix pomatia* Fed on Lettuce and Lettuce Plus Oleic Acid

Fraction	Control snails	Snails fed 18:1
Number of animals	25	25
Fresh wt (g)	478.5	485.5
Total lipids (g)	3.783	4.930
Purified total lipids (g)	3.792	4.948
(percent of fresh wt)	0.79	1.02
Phospholipids		
PL I[a] (g)	0.244	0.227
PL II[a] (g)	1.546	1.563
Neutral lipids (g)	1.968	3.143
NL I[b] (g)	0.594	1.493
NL II[b] (g)	0.852	0.846
NL III[b] (g)	0.077	0.044
NL IV[b] (g)	0.443	0.761

[a]PL I = phosphatidyl serine and phosphatidyl inositol; PL II = other phospholipids (mostly phosphatidyl choline and phosphatidyl ethanolamine).

[b]NL I = glycerides and sterol esters, NL II = free sterols, NL III = dark green pigment, and NL IV = free fatty acids.

lettuce consumption was ca. one leaf/five snails. Every morning nonconsumed food was removed. From each experimental group, 25 specimens were selected randomly and sacrificed by deep-freezing.

Methods

Isolation and purification of total lipids: From both groups of frozen animals, shells and digestive tracts were removed, whereafter total lipids were extracted with chloroform-methanol (23). Besides, total lipids were extracted from a head of lettuce. Sephadex G-25 (Pharmacia, Uppsala, Sweden) was used to separate total lipids from nonlipid contaminants (24).

Isolation of phospholipids and neutral lipids: The purified total lipids were subjected to column chromatography over silicic acid (SilicAR CC4, 100 mesh, Mallinckrodt Chemical Works, St. Louis, Mo.). Neutral lipids, including free fatty acids, were eluted with chloroform. The phospholipids emerged as two separate fractions, viz. a less polar fraction (PL I), consisting of phosphatidyl inositol and phosphatidyl serine, was eluted with chloroform-methanol 4:1 (v/v) and the more polar phospholipids (PL II) with chloroform-methanol 1:2 (v/v).

Fractionation of neutral lipids: Samples (300 mg) of the neutral lipids were fractionated by column chromatography on 30 g Sephadex LH-20 (Pharmacia) (25). Pyrex glass columns with an inner diameter of 2 cm were used, in which the Sephadex was poured as a slurry in chloroform, containing 0.2% glacial acetic acid. To prevent distortion, both ends of the gel bed were filled with 1.5 cm washed sand.

Four sharply defined fractions were obtained with chloroform containing 0.2% glacial acetic acid as the elution solvent. Triglycerides and sterol esters together with small amounts of pigments, diglycerides, and monoglycerides were recovered in the first 60 ml eluate (NL I). Subsequently, a colorless fraction (NL II) of 40 ml was collected, containing the free sterols in a very pure state, except for a minute trace of triglyceride contamination. The following 25 ml contained only a dark green colored pigment (NL III), while in fraction IV (250 ml) the free fatty acids (with a pigment contamination) were obtained (NL IV).

Thin layer chromatography (TLC): During the fractionation procedures, purities of all neutral lipid and phospholipid fractions were checked by one dimensional TLC on Silica Gel G (Merck) with the solvent systems, according to Freeman and West (26). Lipid classes were visualized under UV light after spraying with a 0.005% aqueous solution of Rhodamine 6G. Identification of phospholipid fractions (PL I and PL II) was achieved using the two dimensional TLC separation of Simon and Rouser (27), as described elsewhere in more detail (28).

Isolation of fatty acids: Except for the free fatty acid fraction (NL IV), from all isolated lipid fractions fatty acids were obtained after saponification in 1.5 N methanolic KOH. The trace amounts of fatty acids resulting from the saponified NL II fraction were combined with the other neutral ester fatty acids (NL I). Fraction NL III yielded no fatty acids at all.

Preparation of fatty acid methyl esters:

TABLE II

Quantities of Fatty Acid Methyl Esters Derived from Phospholipid and Neutral Lipid Fractions of *Helix pomatia*

Fatty acid methyl esters from	Control snails		Snails fed 18:1	
	Wt (g)	Percent of fraction	wt (g)	Percent of fraction
PL I	0.117	48.0	0.113	49.8
PL II	0.797	51.6	0.794	50.8
NL I	0.275	46.3	0.615	41.2
NL IV	0.262	59.1	0.421	55.3

Methylation of all fatty acid fractions was effected through the use of diazomethane as described by Schlenk and Gellerman (29). Fatty acid methyl esters were freed from contaminating pigments by column chromatography over silicic acid (Bio-Sil HA, 325 mesh, Bio-Rad Laboratories, Richmond, Calif.) using hexane-diethylether 9:1 (v/v) as the eluens.

Gas liquid chromatography (GLC): GLC of all isolated total and hydrogenated fatty acid methyl ester fractions was performed on either a Becker model 1452 or a Becker Multigraph 2300 instrument, both equipped with dual flame ionization detection. Glass or aluminium columns (1.80 m x 3.8 mm inside diameter), packed with acid and alkaline washed Chromosorb W (60-70 mesh) and coated with 20% polyethyleneglycol adipate, were employed (30). The column oven temperature was kept at 180 C, and the outlet flow of the carrier gas (nitrogen) was 50 ml/min. Identification of fatty acid methyl esters using authentic reference fatty acid mixtures (Sigma Chemical Co., St. Louis, Mo. and Applied Science Laboratories, State College, Pa.), as well as quantitative evaluation was performed essentially as described by van der Horst (21).

RESULTS

Effect of oleic acid ingestion upon lipid level and lipid class distribution: Table I shows that, although feeding of oleic acid did not result in an obvious effect upon the body fresh wt, the total lipid content of the snails increased by 30%. Evidently, this marked enhancement is fully attributable to the elevation of neutral lipids, since the phospholipid content remained remarkably constant.

Sephadex fractionation of the neutral lipids proved this elevation to be restricted to the neutral ester (NL I) and the free fatty acid (NL IV) levels, whereas the free sterol (NL II) content was not affected.

Effect of oleic acid ingestion upon fatty acid compositions of the different lipid classes: Quantities of pure fatty acid methyl esters isolated from the various lipid fractions are given in Table II.

Whereas under normal dietary conditions the major content (63%) of the total fatty acids is derived from phospholipid structures, in the lipids of the snails fed oleic acid, fatty acids from the depot lipid fractions are prevailing. The occurrence of substantial proportions of unbound fatty acids in the lipids of *H. pomatia* (ca. 7% of the total lipids) is not to be looked upon as artifacts caused by hydrolysis of glycerides, since the content, as well as the rather specific composition (Figs. 1-5), is fully reproducible. GLC of the fatty acid methyl esters presented very complicated spectra. In all fractions, ca. 100 different fatty acids were identified, ranging up to 29 carbon atoms. Since within the contemplated scope of our investigation, the presentation of an integral comparison of the percentages of all individual fatty acids seems hardly significant, it is, therefore, decided to compare a selected number of characteristic fatty acids. In Figures 1-5, diagrams of some 15 key fatty acids are given for neutral esters, free fatty acids, and both phospholipid fractions, while a comparable fatty acid composition is given for lettuce.

Although in lettuce nearly 80% of the otherwise low content of total fatty acids is of the C_{18}-series, the presence of oleic acid is restricted to only 1.3%. In the snail lipids, particularly in the neutral esters and the free fatty acids, oleic acid is a more prominent component ranging from 3.8-11.5%, thus suggesting some important function in these lipids.

The phospholipid fractions of *H. pomatia*, which are characterized by high amounts of stearic (18:0), linoleic (18:2ω6), homolinoleic (20:2ω6), and particularly arachidonic (20:4ω6) acid, appear to be nearly insensitive to the excessive oleic acid ingestion. Although close scrutiny of both fractions suggests a tendency for slight variations in a number of fatty acid levels, marked alterations are virtually absent. By contrast, the effect of the ingested oleic acid upon the depot lipids is striking: the fourfold level of oleic acid in the

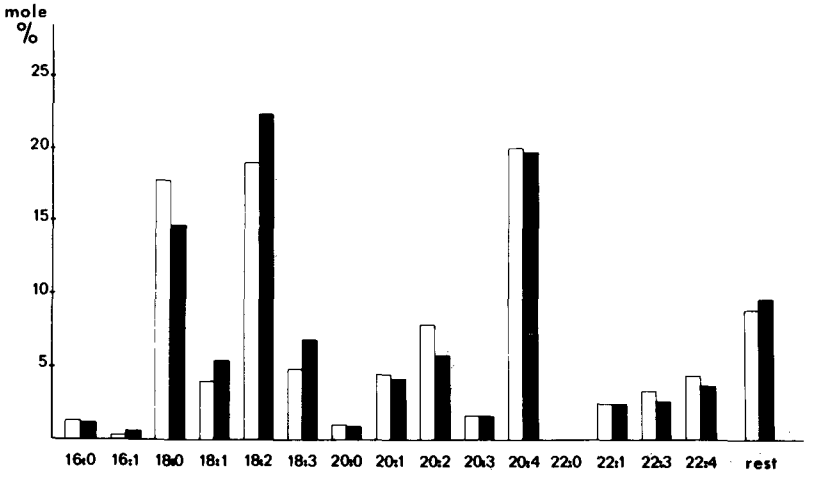

FIG. 1. Effect of oleic acid ingestion upon the fatty acid composition of phosphatidyl inositol and phosphatidyl serine (fraction PL I) of *Helix pomatia*. □ = Control snails and ■ = snails fed 18:1.

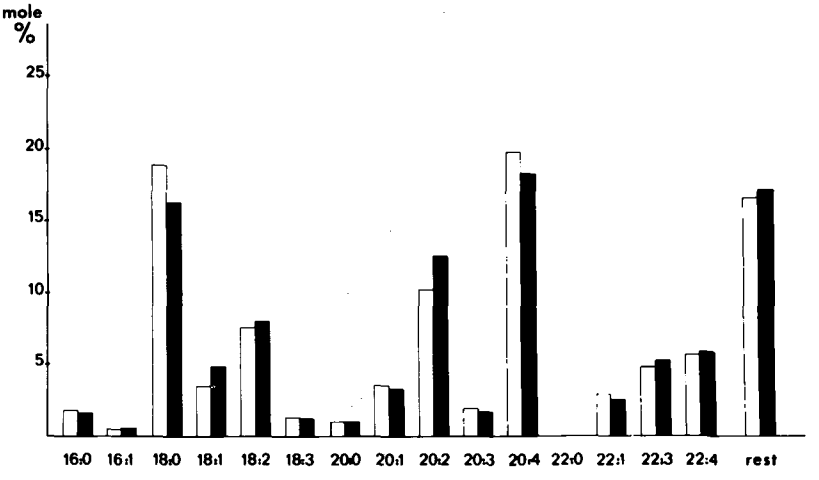

FIG. 2. Effect of oleic acid ingestion upon the fatty acid composition of phosphatidyl choline and phosphatidyl ethanolamine (fraction PL II) of *Helix pomatia*. □ = Control snails and ■ = snails fed 18:1.

free fatty acid fraction is already indicative of an active intestinal resorption of this acid from the dietary supply, whereas the fivefold elevation of oleic acid in the glyceride and sterol ester fraction proves the ingested fatty acid to be substantially combined as neutral esters.

At the same time, importance evidently should be attached to the high figures for linoleic acid in this neutral ester fraction upon feeding oleic acid, suggesting an associate relationship between oleic and linoleic acid which has been demonstrated for a closely allied species (22). This may be equally true for the high level of arachidonic acid in the free fatty acid fractions of animals fed oleic acid. Shorter chain acids, such as palmitoleic (16:1) acid, derived from the oleic acid supply by partial degradation, accumulate in both depot lipid fractions, but chain extension of the latter acid to 20:1 or 22:1 is apparently only occurring to a very limited extent.

DISCUSSION

The food consumption of the 25 snails receiving the oleic acid supplemented diet during a 3 week period resulted in a calculated oleic acid intake of 7 g. In this group, the total lipid increase compared to the control animals amounted to 1.2 g, which was associated exclusively with a rise in the content of depot lipids like glycerides, sterol esters, and free fatty acids. However, despite this nutritive stress, the stability of the content of structural

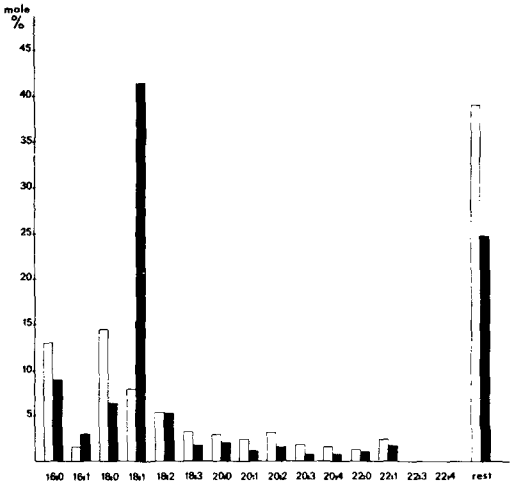

FIG. 3. Effect of oleic acid ingestion upon the fatty acid composition of the glycerides and sterol esters (fraction NL I) of *Helix pomatia*. □ = control snails and ■ = snails fed 18:1.

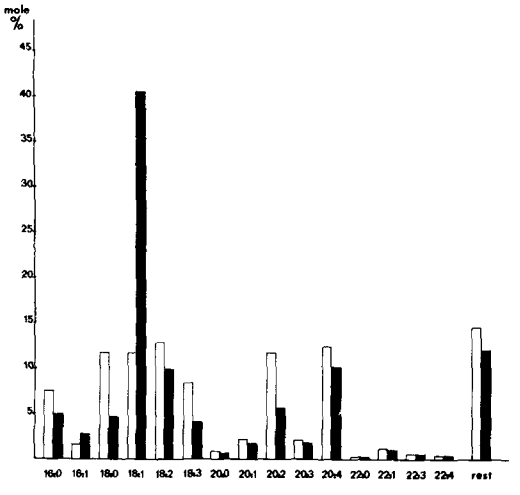

FIG. 4. Effect of oleic acid ingestion upon the free fatty acid composition (fraction NL IV) of *Helix pomatia*. □ = Control snails and ■ = snails fed 18:1.

lipids (phospholipids and free sterols) is striking (Table I).

A curious observation deriving further study is that the supply of oleic acid did not reduce the appetite of the snails, and, though the animals were clearly fed above the maintenance energy requirements, there were no signs of some adaptation of their food consumption.

While the excessive oleic acid ingestion produced only a slight change in the fatty acid patterns of the structural lipids, thus leaving their structural and functional integrity unaffected, the compositions of the free, glyceride, and sterol ester fatty acids were altered drastically (Figs. 1-5). From these results, it may be stated, that the composition of the structural lipids is specifically species oriented (arising from genetic and metabolic differences), whereas both the content and the composition of the depot lipids appear to be substantially governed by the dietary fat intake. These observations agree with current theories (31-33). However, the response of the depot lipids to the various dietary fatty acids is apparently not proportional, since, for instance, linolenic (18:3ω3) acid, the principal fatty acid of lettuce and several other green plants (17), is only a relatively minor fatty acid in the lipids of *H. pomatia* and other pulmonates (19-21,34). Besides, active fatty acid modification and alteration are superimposed on the basic depot fat characteristics. Considering the very low level of lipids in the common diet of this herbivorous snail, in an approach of more physiological conditions the contribution of the latter processing will be more prominent and may, in fact, account for the observed independence of the fatty acid composition of a pulmonate species from that of its plant diet (16).

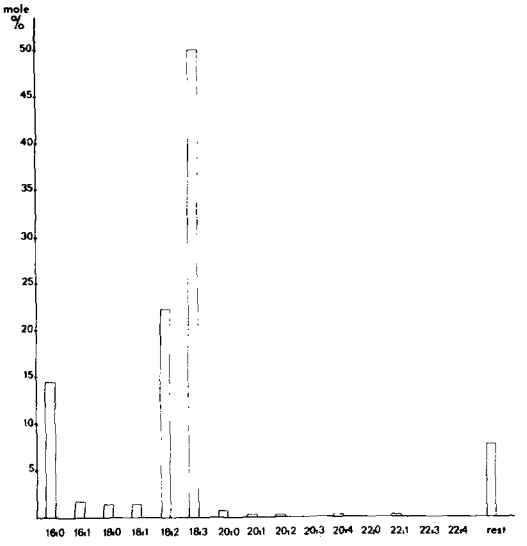

FIG. 5. Fatty acid composition of the total lipids from green lettuce.

Nevertheless, within the perspective of environment induced effects upon the lipid composition, it should be noted that these pulmonates may differ from other poikilothermic animals in accumulating the bulk of their energy reserves as polysaccharide stores, rather than depot lipids (34,35).

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Search for Barley (*Hordeum vulgare* L.) with Higher Lipid Content[1]

JOHN G. PARSONS, Dairy Science Department, and **PHIL B. PRICE,** Plant Science Department[2], South Dakota State University, Brookings, South Dakota 57006

ABSTRACT

The search for a barley with a higher lipid content was concentrated on the USDA Barley World Collection. Seeds of 14,000 entries were examined visually for an embryo size: total seed size ratio greater than the cultivated barley variety Prilar, and 60 entries were selected. Seeds of the 60 entries were assayed for lipid content by NMR spectroscopy, and the 7 entries with the highest oil content, along with Prilar, were prepared for further analysis. Barley lipids were solvent extracted, classified by silicic acid column chromatography, and separated by thin layer chromatography, and the fatty acid composition was determined by gas liquid chromatography. Total lipid contents of the 8 barleys ranged from 3.4% for Prilar to 4.6% for CI 12116. The seven selections had lipid contents which ranged from 9-35% higher than Prilar. Only slight qualitative differences were noted among the lipid classes of the eight barleys analyzed.

INTRODUCTION

The caloric content of barley is lower than maize and sorghum. This deficiency frequently is referred to by animal nutritionists (1), and increased utilization of barley as a feed grain undoubtedly would occur if the energy content was raised. Fats have ca. 2.25 times the caloric energy/unit wt of that contained by carbohydrates and proteins. The most efficient method of increasing the caloric content of barley with the least disturbance in proportions among the biochemical constituents would be to increase the barley lipid content.

A search for a barley with a higher lipid content which would improve the energy level was initiated several years ago by Price (2). The search was necessitated by a lack of sufficient genetic variability in oil content among cultivated barley varieties. The Barley World Collection maintained by the USDA, Beltsville, Md., is a valuable gene pool from which many useful genetic characters can be selected. It was on this collection that the search was concentrated.

Over 90% of the oil in a maize caryopsis is contained in the embryo. Increased oil content in maize lines is achieved by selecting for an increased proportion of embryo wt to total seed wt (3,4).

MacLeod and White (5) previously reported ca. 30% of the total seed lipid was contained in the barley embryo. However, their extraction procedure releases only the ether-soluble materials and does not account for the structural lipids, the phospholipids. Also, these results are based upon only one barley variety, Proctor. A decision was made to use the same procedure as maize breeders in selecting for higher lipid content barleys.

Preliminary screening of six row cultivated barley varieties by NMR spectroscopy indicated seed of the South Dakota variety Prilar contained the highest lipid content among varieties grown in the Upper Midwest. Selection of Barley World Collection entries was based upon an apparent embryo: total seed ratio higher than that found in Prilar seed. Seed of ca. 14,000 entries in the USDA Barley World Collection were examined visually under a 3 X magnifying lamp. Sixty entries in the Collection were deemed to meet that requirement. The 60 were analyzed by NMR, and 7 of these were found to be sufficiently superior to Prilar to warrant further analysis.

Research on these entries was initiated to permit selection of a genome suitable for hybridization with Prilar as a means of achieving quantitative improvement in barley lipids. The lipids of these seven entries, Cereal Investigation (CI) nos. 14233, 5681, 12116, 14234, 14285, 15362, and 14718, along with Prilar (CI 15241) are reported in this study.

MATERIALS AND METHODS

Whole grain samples of the 8 barleys were gound in a Udy[3] cyclone mill to pass a 0.024 in. screen. Total lipids were extracted and purified, as described previously (6), and separated into classes by silicic acid column chromatography (7,8). The lipids in each class were separated by thin layer chromatography (TLC) according to the techniques of Stahl (9), as described by Price and Parsons (6). Typical separations are

[1] South Dakota Experiment Station Paper 1282.

[2] ARS, USDA.

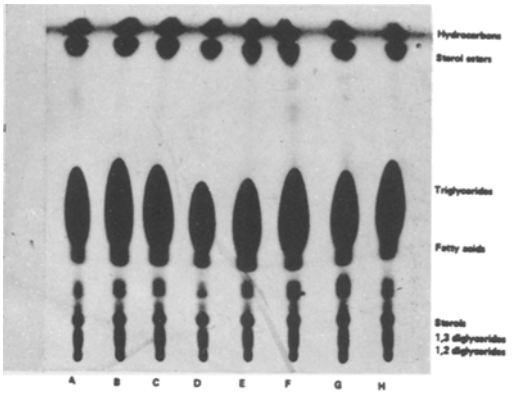

FIG. 1. Thin layer chromatographic separation of neutral lipids from Prilar and seven other World Collection entries: A-Prilar, B-CI 114285, C-CI 14718, D-CI 14234, E-CI 14362, F-CI 15681, G-CI 14233 and H-CI 12116. Absorbent: Silica gel G. Solvent: petroleum ether-diethyl ether-acetic acid (90:10:1). Visualization: charring by heating with sulfuric acid-potassium dichromate (10).

shown (Figs. 1, 2, and 3). The lipids separated by TLC were identified by comparing R_f values with authentic compounds and with published R_f values (11,12), as described previously (6). In addition, the aqueous 20% perchloric acid spray of Lepage (13) gave characteristic color reactions with glycolipids. The α-naphthol-sulfuric acid reagent (14) gave a blue color due to the sugar moiety of the glycolipids. The gas liquid chromatographic (GLC) analyses, the column, operating conditions, and the preparation of methyl esters were described by Price and Parsons (6).

RESULTS

The method of selection for higher lipid content in barley (visual examination of seeds and assay by NMR) provided seven entries for use in this study. The NMR values for lipid content were lower but in the same numerical order as those obtained later by organic solvent extraction. Also, the NMR values were similar to the percent lipid in the neutral lipid class (Table I).

Total lipid content of the 8 barleys used in this study ranged from 3.4% for Prilar to 4.6% for CI 12116 (Table I). The total lipid values for each of the 7 selections exceeded Prilar by 9-35%. Neutral lipids, glycolipids, and phospholipids for the 7 selected entries averaged 70.7, 10.2, and 19.1%, respectively. The figures are similar to those for Prilar: 70.4, 12.5, and 17.1%. There is considerable variation in per-

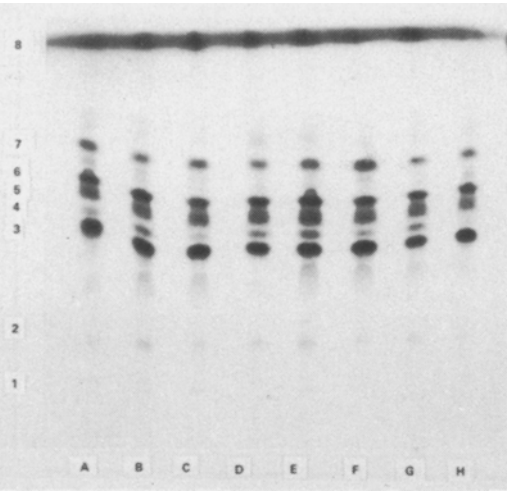

FIG. 2. Thin layer chromatographic separation of glycolipids from Prilar and seven other World Collection entries. Absorbent: Silica Gel H. Solvent: chloroform-methanol-water (75:25:4). Visualization: charring by heating with sulfuric acid-potassium dicromate (10). A-Prilar, B-CI 14284, C-CI 14718, D-CI 14234, E-CI 14362, F-CI 5681, G-CI 14233, and H-CI 12116. The glycolipid spots were identified as follows: 1-origin, 2-sulfatides, 3-digalactosyl diglyceride, 4-unknown, 5 and 6-cerebrosides (6-sterol glycoside), 7-monogalactosyl diglyceride, and 8-solvent front.

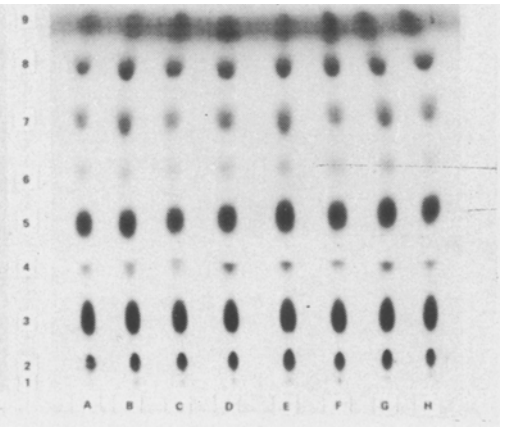

FIG. 3. Thin layer chromatographic separations of phospholipids from Prilar and seven other World Collection entries. Adsorbent: Silica Gel H. Solvent: chloroform-methanol-water-2% aqueous ammonia (65:35:4:0.2). Visualization: charring by heating with sulfuric acid-potassium dichromate (10). A-Prilar, B-CI 14285, C-CI 14718, D-CI 14234, E-CI 14362, F-CI 5681, G-CI 14233, and H-CI 12116. The spots were identified as follows: 1-origin, 2-lysophosphatidyl choline, 3-phosphatidyl choline and phosphatidyl serine, 4-phosphatidyl inositol, 5-phosphatidyl ethanolamine, 6-phosphatidyl glycerol, 7-unknown, 8-diphosphatidyl glycerol, and 9-solvent front.

TABLE I

Total Lipid, Composition, and Lipid in Each Class for Eight Barley World Collection Entries

Barley entry	Percent total lipid (dry wt basis)	Percent of Prilar	Percent composition			Percent lipid by class		
			Neutral lipid	Glyco-lipid	Phospho-lipid	Neutral lipid	Glyco-lipid	Phospho-lipid
Prilar (CI 15241)	3.4	--	70.4	12.5	17.1	2.4	0.4	0.6
CI 14285	3.7	109	69.7	11.7	18.6	2.6	0.4	0.7
CI 14718	3.7	109	73.6	10.6	15.8	2.7	0.4	0.6
CI 14234	3.8	112	69.4	11.0	19.6	2.6	0.4	0.8
CI 14362	3.8	112	65.0	10.2	24.8	2.5	0.4	1.0
CI 05681	4.0	118	74.2	11.8	14.0	3.0	0.5	0.6
CI 14233	4.1	121	64.7	9.4	25.9	2.7	0.4	1.1
CI 12116	4.6	135	78.1	6.9	15.0	3.6	0.3	0.7
Means[a]			70.7	10.2	19.1			

[a]Means are for seven World Collection selections, excluding Prilar, to make comparisons possible.

TABLE II

Fatty Acid Composition of Neutral Lipids of Eight Barley World Collection Entries[a]

Barley entry	Fatty acid					
	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3
Prilar (CI 15241)	0.4	26.0	1.2	18.4	49.9	4.1
CI 14285	0.4	23.4	0.8	18.0	51.6	5.8
CI 14718	0.5	27.6	1.0	17.0	49.4	4.5
CI 14234	0.5	29.3	0.8	15.4	49.6	4.4
CI 14362	0.4	27.6	1.3	14.8	51.0	4.9
CI 05681	0.3	23.8	0.8	20.0	50.5	4.6
CI 14233	0.4	24.5	0.9	17.0	52.0	5.2
CI 12116	0.3	28.8	1.0	18.7	48.2	3.0

[a]Expressed as mole percent and calculated from peak areas of the gas chromatograms. Fatty acids are expressed as number of carbons: number of double bonds.

cent composition of the three lipid classes, and the extremes are expressed by the two entries, CI 14233 and CI 12116, which are highest in lipid content. CI 14233 has the lowest proportion of neutral lipid and the highest of phospholipid. CI 12116 is highest in neutral lipid, lowest in glycolipid, and next to the lowest in phospholipid.

The neutral lipids from the 8 barleys are shown on a typical TLC (Fig. 1). Prilar (A) is qualitatively identical with the seven selections in number and position of the neutral lipid fractions. Triglycerides are the preponderant fraction, comprising more than 50% of the total lipid extract. The spots which migrate ahead of the sterol band, although not identified, were present in all the barleys. The sterol and sterol ester bands were detected by the characteristic colors (red-violet) which develop after the plate is sprayed with 50% sulfuric acid and heated in the oven.

The barley glycolipid fractions (Fig. 2) showed six distinct sugar spots (blue color) with the α-naphthol-sulfuric acid spray reagent (14). The 20% perchloric acid spray (13) revealed the glycolipids as brown spots. Band number 6 was identified tentatively as sterol glucoside from its atypical reddish color. This band and band number 5 also cochromatographed with the cerebroside standard (2 bands) obtained from Analabs, North Haven, Conn.

The phospholipids from barley were chromatographed on Silica Gel H (Fig. 3). The phospholipid fractions of Prilar (A) and the seven selections, give a highly similar chromatographic pattern. The ninhydrin spray reagent (0.2% in ethanol) revealed the presence of phosphatidyl serine completely coalesced with phosphatidyl choline. The specific phospholipid spray of Dittmer and Lester (15) revealed that all the lipids contained phosphorus, including the unknown fraction, band number 7. Individual phospholipids were separated and identi-

TABLE III

Fatty Acid Composition of Glycolipids of Eight Barley World Collection Entries[a]

Barley entry	Fatty acid							
	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Prilar (CI 1524)	1.2	0.9	21.3	0.6	1.4	7.9	59.8	6.9
CI 14285	0.8	0.6	20.8	0.5	0.9	5.0	65.8	5.6
CI 14718	0.6	0.6	20.4	0.5	1.5	5.9	65.8	4.7
CI 14234	1.1	1.4	24.3	1.1	1.9	6.5	58.5	5.2
CI 14362	1.5	0.9	23.9	0.9	1.6	5.4	59.8	6.0
CI 04581	1.1	0.8	20.8	0.6	1.3	4.6	65.9	4.9
CI 14233	1.4	1.1	24.4	0.6	1.4	5.3	59.4	6.4
CI 12116	0.5	0.5	19.8	0.5	1.1	7.5	63.9	6.2

[a]Expressed as mole percent and calculated from peak areas of the gas chromatograms. Fatty acids are expressed as number of carbons: number of double bonds.

TABLE IV

Fatty Acid Composition of Phospholipids of Eight Barley World Collection Entries[a]

Barley entry	Fatty acid					
	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3
Prilar (CI 15241)	1.4	36.9	0.9	14.2	44.9	1.7
CI 14285	0.8	31.0	0.8	14.0	51.0	2.4
CI 14718	1.3	35.2	1.1	13.2	47.5	1.7
CI 14234	1.4	35.7	0.7	10.6	49.5	2.1
CI 14362	0.8	33.4	1.5	11.2	50.2	2.9
CI 05681	1.3	36.7	0.9	11.8	47.3	2.0
CI 14233	0.9	31.4	0.7	11.2	51.9	3.9
CI 12116	1.0	34.2	0.6	15.8	46.4	2.0

[a]Expressed as mole percent and calculated from peak areas of the gas chromatograms. Fatty acids are expressed as number of carbons: number of double bonds.

fied by the two dimensional TLC procedure of Parsons and Patton (16).

The fatty acid composition of the neutral lipids is presented in Table II. The neutral lipids of Prilar are quite similar to those of the seven selections. The fatty acid compositions of the glycolipid and phospholipid classes (Tables III and IV) reveal no major differences among the eight barleys. The phospholipids have the lowest percentage of linolenic acid (C18:3) and the highest percentage of palmitic acid (C16:0) (Table IV). The glycolipids (Table III) appear to be the most heterogeneous. They contain measurable amounts of lauric (C12:0) and palmitoleic (C16:1). These fatty acids, lauric and palmitoleic, were detected previously (6) as trace components of the glycolipids but were not mentioned.

DISCUSSION

The search for a barley with a higher lipid content resulted in the discovery of an entry in the Barley World Collection which contains almost 35% more lipid than the cultivated variety Prilar. The lipid content of CI 12116 is sufficiently higher than commercial varieties as to make it useful to geneticists and plant breeders. As revealed by TLC (Figs. 1, 2, 3), the same fractions are present in the three lipid classes of all eight barleys. The variation which exists is in the amount of lipid present, percent composition in each lipid class, and the percent of each fatty acid. Additional information on the energy and nutritional needs of livestock may indicate that specific lipid components not found in *Hordeum vulgare* L. are needed to promote maximum growth and development. It will be necessary then to conduct a search among other cultivated and wild barley species for qualitative differences in lipid fractions. For the present, an increase in the caloric energy of cultivated barley will reduce one of its more obvious deficiencies.

Reciprocal crosses between Prilar and CI 12116 have been accomplished so that selec-

tions with a higher lipid content can be developed. A study of the inheritance of higher lipid content in barley also is underway.

ACKNOWLEDGMENTS

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Fatty Acid Composition of Liver Lipids during Development of Rat

A.J. SINCLAIR, Nuffield Institute of Comparative Medicine,
The Zoological Society of London, Regent's Park, London, N.W.1, England

ABSTRACT

The fatty acid composition of triglycerides and phospholipids from rat liver was determined throughout the period of growth in the rat. Major changes in the triglyceride fatty acid composition were observed during the period studied. The triglycerides from fetal and newborn rats contained only a small percentage of polyunsaturated acids compared with suckling and weanling rats. During the suckling phase the liver triglycerides were rich in long chain polyunsaturated acids such as 20:4, 20:5, 22:5, and 22:6; once the pups were weaned, the percentage of these acids in the liver triglycerides fell. In these experiments, 18:2 and 18:3 were the only polyunsaturated acids in the maternal diet. However, the stomach contents of the suckling pups contained 18:2 and 18:3, as well as the long chain polyunsaturated acids. Radioactive 18:3 and 22:6 were fed to suckling pups, and the results suggested that the LCP in the rat liver triglycerides during the suckling period were derived from the long chain polyunsaturated acids in the dam's milk, rather than by synthesis from either 18:2 or 18:3 within the pups.

INTRODUCTION

It is now well established that linoleic and linolenic acids cannot be synthesized by mammals. When these acids are included in the diet they can be converted by the animal to longer chain, more unsaturated fatty acids.

Normally, when animals are fed diets in which the only polyunsaturated fatty acids are linoleic and linolenic acids, the long chain derivatives of these acids, e.g. 20:4, 20:5, 22:5 and 22:6, are found only in the phospholipid (PL) fraction of tissues (1). It is known, however, that the feeding of long chain polyunsaturated acids (LCP) such as those found in marine oils, is associated with an increase in the percentage of these acids in tissue triglycerides (TG) (2,3).

Recent studies from this laboratory (4,5) have demonstrated that in suckling rats, aged 1 and 15 days, ca. 25% of the liver triglyceride fatty acids consist of LCP, such as 20:4, 20:5, 22:4, 22:5, and 22:6. In adult rats, these fatty acids account for only ca. 8% of the liver TG fatty acids (5). These studies also revealed that the stomach contents of suckling rats contained linoleic and linolenic acids together with their LCP derivatives, and it was suggested that rat milk was the source of the LCP fatty acids found in the liver TG of the pups (5).

The present report describes in more detail the fatty acid composition of liver TG and PL in suckling rats together with a study of the metabolism of radioactive fatty acids by these animals.

METHODS AND MATERIALS

Animals and Diets

Female rats of the Wistar strain were maintained on a semisynthetic diet (5) which contained 14.4% of its energy as fat; the fat was a mixture of soybean oil and linseed oil (5:1, v/v). This diet will be referred to as the SBOL diet. In these experiments four other diets were also used; three of these diets (diets safflower seed oil [SSO], LCP[1] and LCP[2]) were identical in gross composition to the SBOL diet, except for the type of fat used. In the SSO diet, the fat was supplied by safflower seed oil and in the LCP[1] and LCP[2] diets, 10% and 33%, respectively, of the soybean oil-linseed oil mixture was replaced by Ethive (British Cod Liver oils [Hull & Grimsby] Ltd., Hull, England). Ethive is an ethyl ester concentrate of the polyunsaturated acids of cod liver oil and contains 31% of its fatty acids as 20:5, 2% as 22:5, and 28% as 22:6. The fourth diet was a commercial diet, diet 86 (E. Dixon and Sons, Ltd., Ware, U.K.) which contained 6.7% of its gross energy as fat.

The animals were mated when ca. 4 months old, and, during the first 24 hr after birth, the litter size was reduced to 9 pups. Litters of less than six pups were not used. No more than two pups were used from any one litter; if the size of the litter fell below six during the suckling period the litter was not used. Pups were weaned onto one of the solid diets (above) when 21 days old. Liver TG and PL fatty acid analyses were performed on a minimum of 6 pups selected randomly from at least 3 different litters at 0, 1, 10, 16, 20, 28, and 35 days after birth. The newborn pups (day 0) were all taken within 2 hr of birth.

Fetal tissue was obtained from a rat on SBOL diet killed in midpregnancy. Adult male rats (6 rats) were killed after they had been on the SBOL diet for at least 12 months.

Analytical Methods

The rats were killed by decapitation, and the livers were removed, washed in ice-cold saline, blotted dry, and then weighed. The lipids were extracted into chloroform-methanol (2:1), as described previously (5), and the total amount was estimated gravimetrically. TG and PL were separated by thin layer chromatography (TLC) (5), the lipid fractions being detected with dichlorofluorescein. Methyl esters of TG and PL fatty acids were prepared, as previously described (5), and the total amount of each fraction was determined by gas liquid chromatography (GLC) using n-heptadecanoic acid (Applied Science Labs, State College, Pa.) as an internal standard (6).

Samples of the diets used and the stomach contents of suckling pups were extracted using chloroform-methanol, as described above. The methyl esters of the total fatty acids from the above samples were purified by TLC prior to GLC (6).

GLC was carried out using a Pye series 104 chromatograph (5,6). Two phases were routinely used: (A) 10% EGSS-X on Gas Chrom P (100-120 mesh, Applied Science Labs) and (B) 10% PEGA on Diatomite C-AW (100-120 mesh, W.G. Pye & Co., Cambridge, England), both at 190 C. The use of these two phases enabled the resolution of the following pairs of acids which do not separate when using the EGSS-X phase only, i.e. 18:3 from 20:1, 20:3 from 22:0, 20:4 from 22:1, and 22:4 from 24:1. Peak areas were calculated using an Infotronics CRS 208 digital integrator.

Radioactive Experiments

Radioactive fatty acids were administrated orally to pups in 0.3 ml olive oil, using a 1 ml glass syringe with a blunt 18 gauge needle. The residual oil plus isotope mixture left in the syringe following dosing was washed into a scintillation vial with petroleum ether (bp 40-60 C) and then evaporated to dryness and counted. The fatty acids used were linolenic acid-1-^{14}C (The Radiochemical Center, Amersham, U.K.) and methyl docosahexaenoate-^{14}C (prepared biosynthetically in this laboratory [4]). The radiochemical purity of the isotopes was determined by GLC of the methyl esters; the linolenic acid was >98% pure, and the docosahexaenoic acid was 93% pure, the rest of the carbon 14 being associated with 22:5ω3.

Radioactivity was measured by scintillation counting using a Packard Tri-Carb model 3000 scintillation spectrometer. The scintillation solution consisted of 4 g diphenyloxazole (PPO) and 0.2 g diphenyloxazole-benzene (POPOP)/liter toluene. The counting efficiency, which was normally ca. 75%, was determined by addition of an internal standard of n-hexadecane-1-^{14}C (The Radiochemical Center, Amersham, U.K.). Aliquots of total lipids were counted and the distribution of radioactivity in liver lipid-TLC fractions was determined by elution of the fractions from the silica gel with 1 ml Hyamine hydroxide (1M in methanol) (Koch-Light Labs, Ltd., Colnbrook, U.K.) and 10 ml scintillation fluid. Using this method, the counting efficiency was ca. 50% and a recovery of 95.1 ± 0.2% (mean ± standard error of mean for 19 determinations) was obtained.

The distribution of radioactivity in the methyl esters of TG and PL fatty acids was determined by preparative GLC using 10% EGSS-X on Diatomite C-AW (60-70 mesh, W.G. Pye & Co., Ltd.) at 190 C. With the aid of a stream splitter, fractions were collected into pasteur pipettes which contained a glass wool plug and ca. 1 cm aluminium oxide at the constricted end. The methyl esters, which emerged as a fine vapor from the column, condensed onto the inner surface of the pipette; after collection, they were eluted from this column with either 15 ml petroleum ether (bp 40-60 C): acetone (1:1, v/v) or with 10 ml scintillation fluid. Rechromatography of the collected methyl ethers established that pure fractions had been obtained; also collection and rechromatography of a series of methyl ester standards (from 16:0-22:6) showed that there was no differential loss of the esters during this procedure.

RESULTS

Dietary Fatty Acids

The SBOL diet used contained two polyunsaturated acids, linoleic and linolenic acids; the milk lipids from rats fed this diet were studied by analyzing the stomach contents of the pups, and it was shown that they contained polyunsaturated acids with 20 and 22 carbons, as well as linoleic and linolenic acids (Table I). Individually, these LCP fatty acids in the stomach contents amounted to only a small proportion of the total fatty acids, but, taken as a group, they constituted between 1-2% of the dietary energy available to the pups. The ratio of linolenic to linolenic acid in the maternal diet and in the stomach contents of the pups was 3.3:1. The average ratio of LCPω6 to LCPω3 in the stomach contents was 0.8:1.

Liver Lipid Concentration

The total lipid concentration in the livers was low in fetal and newborn pups, but, during the suckling period, the concentration increased to reach a maximum value in 10 and 16 day old pups (Table II); thereafter, the concentration decreased with the age of the rats. The newborn pups which were killed within 2 hr of birth had empty stomachs, whereas all other pups killed during the first 21 days of life had full stomachs.

The liver TG concentration was extremely low in the newborn pups and within the next 24 hr this value increased 9 times. The maximum TG concentration was observed in the livers of 10 day old pups. In the pups aged 1 day, the TG represented 36% of the total liver lipids, and, with age, this percentage decreased to ca. 23% in the adult rat livers.

The PL concentration was lowest in the livers from newborn pups; in the older animals, this parameter varied between 19-24 mg/g liver. The PL as a percentage of the total lipids decreased from a value of 54% in the newborn pups to around 40% in the livers from all the other rats.

Liver TG and PL Fatty Acids

Marked changes in the fatty acid composition of the liver TG occurred during development. Table III and Figure 1 show that the period of development of the rats could be classified in terms of liver TG fatty acids, into three phases (A) TG low in total polyunsaturation (fetal and newborn rats), (B) TG rich in LCP fatty acids (suckling rats), and (C) TG rich in unsaturation but low in LCP (weaned rats).

The liver TG fatty acids from fetal and newborn rats contained 76% and 60% of 16:0 + 18:1, respectively, and the polyunsaturated acids in these samples (less than 20% of total fatty acids in each case) were predominantly linoleic and linolenic acids.

TABLE I

Fatty Acid Composition[a] of Soybean Oil and Linseed Oil (SBOL) Diet and of the Stomach Contents from Suckling Rats

Fatty acid[b]	Maternal SBOL diet	Stomach contents of pups	
		Aged 10 days[c]	Aged 17 days[d]
10:0	--	8.0	10.3
12:0	0.2	9.6	13.4
14:0	0.5	10.8	14.3
16:0	10.7	21.4	20.6
18:0	3.5	3.2	2.5
20:0	0.1	0.3	0.1
22:0	0.2	0.2	0.1
16:1	0.1	3.0	1.1
18:1	22.4	19.2	13.3
20:1	0.2	1.0	1.2
22:1	0.9	1.6	1.5
24:1	--	0.3	0.5
18:2ω6	46.6	13.9	14.1
20:3ω6	--	0.2	0.1
20:4ω6	--	0.7	0.6
22:4ω6	--	0.3	0.1
18:3ω3	14.2	3.9	4.5
20:5ω3	--	0.4	0.3
22:5ω3	--	0.6	0.3
22:6ω3	--	0.6	0.4
Total ω6	46.6	15.1	14.9
Total ω3	14.2	5.5	5.5

[a]The results are expressed as a percentage of the total fatty acids which were analyzed as methyl esters.

[b]The number before the colon = number of carbon atoms, number after colon = number of double bonds, and number after ω = position of first double bond from CH_3-end.

[c]Mean of results from four pups.

[d]Mean of results from eight pups.

These figures contrast markedly with the liver TG fatty acids for 1 day old rats where polyunsaturated acids accounted for 46% of the total, and, of these, two-thirds were of the long chain variety, i.e. with 20 and 22 carbon atoms. In the older animals, the LCP as a proportion of the total fatty acids fell, the greatest change

TABLE II

Liver Wt and Lipid Concentration in Developing Rats[a]

Age (days)	Liver wt(g)	Lipid concentration (mg/g liver)		
		Total	Triglyceride	Phospholipid
Fetal	0.18 ± 0.01	24.6 ± 1.4	--	--
Newborn	0.27 ± 0.01	21.1 ± 1.3	2.4 ± 0.5	11.4 ± 0.2
1	0.28 ± 0.01	49.5 ± 3.2	17.6 ± 1.1	20.7 ± 0.8
10	0.60 ± 0.06	60.7 ± 3.9	19.8 ± 0.7	22.9 ± 1.0
16	1.02 ± 0.06	59.8 ± 3.1	16.4 ± 1.5	23.5 ± 0.5
20	1.75 ± 0.08	51.7 ± 2.3	12.5 ± 0.9	22.8 ± 0.9
28	4.27 ± 0.22	50.9 ± 6.5	13.6 ± 1.3	22.9 ± 0.6
35	5.64 ± 0.63	49.9 ± 5.1	15.0 ± 1.2	23.0 ± 1.1
Adult	14.9 ± 1.21	44.1 ± 3.5	9.9 ± 0.9	19.4 ± 0.7

[a]The results shown are the mean ± standard errors from six livers at each time point.

TABLE III

Fatty Acid Composition[a] of Liver Triglycerides in Suckling and Weaned Rats[b]

Fatty acid[c]	Fetal	Newborn	1	10	16	20	28	35	Adult
12:0	..[d]	--	0.6 ± 0.1	2.1 ± 0.3	1.4 ± 0.2	1.2 ± 0.2	--	--	--
14:0	2.3	3.3 ± 0.4	1.4 ± 0.1	3.4 ± 0.2	4.3 ± 0.2	3.7 ± 0.6	2.3 ± 0.2	1.6 ± 0.1	1.0 ± 0.1
16:0	25.9	21.6 ± 0.8	24.9 ± 0.7	18.8 ± 1.0	20.5 ± 0.5	20.1 ± 0.7	22.9 ± 0.8	24.3 ± 0.8	22.0 ± 1.1
16:1	5.7	5.4 ± 0.2	2.3 ± 0.2	1.0 ± 0.2	1.1 ± 0.1	2.2 ± 0.2	2.6 ± 0.2	3.5 ± 0.3	4.0 ± 0.1
18:0	7.6	6.8 ± 0.4	2.5 ± 0.06	2.7 ± 0.2	2.6 ± 0.2	2.4 ± 0.2	2.4 ± 0.3	2.3 ± 0.3	1.7 ± 0.3
18:1	40.3	39.5 ± 1.9	19.4 ± 0.9	17.6 ± 1.8	19.4 ± 0.5	26.0 ± 0.9	29.9 ± 1.0	30.7 ± 1.0	27.5 ± 0.8
18:2ω6	8.5	10.1 ± 0.6	14.0 ± 1.0	24.1 ± 1.3	21.6 ± 0.8	23.6 ± 0.5	23.3 ± 1.0	24.0 ± 0.7	28.6 ± 0.4
18:3ω3	0.5	1.3 ± 0.1	1.4 ± 0.1	3.7 ± 0.3	3.0 ± 0.2	4.0 ± 0.5	3.1 ± 0.3	3.7 ± 0.3	3.8 ± 0.4
20:1	--	--	--	--	--	0.8 ± 0.1	0.5 ± 0.07	0.6 ± 0.06	0.9 ± 0.09
20:2	--	--	0.6 ± 0.1	0.6 ± 0.05	0.6 ± 0.1	0.5 ± 0.06	--	--	--
20:3ω6	--	--	1.0 ± 0.07	1.3 ± 0.3	0.9 ± 0.1	0.6 ± 0.1	--	--	--
20:4ω6	0.9	1.1 ± 0.2	9.3 ± 0.6	5.5 ± 0.9	4.5 ± 0.3	2.7 ± 0.1	2.0 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
20:5ω3	1.2	--	3.5 ± 0.2	2.2 ± 0.2	2.4 ± 0.1	1.3 ± 0.2	2.1 ± 0.1	1.5 ± 0.2	1.5 ± 0.2
22:4ω6	0.6	--	2.0 ± 0.2	1.6 ± 0.3	1.2 ± 0.1	0.9 ± 0.09	--	0.5 ± 0.05	0.7 ± 0.1
22:5ω3	--	0.5 ± 0.1	4.0 ± 0.3	6.3 ± 1.0	5.6 ± 0.3	4.1 ± 0.6	2.7 ± 0.3	2.2 ± 0.3	2.3 ± 0.3
22:6ω3	3.9	4.8 ± 1.0	10.3 ± 1.0	7.8 ± 1.0	8.0 ± 0.3	3.9 ± 0.3	3.5 ± 0.3	2.5 ± 0.4	3.0 ± 0.5
ω6/ω3	1.7	1.7	1.4	1.7	1.5	2.1	2.3	2.7	3.3

[a]The results are expressed as a percentage of the total fatty acids which were analyzed as the methyl esters.

[b]The results from six livers at each time point (days) are presented as the mean ± standard errors except in the case of the fetal livers which were pooled prior to analysis.

[c]The number before the colon = number of carbon atoms, number after the colon = number of double bonds, and number after ω = position of first double bond CH_3-end.

[d]Less than 0.5%.

TABLE IV

Fatty Acid Composition[a] of Liver Phospholipids in Suckling and Weaned Rats[b]

Fatty acid[c]	Newborn	1	10	16	20	28	35	Adult
14:0	1.3 ± 0.2	0.5 ± 0.06	0.8 ± 0.05	0.6 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	--[d]	0.4 ± 0.04
16:0	23.7 ± 0.8	28.1 ± 1.4	23.9 ± 0.7	23.9 ± 0.6	20.9 ± 0.5	21.6 ± 0.7	21.8 ± 0.6	20.8 ± 1.0
16:1	3.1 ± 0.2	0.8 ± 0.09	0.6 ± 0.1	0.5 ± 0.04	1.1 ± 0.06	1.0 ± 0.3	0.8 ± 0.1	1.9 ± 0.1
18:0	18.8 ± 0.5	19.2 ± 0.5	21.9 ± 0.3	20.0 ± 0.3	23.9 ± 0.5	22.8 ± 0.7	22.6 ± 0.7	19.4 ± 0.5
18:1	14.9 ± 0.4	7.0 ± 0.5	3.7 ± 0.2	4.4 ± 0.4	4.9 ± 0.3	6.6 ± 0.6	6.4 ± 0.6	8.8 ± 0.6
18:2ω6	10.0 ± 0.5	8.1 ± 0.6	10.8 ± 0.6	9.3 ± 0.6	12.5 ± 0.9	13.2 ± 0.7	12.8 ± 0.9	14.9 ± 0.4
20:3ω6	0.7 ± 0.05	0.5 ± 0.09	0.5 ± 0.09	0.9 ± 0.1	0.6 ± 0.1	0.5 ± 0.05	--	1.0 ± 0.09
20:4ω6	12.9 ± 0.5	21.6 ± 0.7	20.9 ± 0.3	19.1 ± 0.4	18.9 ± 0.9	19.6 ± 0.9	18.3 ± 0.6	19.5 ± 0.5
20:5ω3 + 22:1	0.7 ± 0.1	--	--	--	0.6 ± 0.1	0.6 ± 0.09	0.9 ± 0.08	0.7 ± 0.04
22:4ω6	--	--	--	0.5 ± 0.1	--	--	0.5 ± 0.1	--
22:5ω6 + 24:0	0.5 ± 0.1	--	--	--	--	--	0.8 ± 0.06	0.5 ± 0.05
22:5ω3 + 24:1	0.5 ± 0.2	1.4 ± 0.03	2.3 ± 0.4	3.0 ± 0.1	2.3 ± 0.3	1.8 ± 0.1	2.6 ± 0.1	1.6 ± 0.07
22:6ω3	10.9 ± 0.4	11.4 ± 0.9	13.4 ± 0.6	14.7 ± 0.7	11.0 ± 0.5	10.7 ± 0.8	11.0 ± 0.7	8.5 ± 0.5
ω6/ω3	2.0	2.4	2.0	1.8	2.3	2.6	2.5	3.3

[a]The results are expressed as a percentage of the total fatty acids which were analyzed as methyl esters.

[b]The results from six livers at each time point (days) are presented as mean ± standard errors. Linolenic acid was present in all samples but usually at less than 0.1%.

[c]The number before the colon = number of carbon atoms, number after the colon = number of double bonds, and number after ω = position of first double bond from CH_3-end.

[d]Less than 0.5%.

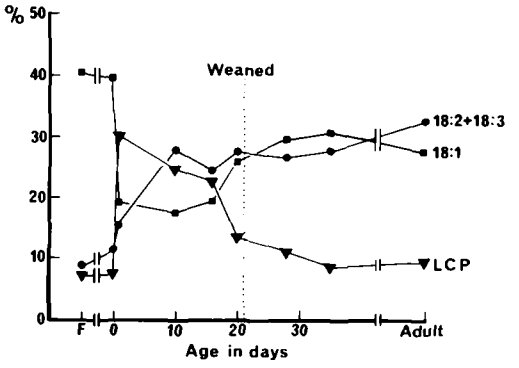

FIG. 1. Changes in the percentage of certain fatty acids in rat liver triglycerides during development. Long chain polyunsaturated acid (LCP) = 20:3 + 20:4 + 20:5 + 22:4 + 22:5 + 22:6; F = fetal; 0 = newborn.

taking place in rats between 16-20 days of age. Throughout the period studied, the percentage of linoleic, plus linolenic acid, in the liver TG increased from 9% of the total fatty acids in fetal rats to 32% in the adults. The total $\omega 6/\omega 3$ ratio in liver TG of rats up to 16 days old was less than 2:1 and in the older rats the ratio was greater than 2:1.

In postnatal life, there were progressive increases in the percentage of 16:1 and 18:1 in liver TG and a tendency for 12:0 and 14:0 to decrease with the age of the animals.

There were less marked changes in the liver PL fatty acids compared with TG fatty acids during postnatal development (Table IV). Leaving aside the values for newborn rats, the major changes that occurred in percentage composition of liver PL fatty acids were increases in the percentage of 18:1 and 18:2 and decreases in the percentage of 16:0 and 22:6. The values for newborn rats differed in two major respects from 1 day old pups: in the newborn pups, the oleate percentage was double and the arachidonate percentage half of the respective values for 1 day old pups. The $\omega 6/\omega 3$ ratio increased from ca. 2:1 in the suckling rats to over 3:1 in the adult rats.

Despite the fact that the liver PL concentrations exceeded that of the TG (Table II), the LCP content of the liver TG in the suckling pups represented a significant percentage of the total LCP found in the liver lipids (TG plus PL). For example, the amount of 22:6 in liver TG and PL was calculated from GLC traces using n-heptadecanoic acid as an internal standard, and it was found that 30%, 11%, and 4% of the 22:6 in the liver was associated with the TG fraction for pups aged 16, 28, and 35 days, respectively.

Tissue TG Fatty Acids

When rats are fed diets rich in $\omega 3$ LCP acids, it has been shown that these fatty acids appear in both liver and adipose TG (2,3). In the present experiments, the diet and the liver TG of the suckling pups contained LCP acids (Tables I and III), but, as shown in Table V, the TG fatty acids from heart, skeletal muscle, and adipose tissue of 16 day old pups contained significantly less LCP compared with liver TG. This was apparent when the results were expressed either as a percentage of the total fatty acids (as in Table V) or as a percentage of the total polyunsaturated acids.

The proportion of LCP in plasma TG of these pups was greater than in the other tissues but less than in the liver TG. The LCP content of adult rat plasma TG was ca. the same as was found in the plasma TG from the suckling rats (Table V).

Effect of Weaning Pups onto a Diet Rich in LCP

The results in Table II demonstrated that the

TABLE V

Polyunsaturated Fatty Acids[a] of Tissue Triglycerides from 16 Day Old Rats

Tissue[b]	Triglyceride polyunsaturated fatty acids			
	18:2ω6	18:3ω3	LCPω6[c]	LCPω3[d]
Liver	20.1	3.3	5.6	16.5
Adipose	9.6	3.1	1.7	0.8
Heart	12.5	3.0	2.7	2.1
Skeletal muscle	13.2	4.0	2.5	2.2
Plasma	19.1	3.8	5.3	5.8
Plasma (adult)	30.3	5.0	3.2	7.7

[a]The results are expressed as a percentage of the total triglyceride fatty acids which were analyzed as methyl esters.

[b]Tissue lipids from five rats (16 days or adult) were pooled prior to thin layer chromatographic and gas liquid chromatographic analyses.

[c]LCPω6 = 20:3 + 20:4 + 22:4.

[d]LCPω3 = 20:5 + 22:5 + 22:6.

TABLE VI

Liver Triglyceride and Phospholipid Fatty Acids of Rats Weaned onto a Diet Rich in 20:5 and 22:6[a]

	18 days	28 days		38 days	
	Milk	SBOL	LCP(1)	SBOL	LCP(2)
No. of pups	4	2	2	2	2
Percent dietary energy as LCPω3[b]	0.66	0	0.87	0	2.93
Percent LCPω3 in liver TG[c]	8.2 ± 0.8[d]	3.6	10.5	1.7	15.0
Percent LCPω3 in liver PL[e]	17.8 ± 0.8	12.4	15.7	13.9	20.5

[a]Twelve pups from two litters, whose mothers were fed the soybean oil and linseed oil (SBOL) diet, were weaned on the eighteenth day after birth. Four pups were killed at this time. Of the remaining pups, two received the SBOL diet for 10 days and two for 20 days. The other four pups were fed the long chain polyunsaturated acids (LCP)(1) diet for 10 days. After this time, two of these pups then were fed the LCP(2) diet for 10 days.

[b]LCPω3 = 20:5 + 22:5 + 22:6.

[c]Percent of liver triglyceride fatty acids as 20:5 + 22:5 + 22:6.

[d]Mean ± standard error of mean.

[e]Percent of liver phospholipid fatty acids as 20:5 + 22:5 + 22:6.

LCP content of liver TG decreased after weaning the pups onto a solid diet. Weaning of the pups onto the SBOL diet changed at least two factors in the pups' diet: first, there was a reduction in the fat calories percentage (ca. 60% in milk [7] and 14.4% in SBOL diet), and second, weaning was associated with a restriction of the dietary polyunsaturated acids to 18:2 and 18:3 only. To test the effect of weaning pups onto a diet containing LCP, groups of rats were weaned onto either the SBOL diet or a diet containing the same amount of fat, but in which some of the 18:2 and 18:3 were replaced by 20:5, 22:5, and 22:6 (Table VI). In the rats weaned onto the LCP diets for 10 and 20 days, the percentages of ω3 LCP in both liver TG and PL were maintained at the levels observed for the suckling rats. In the rats weaned onto the SBOL diet, there was a decrease in the percentage of ω3 LCP in liver TG and PL. At 38 days, 27% of the liver 22:6 was associated with the TG fraction in the pups weaned onto the LCP diet compared with a value of ca. 4% for the pups weaned onto the SBOL diet.

TABLE VII

Incorporation of 18:3-1-^{14}C and 22:6-^{14}C into Liver Lipids of 17 Day Old Rats (Recovery of Carbon 14 in Liver Lipids)[a]

	Percent dose in liver lipids	
Liver lipid fraction	18:3-1-^{14}C	22:6-^{14}C
Total	3.16 ± 0.26[b]	19.8 ± 1.32
Triglycerides	2.53 ± 0.20	5.50 ± 0.26
Phospholipids	0.45 ± 0.03	13.9 ± 0.61

[a]The isotopes were administered orally to 17 day old pups which were killed 22 hr later.

[b]Mean ± standard error of mean for four animals in each group.

Incorporation of Radioactive Fatty Acids into Liver TG and PL

Radioactive linolenic and docosahexaenoic acids were given orally to suckling rats (17 days old) and the pups were killed 22 hr later. It was found that both isotopes were incorporated into liver TG and PL (Table VII), but the distribution of the isotopes between these frac-

TABLE VIII

Incorporation of 18:3-1-^{14}C and 22:6-^{14}C into Liver Lipids of 17 Day Old Rats (Percentage distribution of carbon 14 in liver fatty acids)

Isotope fed	18:3-1-^{14}C		22:6-^{14}C	
Fatty acid	Triglyceride	Phospholipid	Triglyceride	Phospholipid
18:3	80[a]	8.8	2.1	1.4
20:4	3.3	5.9	1.1	0.2
20:5	7.5	11	1.9	1.0
22:5	5.6	26	8.3	2.9
22:6	1.8	36	86	90

[a]Mean percentage distribution of carbon 14 in total fatty acids of triglyceride or phospholipid.

TABLE IX

Polyunsaturated Fatty Acids[a] of Maternal Diets and the Stomach Contents and Liver Triglycerides from Suckling Pups

Diet	18:2ω6	18:3ω3	LCPω6	LCPω3
Diet SBOL[b]	46.6	14.2	--	--
Stomach contents	14.1	4.5	0.9	1.0
Liver triglycerides	21.6	3.0	5.5	16.0
Diet safflower seed oil[c]	66.7	1.4	--	--
Stomach contents	40.3	0.2	2.3	--
Liver triglycerides	35.1	--	11.7	--
Diet 86[d]	43.3	4.4	0.3	4.1
Stomach contents	9.8	0.6	0.7	1.1
Liver triglycerides	13.8	0.5	5.0	14.4

[a]The results are expressed as a percentage of total fatty acids which were analyzed as methyl esters. For abbreviations (LCPω6 and ω3) see Table V.

[b]Results for 16 day old soybean oil and linseed oil (SBOL) rats taken from Tables I and III.

[c]Mean of results from three pups aged 16 days.

[d]Results from five pups aged 10 days; tissues were pooled prior to analysis.

tions was different. Radioactivity from linolenic acid-1-^{14}C was incorporated preferentially into liver TG compared with PL, whereas radioactivity from 22:6-^{14}C was found to be associated predominantly with the PL. The ratio of the recovery of the radioactivity in the liver lipids (22:6-^{14}C: 18:3-^{14}C) was 6.3, 2.2, and 31 for the total lipids, TG, and PL, respectively.

The distribution of radioactivity in the liver TG and PL fatty acids is shown in Table VIII. For linolenic-1-^{14}C, most of the activity in the TG was associated with 18:3, whereas in the PL fraction, most of the radioactivity was associated with 20:4, 20:5, 22:5, and 22:6, i.e. metabolites of 18:3. In the case of 22:6-^{14}C administration, the radioactivity in the liver TG and PL fatty acids still was associated with 22:6.

Effect of Maternal Diet on Liver TG Fatty Acids of Suckling Rats

The effect of maternal dietary fatty acids on the fatty acid composition of liver TG in suckling pups is shown in Table IX. Three diets were used: (A) a diet containing linoleic and linolenic acids (SBOL diet), (B) a diet rich in linoleic acid and poor in linolenic acid (SSO diet), and (C) a diet in which there was linoleic, linolenic, and LCP acids from both ω6 and the ω3 series (diet 86). In the base of the SSO diet, the fatty acids of the stomach contents and liver TG were rich in linoleic acid and the only 20 and 22 carbon acids which were detected were derived from linoleic acid. The commercial diet was the only one of the three used which contained LCP acids (mostly of the ω3 type) and the stomach contents and liver TG fatty acids reflected this. The stomach contents of the pups whose mothers were fed the diet 86 contained the lowest percentage of polyunsaturated acids; this could be due to lower percentage of fat in this diet compared with the SBOL and SSO diets (8).

DISCUSSION

Pronounced changes in the fatty acid composition of the liver have been shown to occur during the postnatal development of the rat. This was especially true for the polyunsaturated acids. During the suckling period between 14-30% of the TG fatty acids were long chain acids, such as 20:4, 20:5, 22:4, 22:5, and 22:6. The presence of large quantities of these acids in tissue TG is unusual, since usually they are found concentrated in tissue PL. The nature of the polyunsaturated acids that accumulated in the liver TG of suckling rats was dependent upon the nature of the fatty acids in the stomach contents; these fatty acids, in turn, reflected the type of fatty acids in the dam's diet (Table IX). The accumulation of LCP in liver TG was associated with the period when the pups had access to a diet which contained LCP, i.e. the milk. Both before suckling (fetal and newborn rats) and after suckling (weaned rats), the LCP content of the liver TG was considerably less than during the suckling period. Although the association between the presence of LCP in the liver TG and their presence in the diet (milk) is to be expected, the fall in LCP content of liver TG from the first day to the twentieth day is curious. A possible explanation for this could be the decrease that takes place in the percentage of LCP in milk fatty acids during the suckling period in the rat (M.A. Crawford, unpublished observations). Also, we have observed that rat pups eat the solid diet (free from LCP) 2-3 days before they are weaned on day 21.

After weaning, most of the LCP derivatives in the liver were associated with the PL fraction. However, when pups were weaned onto a diet containing LCP, it was seen that the level of LCP in the liver TG was maintained at the preweaning level (Table VI).

These results suggest that the LCP acids in the liver TG were of dietary origin; this hypothesis was supported by the fact that, when 22:6-^{14}C was fed to suckling rats (Tables VII and VIII), it was found that the 22:6-^{14}C was incorporated into liver TG. Also, it has been shown previously that when 20:4-^{3}H is fed to suckling pups it is incorporated into liver TG (9).

In rats maintained on diets in which the only polyunsaturated acids are 18:2 and 18:3, it is known that the derivatives of these fatty acids are found in tissue lipids. These derivatives, e.g. 20:4 and 22:6, are predominantly found in the β-position of PL (3). In animals fed diets containing LCP, these fatty acids are found both in the β-position of PL and in the TG (3). As with PL, there is an asymmetry in the distribution of fatty acids into the different positions of the glycerol moiety of the TG: the order of preference of 20:5, 22:5, and 22:6 for the three positions of the glycerol moeity being $\alpha^1 > \beta > \alpha$ (3).

It might be postulated that exogenous and endogenous LCP have different fates in tissue lipids, e.g. exogenous or dietary 22:6 to TG and endogenous (from 18:3 in animal) to PL. However, the results of Tables VII and VIII do not support this conclusion. Where 18:3-1-^{14}C is fed to the pups, the label is found almost exclusively as 18:3 in TG and the endogenously synthesized products (20:5, 22:5, 22:6) are restricted to the PL. When 22:6-^{14}C is fed, the label is found in both liver TG and PL, but with a preference for the PL. That is both endogenous and exogenous 22:6 is found in the PL.

Another explanation for the presence of LCP in TG could be that there is a limit to the amount of LCP which can be taken up by PL. Since LCP are found in the β-position only of the PL, the uptake of these fatty acids will depend upon the turnover of fatty acids in this position and the replacement of fatty acids from the ω6 series with fatty acids from ω3 series or vice versa. Clearly, by feeding 22:6, it is possible to increase the level of 22:6 in liver PL (Table VI), but there is a limit to this uptake. The results presented suggest that when rats are fed ca. 6% of the dietary energy as 18:2 and 18:2, the amount of endogenously synthesize LCP derivatives does not exceed the capacity of the liver PL to incorporate these acids but that this capacity is exceeded when the diet contains ca. 5.4% of its dietary energy as 18:2 and 18:3 and 0.9% of its energy as LCP. Under these conditions the LCP accumulate in the liver TG.

Of the tissues studied in the suckling rats, that with the greatest percentage of LCP in the TG fraction was the liver. In adult rats, the LCP content of the liver TG was considerably lower than in suckling rats (Table III), and yet the proportion of LCP in the plasma TG from suckling and adult rats was ca. the same (Table V). Plasma TG originate from diet and from liver, and the fatty acids of plasma TG are precursors of adipose tissue fatty acids and presumably of other tissue TG fatty acids. The fact that, in the suckling rats, the LCP did not accumulate in the muscle and adipose TG suggests that the LCP were being retained in the liver to a certain extent. In fact, in the experiment reported in Tables VII and VIII, the recovery of the carbon 14 in the liver lipids as a percentage of the carbon 14 in the whole carcass, including liver, was 31.3% for 22:6-^{14}C and 6.3% for 18:3-^{14}C (A.J. Sinclair, unpublished observations). This apparent retention of LCP by the liver also is found if one compares the incorporation of 20:4-^{3}H with 18:2-1-^{14}C in suckling rats (A.J. Sinclair, unpublished observations).

The fatty acid composition of tissue TG and PL in developing rats was reported in 1964 by Dobiasova, et al. (10). However, since the dietary fatty acids differed from those used in this study, a comparison of the two sets of results is difficult. Their diet was rich in linoleic acid and lacked linolenic acid (similar to the SSO diet used, Table IX), but they did not observe an increase in the percentage of 20:4 in liver TG during the suckling period. A possible explanation for this result could be that their diet contained ω3 LCP (as in diet 86, Table IX), and, since they do not report any LCP with retention times greater than 20:4, the LCP of the ω3 series would be excluded from their results.

A more recent paper (11) on the neutral lipids and PL fatty acids from heart tissue of developing rats revealed similar changes to those reported here.

It has been shown that the major accumulation of 20:4 and 22:6 in the rat brain takes place during the suckling period (6,12). The origin of these LCP in the brain is not know, but, clearly, they could be derived from dietary fatty acids, from liver fatty acids, or by synthesis within the brain (4). Although there is a clear association in time with the accumulation of LCP in the brain and in the liver TG of suckling rats, the relative contribution of the pool of LCP in the liver to brain LCP awaits further experimentation.

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Glycosphingolipids of Human Thyroid

JOANNA N. KARLI and **GABRIEL M. LEVIS**, Biochemical Research Laboratory, Department of Clinical Therapeutics, School of Medicine, University of Athens, Alexandra Hospital, Athens 611, Greece

ABSTRACT

Glycosphingolipids were isolated from total lipids of female and male human thyroids by alkaline hydrolysis, silicic acid, diethylaminoethyl-celluose and thin layer chromatography and analyzed by gas liquid chromatography. On the basis of their mobility in two dimensions on thin layer chromatography, IR analysis, and of sugar molar ratio, four neutral glycolipids, a sulfatide, and a hematoside fraction were identified. Glucosyl, plus galactosyl ceramide, and trihexosyl ceramide were the major fractions and accounted for 33% and 28% of total neutral glycolipids, respectively. Dihexosyl ceramide was a mixture of lactosyl and digalactosyl ceramide. The acidic lower phase glycolipids comprised ceramide galactosyl sulfate as the major component of male thyroids. Hematoside was identified tentatively as a minor component of the thyroids of both sexes. Major fatty acids of all neutral glycolipid fractions were 20:0, 22:0, 24:0, and 24:1; 24:0 and 24:1 for sulfatides. Low proportions of α-hydroxy fatty acids were identified. Total neutral glycosphingolipids of male thyroids were comparable in quantities with human liver but lower than kidneys, leucocytes, and platelets. Male thyroids comprised higher quantities of neutral glycosphingolipids (4.04 ± 0.32 μmoles/g total lipid) as compared to females (2.34 ± 0.21 μmoles/g total lipid), and much higher sulfatide than the females. These marked differences may suggest that the biosynthesis of the glycosphingolipids in the thyroid gland is under hormonal control. Similarities in glycosphingolipid composition of human thyroid and kidney are discussed in relation to a possible role played by glycolipids in ion transport, which is a common feature of the two organs.

INTRODUCTION

Neutral glycosphingolipids with one-four sugar units and, in certain cases, sulfatides have been identified as minor constituents of the lipid fraction of most mammalian tissues. These compounds are more concentrated in the plasma membrane of the cell (1-3) and, therefore, it is reasonable to suspect that they may play some role in membrane functions.

In this connection, human thyroid has been examined for the presence and composition of glycosphingolipids, since membrane of the thyroid cell may be the locus of the mechanism responsible for the iodine trapping capacity of the gland. In addition, several investigators have tried to correlate complex lipids and, more specifically, phospholipid metabolism and composition with iodine uptake by the gland in vivo and in vitro (4-9 and J.N. Karli, personal communication). To our knowledge, the thyroid gland has not been examined for glycosphingolipids.

MATERIALS

Thyroids were obtained from postmorten individuals soon after death (not later than 12 hr) and kept frozen at -20 C until extracted. Thyroids were examined for normalcy, and tissues with pathological findings were discarded.

All solvents were analytical grade and distilled before use. Silica Gel H used for separation of glycolipids by thin layer chromatography (TLC) was washed with chloroform: methanol 2:1 v/v), dried, and heated for 6 hr at 90 C. Reference glycosphingolipids were isolated from human (11) and pig leucocytes (12) and erythrocytes. Reference normal and 2-hydroxy fatty acid esters were obtained from rat brain glycolipids (13). A Pye-Unicam model gas liquid chromatograph (GLC) was used for GLC analyses. Hexamethyl-disilazane and trimethylchlorosilane were obtained from Macherey, Nagel and Co., Düren, West Germany.

METHODS

Extraction of lipids took place, according to Folch-Pi, et al. (14). Total lower phase lipids were weighed and subjected to mild alkaline hydrolysis (11). Total glycolipids were isolated from the alkali-stable fraction by column chromatography on silicic acid (BIO-RAD special for lipid chromatography). Fatty acid esters and other nonpolar nonsaponifiable lipids were washed with chloroform and then with ethyl acetate; glycosphingolipids were obtained with acetone: methanol 9:1 v/v (15,16). Neutral and

TABLE I

Total Content and Composition of Lower Phase Glycosphingolipids of Human Thyroid

Glycosphingolipids	Female	Male
	μmoles/g total lipid	
Total neutral glycosphingolipids[a]	2.34 ± 0.21(7)	4.04 ± 0.32(11)
Total acidic glycosphingolipids[b]	Not detected	(A)1.98(3) (B)1.20(5)
	Composition of neutral glycosphingolipids in μmoles/g total lipid[c]	
Glucosyl ceramide	0.65	1.27
Galactosyl ceramide	0.22	0.61
Dihexosyl ceramide	0.21	0.46
Trihexosyl ceramide	0.47	1.06
Aminoglycolipid	0.20	0.36
	Molar ratio gas liquid chromatography: galactose	
Glucosyl ceramide	1.0:0	1.0:0
Galactosyl ceramide	0:1.0	0:1.0
Dihexosyl ceramide	1.0:3.0	1.0:2.9
Trihexosyl ceramide	1.0:1.7	1.0:1.8
Aminoglycolipid	1.0:2.1	1.0:1.8
	Composition of acidic glycosphingolipids in μmoles/g total lipid[d]	
Sulfatide	(A)1.87	(A) ?
	(B)1.06	(B)1.0
Hematoside	(A)0.11	(A)1.0
	(B)0.14	(B)1.1

[a]Total neutral glycolipids from thyroids of individuals aged 10-80 years were analyzed by gas liquid chromatography, as described in the text; μmoles were calculated from the area of the glucose peaks corrected, as described in the text. Data are mean values ± standard error for the number of thyroids given in parentheses.

[b]Total acidic glycolipids from thyroids of 2 groups of male individuals aged (A) 26-45 years and (B) 60-70 years, analyzed by gas liquid chromatography, as described in the text; μmoles are sum of sulfatide galactose and the hematoside glucose. Figures in parentheses are number of thyroids pooled and analyzed. The fraction from females was not analyzed due to insufficient quantities.

[c]Neutral glycosphingolipids from 2 groups of thyroid from individuals aged 20-60 years were pooled and separated into fractions by preparative thin layer chromatography. Each fraction was analyzed for carbohydrate content by gas liquid chromatography. For aminoglycolipid, glucose:galactose ratio was 1.0:0.9.

[d]Acidic glycosphingolipids from two groups of male thyroids were separated, as described in the text, and analyzed by gas liquid chromatography; μmoles for sulfatides were calculated from the galactose peak and for hematoside from the glucose peak in the chromatograms.

acidic glycosphingolipids were separated from each other by chromatography on a column of diethylaminoethyl-cellulose (17). The isolated crude glycosphingolipid fractions were checked for purity by TLC and phosphorus determination, and, if needed, were rechromatographed, as described previously. Total neutral glycolipids were separated into four fractions (monohexosyl ceramide[GL-1], dihexosyl ceramide [GL-2], trihexosyl ceramide [GL-3], and aminoglycolipid) by preparative TLC on plates coated with washed Silica Gel H, which were developed twice with chloroform-methanol-H_2O (65:25:4 v/v/v) (18). Reference standards were run on the same plate for identification. The glycolipids were located and extracted from the plates, as described previously (12). The purity and further identification of the isolated neutral glycosphingolipids were performed by TLC (18), by IR analysis (19), and GLC analysis of the carbohydrate moieties of each fraction, as described below. Sulfatides also were identified by IR analysis (20). Identification of glycosyl and galactosyl ceramide in the GL-1 fraction was performed on Silica Gel H borate plates (12). Carbohydrates were estimated by GLC analysis of the trimethylsilyl derivatives of the O-methylglycosides at 165 C in a glass column (6 ft x 1/4 in.) packed with 3% SE-30 on Diatomite CQ, 60-80 mesh, using mannitol as an internal standard (21,22). To acetylate the galactosamine, the O-methyl

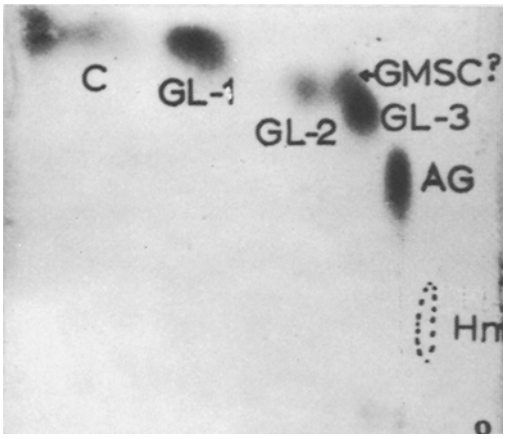

FIG. 1. The separation by two dimensional thin layer chromatography of the lower phase total glycosphingolipids from female thyroid. Plates coated with Silica Gel H. Solvents: (first direction) chloroform-methanol-water (65:24:4, v/v/v) and (second direction) tetrahydrofuran-methylal-methanol-4 N aqueous ammonia (10:5:4:1, v/v/v/v). Spray, 50% H_2SO_4 v/v. GL-1 = monohexosyl ceramide, GL-2 = dihexosyl ceramide, GL-3 = trihexosyl ceramide, AG = aminoglycolipid, Hm = hematoside, GMSC = sulfatide, C = contaminants at solvent front, and O = origin. Lipid sample was ca. 180 μg. Note small quantity of tentatively MGSC in female thyroid.

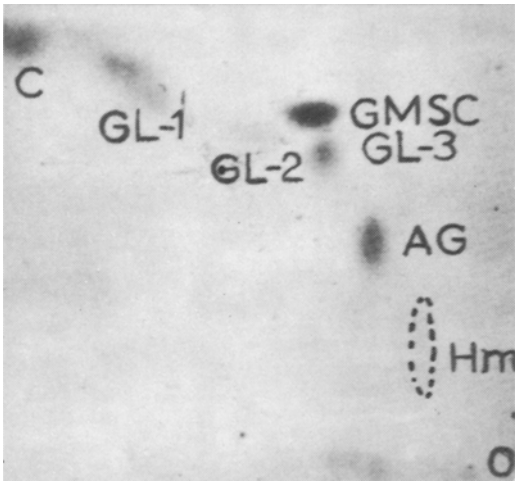

FIG. 2. The total glycosphingolipids of the lower phase from male thyroid. Two dimensional thin layer chromatography on plates coated with Silica Gel H. Solvents: (first direction) chloroform-methanol-water (65:25:4, v/v/v) and (second direction) tetrahydrofuran-methylal-methanol-4 N aqueous ammonia (10:5:4:1, v/v/v/v). Spray, 50% H_2SO_4 v/v. Abbreviations as in Figure 1. Lipid sample was ca. 60 μg.

glycosides, obtained from the animoglycolipid, were treated with acetic anhydride in methanol in the presence of pyridine. Results of total glycolipids were expressed in μmoles calculated from the area of the glucose peaks of the methyl glycosides taking into account the galactosyl and digalactosyl content of the tissue. Normal fatty acids were analyzed as methyl esters by GLC on a 5% ethylene glycol succinate column at 180 C. The methyl esters of 2-hydroxy fatty acids were analyzed together with the normal ones on a 3% SE-30 column at 215 C. The columns were 6 ft x 1/4 in. and the supporting material was Diatomite CQ 60-80 mesh.

Fatty acid esters were recovered from the methanolysis mixture by extraction with redistilled hexane. The efficiency of the methanolysis procedure was tested with known quantities of Cytolipin H (lignoceryl derivative, Miles Laboratories, Elkhart, Ind.), which were treated under the above described conditions. In a triplicate experiment, the recoveries of both carbohydrates and fatty acids, the latter assayed quantitatively by GLC using an internal standard of C_{22}, were in the range of 87-95%.

Identification of the peaks was achieved by comparison with authentic standards of normal fatty acids (Applied Science Laboratories, State College, Pa.) or 2-hydroxy fatty acids isolated from brain glycolipids. When needed, the fatty acid esters also were chromatographed on a 5% EGS column at 170 C (support Diatomite CQ 100-120 mesh) before and after hydrogenation in a stream of hydrogen over platinum oxide.

RESULTS AND DISCUSSION

Total neutral and acidic glycolipids of the lower chloroform phase expressed in μmoles/g total lipids are shown in Table I. The lipid fraction of male thyroids comprises neutral glycosphingolipids in significantly higher amount ($p<0.02$) as compared to females. The four neutral glycosphingolipids usually encountered in nonnervous mammalian tissues were found in the thyroid of both sexes, with the major constituent being GL-1. This glycolipid fraction contained glucosyl ceramide and considerable quantities of galactosyl ceramide. It is evident from the increased galactose content that GL-2 was similarly a mixture of lactosyl and digalactosyl ceramide.

The acidic fractions of the lower chloroform phase from male thyroids comprised two fractions with mobilities on TLC of ceramide galactosyl sulfate (sulfatide) and hematoside (Table I). The sulfatide fraction was characterized by IR analysis and was pure when analyzed by TLC in two dimensions. However, the gas chromatograms obtained from the trimethylsilyl derivatives of the O-methyl glycosides indi-

TABLE II

Percentage Composition of Normal Fatty Acids in Neutral Glycolipids of Human Thyroid[a]

Normal fatty acid	GL-1		GL-2		GL-3		Aminoglycolipids	
	Male	Female	Male	Female	Male	Female	Male	Female
12:0	4.7	---	1.1	4.8	---	---	---	---
14:0	3.4	trace	3.3	---	---	2.9	trace	1.9
15:0	---	---	0.5	---	---	2.5	---	---
16:0	12.2	16.1	24.4	23.7	8.4	9.7	12.5	8.9
16:1	1.9	0.8	2.9	5.5	0.4	1.3	---	1.5
17:0	1.1	---	0.5	---	6.5	---	---	---
17:1	0.6	---	---	---	---	---	---	---
18:0	4.7	9.3	5.2	10.1	3.9	17.2	4.6	3.6
18:1	2.6	3.7	2.5	7.7	---	---	1.5	1.9
19:0	2.0	---	1.8	---	0.2	---	---	0.6
19:1	1.3	---	---	---	---	---	---	Trace
20:0	8.0	15.5	5.3	5.7	4.9	3.3	3.6	4.7
21:0	2.2	2.6	0.8	1.2	0.6	---	0.4	0.9
21:1	2.3	---	0.9	1.2	---	---	---	---
22:0	21.1	25.4	12.4	10.1	17.7	8.4	16.3	19.2
22:1	5.4	7.5	2.2	3.5	1.9	6.6	1.9	0.7
23:0	7.0	---	3.1	3.3	3.4	8.2	4.2	6.3
23:1	1.1	---	---	---	---	---	---	---
24:0	9.5	9.5	14.7	8.2	22.9	9.3	23.6	23.9
24:1	8.0	9.1	18.2	14.2	28.7	30.6	30.6	25.3

[a]Glycosphingolipid fractions were separated by thin layer chromatography and the glycolipid fatty acid methyl esters were analyzed by gas liquid chromatography as described in the text.

cated the presence of glucose. This finding needs further confirmation. Hematoside tentatively was identified by its glucose:galactose ratio and from the positive color reaction it gave for sialic acid. A part of hematoside may be lost in the upper phase (23). The analysis of this fraction is now in progress.

The acidic fraction from female thyroids was very poor. Due to the small quantities available, it was not possible to obtain accurate data on this fraction. The differences in total glycosphinolipids of the lower chloroform phase of one group of female and one group of male thyroids separated by two dimension TLC are depicted in Figures 1 and 2.

The composition of the normal fatty acids (Table II) of the neutral glycolipids is similar in males and females with major constituents the fatty acids with chain length from C_{20}-C_{24}. However, GL-1 and GL-2 of both sexes contain lower proportions of the C_{24} fatty acids as compared to GL-3 and aminoglycolipids. In addition other characteristic differences, such as the absence of oleic acid from the GL-3 and the increased palmitate of the GL-2, were observed between the isolated glycolipids. Hydroxy fatty acids accounted for ca. 10% of the total fatty acid content in the GL-1 fraction and less than that in the other glycosphingolipid fractions. Fatty acids from sulfatides and hematosides also were analyzed. It was found that C_{24} and C_{18} were the major fatty acids of the two acidic glycolipids, respectively.

The data presented in this article demonstrate that the lipids of human thyroid gland comprise neutral glycosphingolipids and sulfatides. On the basis of a mean mol wt of glycosphinolipids of 980, it can be calculated that the neutral fraction of these lipids constitutes 0.22 and 0.39% of total lipids for females and males, respectively. These values are very close to those reported for human liver (16) but lower than those of human kidneys (24) and platelets (25) and much lower than that of human leucocytes (11).

The pattern of glycolipids of human thyroid differs considerably from that of other nonnervous human tissues. The major component of human liver (16), leucocytes (11), platelets (25), serum (15,19), and spleen (26) is the ceramide dihexoside, while that of kidneys (24) and erythrocytes (27) is aminoglycolipid. Contrary to the above, GL-1 and GL-3 are the predominant constituents of human thyroid glycolipids. GL-1 of thyroids was found to consist of a mixture of glucosyl and galactosyl ceramide. Galactose also has been found in the GL-1 fraction of human liver, but to a much lower extent than in the thyroid (16), while in human kidney half of the GL-1 was found to be galactosyl ceramide (24). GL-2 of human thyroids also consists of a mixture of lactosyl and digalactosyl

ceramides. Similar results were reported for human kidneys (24) and not for liver and platelets (25).

It seems, therefore, that human thyroid, though it comprises lower quantities of total glycolipids than kidney, does show similarities with respect to its galactosphingolipid content, which is present in similar quantities in the two tissues and is almost absent from other mammalian tissues. This may indicate that the role played by these lipids is common for the two tissues, the functions of which comprise an active ion transport.

The fatty acid composition of the four glycolipids was not very similar. These differences do not exclude a metabolic relationship between the four glycolipid fractions. It is possible that they may result from a substrate preference of the glycosyl transferases in the pathway from GL-1 to aminoglycolipid. For example, the increased proportion of the C_{24} fatty acids found in the larger glycolipids may derive from a preferential utilization of GL-1 species with C_{24} fatty acids for carbohydrate chain elongation. Such a specificity for GL-1 species with 2-hydroxy fatty acids has been shown to occur in mouse kidney preparations (28). Such enzymes with various specificities must be unequally distributed in various human tissues since a high variation occurs in the fatty acid composition of their glycolipids. For example liver lactosyl ceramide comprises very low proportions of the C_{20}, C_{22}, and C_{24} acids which, however, constitute a large proportion of the normal fatty acids of the kidney and thyroid glycolipids.

In relation to the quantitative difference found in neutral glycosphingolipids between males and females, it is worth mentioning that such changes, but in the opposite direction, were reported for the glycolipids of human liver (16), and that the biosynthesis of the glycolipids in kidneys of the mouse seems also to be affected by sex (28). In this respect, it has been shown that testosterone stimulates the synthesis of glycolipids in the kidneys of female mice (30). It could, therefore, be suggested that the biosynthesis of glycosphingolipids in the thyroid gland is under hormonal control. Furthermore, it has been shown that the phospholipid content of the gland is affected by the sex (4). Since these two lipid classes are structural units of the cell membranes, it is possible that the functions of these membranes are controlled by sex hormones.

In male thyroids, sulfatide represents (on a molar basis) ca. 28% of total lower phase glycolipid, which compares with 14% by wt of human kidney (30), irrespective of sex. This is another aspect in which thyroidal glycosphingolipids resemble those of kidney, and it is worth mentioning that, in the literature, the occurrence of sulfatide has been discussed in relation to the ion transport system of the avian salt gland (31) and of the outer part of the bovine kidney medulla, where the corticosteroid dependent Na^+ transport system is located (32). In the thyroid, this system is involved indirectly in the iodide transport (33). On the other hand, the increased sulfatide content of male thyroid, as compared to galactosyl ceramide, may indicate an active sulfatide biosynthesis needed for the functions of thyroidal plasma membranes.

The finding that sulfatides are almost absent from female thyroids is a peculiar one and needs further confirmation with fresh thyroids from humans and other species. It would be rather improbable that sex difference would result in such a dramatic alteration of the composition of the gland unless sulfatide is substituted by a more polar acidic glycolipid found in the upper phase. Nevertheless, similar profound changes due to sex differences have been reported in the glycosphingolipids composition of the mouse kidneys. Thus, the kidneys of males of several strains examined contained significant amounts of diglycosyl ceramide, but those of females contained, at the most, only traces (34).

A more detailed study is needed in human thyroids of young ages where the physiopathology of the gland shows differences between the two sexes (35).

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Earthworm Lipids: Occurrence of Phytanic, Pristanic and 4,8,12-Trimethyltridecanoic Acids

ABSTRACT

Phytanic, pristanic, and 4,8,12-trimethyltridecanoic acids were found to comprise from 2.7% (winter) to 13.3% (spring) of the fatty acids of the neutral lipids of earthworms collected in different seasons from agricultural pasture land. Only small proportions of these isoprenoid acids were present in the phospholipids and glycolipids. It is postulated that these acids originated by microbial action on phytol and were assimilated in the gut of the earthworms from living and dead microorganisms and other fauna in ingested soil.

INTRODUCTION

The isoprenoid fatty acids, phytanic (3,7,11,15-tetramethylhexadecanoic), pristanic (2,6,10,14-tetramethylpentadecanoic), and 4,8,12-trimethyltridecanoic (4,8,12-TMTD) are known to occur in small proportions in the lipids from many different sources, including those of ruminants, terrestrial and marine mammals, fish, zooplankton, some crude petroleum oils and certain geological deposits (1). Also, rumen bacterial lipids have been found to contain phytanic acid, but not pristanic or 4,8,12-TMTD acids (2). In freshwater fish oils, the combined contents of these fatty acids ranged from 0.20%-0.73% of the total fatty acids (3). A current investigation of the fatty acids of the lipids of earthworms from agricultural pasture land has shown that these three multibranched acids were present in the neutral lipids in proportions ranging from 2.7% of the fatty acids in winter to 13.3% in spring. The occurrence of isoprenoid fatty acids in earthworms does not appear to have been reported previously (4,5), but the presence of isoprenoid hydrocarbons in these organisms recently has been established by Nooner, et al. (6).

EXPERIMENTAL PROCEDURES

As indicated in another communication reporting the occurrence of triglycerides (7), the earthworms investigated in this project were identified as a mixture of *Lumbricus rubellus* and *Allolobophora caliginosa* and were collected at different seasons from the same area of predominantly rye grass-clover pasture grazed by dairy cows. Worms were dug and hand-sorted from soil down to ca. 6 in. below the surface of the ground. Immediately after collection, the worms were washed (7), and the total lipids were extracted with a mixture of chloroform:methanol (2:1 v/v) and resolved into neutral lipids, phospholipids, and glycolipids by column chromatography (7). Aliquots of total lipids and neutral lipids respectively were saponified with 0.5 N alcoholic KOH for 6 hr, freed of unsaponifiable matter, acidified, and converted to methyl esters with diazomethane. Phospholipid fractions were saponified with 0.5 N methanolic NaOH for 2 hr, and the fatty acid methyl esters were prepared by methanolysis with 14% BF_3-methanol reagent. The glycolipid fraction was acid hydrolyzed (8), and the fatty acids, after purification by preparative thin layer chromatography (TLC), were esterified with diazomethane. Fatty acid methyl esters were analyzed by gas liquid chromatography (GLC) using a Pye Series 104 chromatograph fitted with dual flame ionization detectors. Both polar and nonpolar columns were employed, the former containing 10% EGSS-X and the latter either 3% or 5% Apiezon L. The 10% EGSS-X and the 3% Apiezon L columns were operated at 180 C and the 5% Apiezon L at 200 C. Identification of fatty acid constituents was based upon close agreement in equivalent chain length (ECL) values with those of authentic fatty acid methyl esters run on the chromatograph under corresponding conditions. These reference standards included the methyl esters of phytanic, pristanic, and 4,8,12-TMTD acids which had earlier been isolated and conclusively identified (9-11). To help confirm identification of fatty acid methyl esters, fractions were hydrogenated and their saturated products reexamined by GLC. In addition, concentrates of isoprenoid and other branched chain fatty acid methyl esters were prepared from a neutral fraction of spring worms by urea-adduct forma-

TABLE I

Contents of Isoprenoid Fatty Acids in Neutral Lipids of Earthworms at Different Seasons and Data Relevant to Lipids Analyzed[a]

Quantitative data	Spring (Oct. 14, 1971)	Spring (Nov. 14, 1973)	Summer (Feb. 20, 1973)	Autumn (March 21, 1973)	Winter (Aug. 30, 1972)
Isoprenoid fatty acids	%	%	%	%	%
Phytanic	7.9	5.2	2.4	2.2	1.9
Pristanic	0.6	0.4	0.7	0.7	0.5
4,8,12-Trimethyltridecanoic	4.8	2.0	0.1	0.2	0.3
Totals	13.3	7.6	3.2	3.1	2.7
Wt freshly washed worms (g)	1050.0	206.3	178.0	137.0	249.0
Wt total lipids (g)	17.70	3.23	2.14	3.38	4.90
Total lipid content (% wt of fresh worms)	1.7	1.6	1.2	2.5	2.0
Total lipid content (% dry matter basis)	10.3	8.5	11.1	11.1	9.1
Neutral lipids (% wt of total lipids)	39.3	37.9	28.5	29.5	31.0

[a]Isoprenoid acids expressed as percentage of total neutral fatty acids.

tion of the straight chain components, followed by filtration. These concentrates of branched chain esters were examined by GLC-MS-mass spectrometry (MS) using an AEI MS 30 W instrument.

Comparison of the MS data obtained with that reported in the literature (9-12) confirmed the presence of only the three isoprenoid fatty acids pertinent to this communication, namely phytanic, pristanic, and 4,8,12-TMTD. The proportions of these acids found in the neutral lipids of earthworms collected at different seasons and the data relevant to the lipids analyzed are recorded in Table I. Only very small amounts of these isoprenoid acids were detected in the phospholipid and glycolipid fractions.

DISCUSSION

As the lipids investigated in this study were extracted from whole earthworms, including body tissues, organs, bacteria, and other microbia, together with gut contents, the origin of the isoprenoid fatty acids reported is uncertain. The biochemistry of phytanic acid and related acids recently has been reviewed by Lough (1), and it appears to be generally accepted that phytanic acid is derived from the phytol moiety of chlorophyll and that it is catabolized by α-oxidation to yield, first, α-hydroxyphytanic acid and then pristanic acid. 4,8,12-TMTD is presumed to have resulted from the initial degradative step in the β-oxidation of pristanic acid. Although in this present work a search was made for other degradation products known to follow in the catabolic sequence from phytanic acid (1), the only one detected was 2-methylbutyric acid, which was found in small proportions along with acetic and propionic acids in the aqueous extraction solutions.

Earthworms in this locality of New Zealand have been reported to feed principally upon dead pasture roots and, to a lesser extent, on dead herbage and dung (14). Their wt/acre has been estimated at ca. 2000 lb (14). In burrowing, earthworms ingest substantial amounts of soil, together with its associated plant debris, plant seeds (15), organic matter, and living and dead microbia and other organisms. It has been calculated that the earthworms inhabiting an acre of fertile land pass from 4-36 tons of soil through their alimentary tracts in the course of a year (16). Isoprenoid acids were not detected in rye grass-clover hay (2) or in fresh white clover (D.R. Body, unpublished data), and they have not been reported in other plant materials. Phytanic acid, however, has been found to comprise 2.8% of the total fatty acids of rumen bacteria (2) and presumably this was biosynthesized by the bacteria from the phytol moiety of chlorophyll (1). It appears probable, therefore, that the isoprenoid fatty acids identified in the lipids of earthworms have been assimilated in the digestive tract predominantly from bacteria and other microorganisms, as well as from dead worms and fauna which had earlier populated the ingested soil. Disintegrated bacteria from the gut of the earthworms sampled also may have contributed to the accumulation of isoprenoid acids, but quantitative considerations preclude this source as the chief one. Consistent with this postulate is the observation of Dawson (17) that the ingestion of soil by earthworms resulted in a temporary decrease in

bacterial content of the soil eaten. From the investigation now reported, it may be inferred that the food of earthworms is furnished appreciably by the biomass of the soil.

It is not apparent from this investigation why the isoprenoid acids of worms collected in spring comprised 13.3% of the fatty acids of the neutral lipids in one sample and 7.6% in another, as compared with those collected in summer, 3.2%, autumn, 3.1%, and winter, 2.7%. It is probable, however, that climatic conditions are most favorable to earthworms and to microflora reproduction in spring, and, as a result of their metabolic activities then being at an optimum, worms ingest increased quantities of soil and soil microorganisms containing isoprenoid fatty acids.

R.P. HANSEN
ZOFIA CZOCHANSKA
Applied Biochemistry Division
Department of Scientific and
Industrial Research
Palmerston North
New Zealand

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Gas liquid chromatography-mass spectrometry operation carried out by L.N. Nixon and P.D. Mintoft.

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Individual Phospholipid Contents and Their Fatty Acid Compositions in Early Normal Human Embryonic Lung Tissue

ABSTRACT

The concentrations of the individual phospholipids and their fatty acid compositions were determined in lung tissue obtained from 10-11 and 14-15 week pregancies. In this early stage of pregnancy, the amounts and fatty acid compositions of the individual phospholipids did not change substantially. Our human embryonic lung tissue results were compared with the corresponding data for infants and adults.

INTRODUCTION

It has been demonstrated that the lungs of infants dying of hyaline membrane disease (HMD) or idiopathic respiratory distress syndrome have abnormal surface properties (1) and low levels of pulmonary phospholipids (2-6). It also has been shown that a large amount of phosphatidyl choline (PC) is liberated into the alveolar space of the newborn infant after the onset of respiration (7). The literature data (2,4) indicate that, not only the concentrations of the pulmonary phospholipids, but also the phosphatidyl choline (surfactant) fatty acid composition is important.

These data show that the pulmonary phospholipids play an important part in the respiratory diseases. Bearing this aspect in mind, we have carried out a study of the concentrations of the individual phospholipids in normal human embryonal lung tissue, and of their fatty acid compositions, in the early stages of pregnancy.

MATERIALS AND METHODS

Normal human embryonic lung tissue originating from legal termination of pregnancy was used in the study. The period of pregnancy was established from the time elapsed since the last menstruation and from the size of the embryo.

bacterial content of the soil eaten. From the investigation now reported, it may be inferred that the food of earthworms is furnished appreciably by the biomass of the soil.

It is not apparent from this investigation why the isoprenoid acids of worms collected in spring comprised 13.3% of the fatty acids of the neutral lipids in one sample and 7.6% in another, as compared with those collected in summer, 3.2%, autumn, 3.1%, and winter, 2.7%. It is probable, however, that climatic conditions are most favorable to earthworms and to microflora reproduction in spring, and, as a result of their metabolic activities then being at an optimum, worms ingest increased quantities of soil and soil microorganisms containing isoprenoid fatty acids.

R.P. HANSEN
ZOFIA CZOCHANSKA
Applied Biochemistry Division
Department of Scientific and
Industrial Research
Palmerston North
New Zealand

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Individual Phospholipid Contents and Their Fatty Acid Compositions in Early Normal Human Embryonic Lung Tissue

ABSTRACT

The concentrations of the individual phospholipids and their fatty acid compositions were determined in lung tissue obtained from 10-11 and 14-15 week pregancies. In this early stage of pregnancy, the amounts and fatty acid compositions of the individual phospholipids did not change substantially. Our human embryonic lung tissue results were compared with the corresponding data for infants and adults.

INTRODUCTION

It has been demonstrated that the lungs of infants dying of hyaline membrane disease (HMD) or idiopathic respiratory distress syndrome have abnormal surface properties (1) and low levels of pulmonary phospholipids (2-6). It also has been shown that a large amount of phosphatidyl choline (PC) is liberated into the alveolar space of the newborn infant after the onset of respiration (7). The literature data (2,4) indicate that, not only the concentrations of the pulmonary phospholipids, but also the phosphatidyl choline (surfactant) fatty acid composition is important.

These data show that the pulmonary phospholipids play an important part in the respiratory diseases. Bearing this aspect in mind, we have carried out a study of the concentrations of the individual phospholipids in normal human embryonal lung tissue, and of their fatty acid compositions, in the early stages of pregnancy.

MATERIALS AND METHODS

Normal human embryonic lung tissue originating from legal termination of pregnancy was used in the study. The period of pregnancy was established from the time elapsed since the last menstruation and from the size of the embryo.

TABLE I

Concentrations of Individual Phospholipids (PL) in Normal Human Early Embryonic Lung Tissue[a]

Phospholipid[b]	10-11 Week tissue (% total PL)	14-15 WEek tissue (% total PL)	Normal infant (% total PL)		Normal adult[c] (% total PL)
DPG	3.5±0.3	4.1±0.4			
PE	15.6±1.8	17.2±1.8	14[d]		21.4±9.1[c]
PC	43.4±4.7	41.4±4.9	54	58.8±2.5[e]	50.9±7.2
Sph	16.2±1.9	15.5±1.7	14		6.5±3.5
PI	6.9±0.9	5.8±0.7			18.4±7.2 $\bar{X}$
PS	13.9±1.2	14.9±1.5			
PA	0.6±0.2	1.1±0.3			
Total PL mg/g wet wt	17.3±1.7	17.4±1.9	18.1	22.3±1.1	18.4

[a]The standard deviation was calculated from 10 parallel determinations in each case.

[b]DPG = diphosphatidyl glycerol, PE = phosphatidyl ethanolamine, PC = phosphatidyl choline. Sph = sphingomyelin, PI = phosphatidyl inositol, PS = phosphatidyl serine, and PA = phosphatidic acid.

[c]Harlan, et al. (12). $\bar{X}$ = PI+PS. The values are given as means ± standard deviation.

[d]Adams, et al. (4).

[e]Brumley, et al. (2).

The examinations were performed in each case on 10 samples of lung tissue obtained from 10 different 10-11 and 14-15 week normal human embryos.

The lipids were extracted and purified from the tissues by the method of Folch-Pi, et al. (8). The individual phospholipids were separated by the method of Turner and Rouser (9) and determined quantitatively by the procedure of Kahovcova and Odavic (10). The fatty acid compositions of the individual phospholipids were determined by means of gas chromatographic separation (11).

RESULTS AND DISCUSSION

Table I shows the concentrations of the individual phospholipids in the lung tissue. The data reveal that the PC is present in the greatest amount, while the quantities of phosphatidyl ethanolamine (PE), sphingomyelin (Sph), and phosphatidyl serine (PS) are almost the same. Phosphatidic acid is found in lowest amount. Significant differences were not observed in the concentrations of the individual phospholipids in the lung tissues from the 10-11 and from the 14-15 week pregnancies.

It also appears from Table I that, in the early human embryonic lung tissue, the PC may be a little lower, while the Sph is significantly higher than in the infant or the adult.

The data relating to the fatty acid compositions are listed in Table II. The unsaturated fatty acids present in greatest amounts in the PE are oleic acid and arachidonic acids, while the most abundant saturated fatty acids are stearic acid and palmitic acid. While the PE contains an excess of unsaturated fatty acid, ca. 50% of the total acid in PC consists of palmitic acid. Also present in a major amount, in addition to the palmitic acid, is oleic acid. The percentage palmitic acid content is of importance from the point of view of the surfactant, since the degree of saturation of pulmonary PC decreases in infants suffering from the HMD syndrome (2,4). Of the individual phospholipids, Sph contains the most saturated fatty acid. More than 50% of the total fatty acid consists of palmitic acid and 10% of stearic acid.

It is also clear from the data that, of the fatty acids with more than 20 carbon atoms in their chains in the Sph, those present in greatest amounts are 22:6ω3 and 22:5ω3. Of the phospholipid components, the PS and the phosphatidyl inositol (PI) contain little palmitic acid but the most stearic acid. A considerable amount of arachidonic acid is found in the PI and of oleic acid in the PS.

It is interesting that, of the arachidonic acid isomers, only the 20:4ω6 occurs in any of the individual phospholipids.

Comparison of the embryonic and the adult human lung tissue data in Table II leads to the following findings. The 16:1 fatty acids contents of the PE, PC, PI, and Sph are ca. the same in both types of tissue. With the exception of Sph, the 16:0 fatty acid contents are also the same. The 18:0 fatty acid content of the PC is much lower in the early embryonic lung tissue than in the adult. The 18:1 fatty acid is found in higher amounts in PE, PC, and

TABLE II

Fatty Acid Compositions of Individual Phospholipids in Human Early Embryonic and Adult Lung Tissue

	PE		Adult[b]	PC		Adult[b]	Sph		Adult[b]	PI		PS		Adult[b]
Fattyacid	10-11 Week	14-15 Week	human	10-11 Week	14-15 Week	human	10-11 Week	14-15 Week	human	10-11 Week	14-15 Week	10-11 Week	14-15 Week	human PI+PS
12:0			2.9±1.7											
14:0			3.3±1.7	0.6	tr	4.0±1.8	0.7		4.6±2.1					1.5±0.8
15:0			12.6±3.3		0.1		tr	tr					0.4	
15:1					0.2		tr	0.9		0.4	0.4	0.7	0.3	
16:0	17.3±2.1	16.2±2.2	17.7±4.2	46.6±6.9	46.2±6.7	51.4±8.9	53.3±5.2	59.4±6.1	28.3±7.7	6.1±0.7	7.1±0.8	2.7±0.3	4.5±0.6	10.7±3.2
16:1	1.4±0.2	1.6±0.3	2.7±0.9	5.5±0.6	2.5±0.3	5.5±0.8	2.3±0.3	2.5±0.4	1.6±0.9	0.9	0.3	0.6	1.3±0.2	2.4±1.6
	x0.9	x1.9					x0.5			x0.9	x0.3	x0.2		
16:2	0.8	1.0±0.2		0.2	0.2		0.5	0.8			0.2	0.2	0.4	
16:3	1.5±0.2	1.6±0.3		0.3	0.2			1.2±0.2		0.4	1.0±0.2		0.7	
			17:0 7.5±3.8											
18:0	23.4±3.1	24.2±3.9	19.3±5.1	4.9±0.5	5.2±0.4	14.5±4.2	10.9±1.5	10.1±1.4	8.3±2.6	43.7±4.5	55.2±5.7	45.7±4.6	41.0±4.2	57.7±4.9
18:1	30.8±5.1	27.7±5.2	17.4±5.5	37.7±4.1	40.4±5.2	16.8±3.9	4.5±0.3	6.3±0.5	2.1±0.6	20.5±1.8	17.8±2.1	43.0±4.3	48.3±5.1	21.4±5.9
18:2ω6	1.1±0.3	0.8	3.3±0.9	0.9	1.2±0.3	4.0±1.6	1.5±0.3	tr		tr	0.7	1.0±0.2	0.9	1.9±0.8
18:3ω6	0.4	tr									0.4			
18:3ω3				0.1									0.6	
18:4ω3	0.3	tr					1.9±0.2	2.1±0.1			0.3	1.0±0.1	1.0±0.2	
								x1.5	20:0 3.6±0.6		x1.0			
20:1	0.5	tr		0.2								1.7±0.3	0.6	
20:2ω6	0.8	tr		0.1			1.3±0.2	1.8±0.3		3.3±0.2	0.7	0.2		
20:4ω6	19.7±2.6	25.0±4.8	9.9±3.4y	2.9±0.3	3.7±0.4	3.0±0.7y	1.5±0.2	1.5±0.3	8.1±0.2	23.5±2.7	14.4±1.8	1.2±0.2	tr	4.5±2.1y
20:5ω3							4.6±0.5	4.6±0.4				1.1±0.2	tr	
									22:0 9.2±0.6			xl.5		
22:4ω6	1.1±0.3	tr												
22:5ω3							2.4±0.3	2.3±0.2						
22:6ω3							14.1±2.1	4.9±0.6						
									24:0 17.6±6.4					

[a]PE =phosphatidyl ethanolamine, PC = phosphatidyl choline, sph = sphingomyelin, PI = phosphatidyl insitol, PS = phosphatidyl serine, tr = in trace, x = not identified, and y = identified as 20:4. Data were calculated from five parallel determinations in each case. Mean values and standard deviations are given.

[b]Harlan, et al. (12).

Sph of the embryonic tissue. Compared with the adult, the 20:4ω6 fatty acid content is higher in the embryonic tissue PE, lower in the Sph, and roughly the same in the PC. The embryonic tissue Sph contains the 22:6ω3 component, whereas the adult tissue Sph does not.

The data reveal that the fatty acid compositions of the individual phospholipids in the embryonic and adult lung tissues differ. This presumably implies a structural, as well as a functional, difference.

VELIMIR NIKOLASEV
Department of Pathophysiology
University Medical School
Szeged, Hungary, H-6701

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Tumor Lipids: Long Chain Dienoic Acid in Sphingomyelin

ABSTRACT

The fatty acids derived from sphingomyelin fractions of 10 rat and mouse tumors were examined quantitatively. All tumors, except a nontransplantable fibroadenoma, contained significant levels of a C-24 dienoic acid which is absent from sphingomyelin of normal tissues or present in only trace amounts. The data suggest an abnormality in the metabolism of sphingolipids of tumor cells.

INTRODUCTION

A 24:2 fatty acid has been shown to represent 9% of the sphingomyelin fatty acids derived from Ehrlich ascites cells (1). The acid was characterized and identified as Δ^{15}, Δ^{18}-tetracosadienoic acid and later shown to be present in a membranous fraction isolated from the same tumor cells (2). Recent studies with hepatoma cells 7288C cultured on a medium supplemented with 5% lipid-free fetal calf serum plus linoleic acid revealed that ca. 25% of the sphingomyelin fatty acids was a 24:2 acid (3). Lower concentrations of these acids also were found in sphingomyelin of hepatoma 7288CTC grown in host animals (Wood, unpublished data). In the present study, the sphingomyelin fraction from several rat and mouse tumors was analyzed to determine if the elevated levels of the 24:2 acid is characteristic of a wide variety of tumors.

METHODS

Total phospholipid fractions, isolated from the various rat and mouse tumors previously (4), were resolved into individual classes by thin layer chromatography (TLC). The sphingomyelin band was scraped from preparative chromatoplates, methyl esters prepared and purified by TLC, and the esters analyzed quantitatively by gas liquid chromatography (GLC), as described previously (3). Fatty acids were identified by comparison of relative retention times with standard values, cochromatography with standards, and analysis of esters before and after sample hydrogenation.

RESULTS AND DISCUSSION

The quantity and fatty acid composition of sphingomyelin derived from 10 rat and mouse tumors is given in Table I. A number of studies with hepatomas (5, 6, and R. Wood, et al., unpublished data), where a valid comparison can be made with the tissue of origin (liver), has shown that sphingomyelin levels were elevated. The present data show that sphingomyelin of four tumors represented more than 10% of the

Sph of the embryonic tissue. Compared with the adult, the 20:4ω6 fatty acid content is higher in the embryonic tissue PE, lower in the Sph, and roughly the same in the PC. The embryonic tissue Sph contains the 22:6ω3 component, whereas the adult tissue Sph does not.

The data reveal that the fatty acid compositions of the individual phospholipids in the embryonic and adult lung tissues differ. This presumably implies a structural, as well as a functional, difference.

VELIMIR NIKOLASEV
Department of Pathophysiology
University Medical School
Szeged, Hungary, H-6701

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total phospholipids.

Sphingomyelin from all the tumors exhibited the characteristic fatty acid composition; saturated acids ranging from 16:0-24:0 and monounsaturated acids, primarily 24:1. The quantity of 23:1 was essentially undetectable in all samples, and, except for one sample, the level of 23:0 was less than 0.5% of the total sphingomyelin fatty acids of all transplantable tumors. The concentration of these two acids is reduced considerably relative to the values reported for a number of normal tissues and fluids and pathological samples (7,8). In addition to the fatty acids normally found in sphingomyelin, all the transplantable tumors contained measurable levels of a C-24 dienoic acid. The nontransplantable fibroadenoma did not contain detectable quantities of this acid. Sphingomyelin from the 2 tumors that contained slightly less than 1% of the 24:2 acid also had the lowest level of sphingomyelin (Table I). Sphingomyelin of Ehrlich ascites cells (1), hepatoma cells cultured on certain medium (3), and host grown hepatoma (R. Wood, unpublished data) examined previously have contained a 24:2 acid. This long chain dienoic acid is not specific for neoplastic tissue. It has been detected in trace amounts in sphingomyelin from brain (8, 9) and serum or plasma (3, 10). The presence of the 24:2 acid in tumor sphingomyelin at concentrations several times that found in normal tissue and its occurrence in all host grown transplantable tumors examined thus far may be significant. It would be of interest to determine whether sphingomyelin containing the 24:2 acid is released into the host animal's circulation which might have diagnostic possibilities. The origin of this acid is obviously important. Recent studies by Coles and Foote (11) on rabbit plasma and red blood cells have established the presence of a 24:2 acid in several glycosphingolipid fractions. Since glycosphingolipid metabolism is known to be altered in neoplastic cells (12-14), the increase in the level of sphingomyelin containing a 24:2 acid reported here may result from the build-up of sphingomyelin precursors that normally are used for glycosphingolipid biosynthesis.

TABLE I

Fatty Acid Composition of Sphingomyelin Derived from Several Transplantable Rat and Mouse Tumors

Tumors[a]	Percent of total phospholipid phosphorus	Fatty acid percentages[b]								
		16:0	18:0	18:1	20:0	22:0	23:0	24:0	24:1	24:2
Walker carcinosarcoma 256	15.5	42.8	9.8	T	1.3	4.6	T	14.3	23.6	2.5
R3259/96A sarcoma	6.0	41.9	17.3	1.3	2.5	4.2	T	13.7	17.3	0.9
Nontransplantable fibroadenoma[c]	8.7	38.2	14.1	T	2.2	6.7	3.0	15.0	19.2	-
Sarcoma 180	7.1	59.2	7.4	T	T	2.3	T	3.6	23.8	3.1
Mammary tumor KHZ	7.6	58.8	5.6	0.8	T	4.4	T	6.5	19.5	3.9
Sarcoma T241	8.5	52.6	14.2	T	T	1.7	-	4.0	26.3	4.9
Taper liver tumor	12.5	50.9	3.8	T	T	4.6	T	10.2	23.1	7.1
Melanoma B16	4.0	33.7	9.9	0.6	7.2	12.9	T	21.6	12.8	0.8
Adenocarcinoma E0771	11.2	31.5	4.9	T	T	7.6	T	11.6	42.2	1.8
Friend virus leukemia	14.7	25.6	3.8	T	1.2	8.3	1.3	23.4	31.4	4.9

[a]The first three are rat tumors and the remainder are mouse tumors.

[b]Percentages represent the analysis of sphingomyelin isolated from a single composite sample composed of lipid from many tumors. Some minor acids are omitted. T denotes less than 0.5%.

[c]Occurred spontaneously in a few percentage of rats ca. 1 year after 800R total body irradiation.

RANDALL WOOD
Departments of Medicine and Biochemistry
University of Missouri School of Medicine
Columbia, Missouri 65201

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LETTER TO THE EDITOR

Alleged Role of Boric Acid in Detritylation of Diglyceride-α-trityl Ethers by Silicic Acid

Sir: In recent years, two publications by D. Buchnea (*Lipids* 6:734 [1971] and 9:55 [1974]) have appeared describing the preparation of mixed acid, α,β-diglycerides via their trityl ethers. The removal of the trityl group was accomplished by treating the diglyceride-α-trityl ethers with a mixture of silicic acid and boric acid that had been activiated (Buchnea, *Lipids* 9:55 [1974]) at 115-120 C for 24 hr. In view of the well known fact that boric acid at 100 C loses readily 1 mole of water to form metaboric acid (A.F. Holleman and H.C. Cooper, in *Textbook of Inorganic Chemistry*, Seventh Edition, John Wiley and Sons, London, England, 1927, p. 463; F.P. Treadwell, in *Lehrbuch der Analytischen Chemie*, Vol. 1. Sixteenth Edition, Franz Deuticke, Leipzig, Germany, 1939, p. 361), it seemed unlikely that the boric acid of the mixture, when activated as described above, would remain unchanged. To clarify this point, weighed amounts of boric acid, silicic acid, and mixtures of silicic acid and boric acid (9:1, w/w) were heated on open dishes in an electric oven at 115 C for periods ranging from 1-24 hr. The loss of wt (water) by boric acid both in the presence and absence of silicic acid after 1 hr was found to exceed slightly the amount required for the formation of metaboric acid, i.e. 29.1%, and increased gradually to reach, after 24 hr, an amount equal to the formation of tetraboric acid (36.4%). These experiments show beyond doubt that given the conditions specified for the preparation of the silicic acid-boric acid mixture (Buchnea, *Lipids* 9:55 [1974]), the boric acid is converted to metaboric acid or tetraboric acid. Thus, to speak of detritylating diglyceride-α-trityl ethers by means of silicic acid-boric acid instead of its anhydrides gives an erroneous impression of the role of boric acid in the detritylation procedure and needs to be corrected.

This applies also to the chromatographic separation of monoglycerides (A.E. Thomas, J.E. Scharoun, and H. Ralston, *J. Amer. Chem. Soc.* 42:789 [1965]), sugars (H. Prey, H. Berbalk, and M. Kausz, *Microchim. Acta* 968 [1961]), and threo and erythro-dihydroxy acids (L.J. Morris, *Chem. Ind.* 1238 [1962]) by silicic acid-boric acid mixtures that have been activated at 100-110 C. It is most likely that the metaboric acid formed at this temperature yields the boric acid complexes leading to the separation of the monoglycerides, sugars, and dihydroxy acids.

ERICH BAER
Bant and Best Department
of Medical Research
University of Toronto
Toronto, Canada

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Quantitative and Qualitative Analyses of Isolated Lipid Droplets from Interstitial Cells in Renal Papillae from Various Species

INGE BOJESEN, Institute of Experimental Hormone Research, University of Copenhagen, Copenhagen Ø, Denmark

ABSTRACT

The lipid droplets of renal papillae homogenates from four different species were obtained by ultracentrifugation. Ca. 80-98% of the lipids (triglycerides, phospholipids, free fatty acids, and cholesterol esters) consist of triglycerides. The triglycerides were fractionated by argentation thin layer chromatography and each fraction characterized by gas liquid chromatography. No fraction contained any unique triglyceride. The fatty acid composition of the total triglycerides, as analyzed by gas liquid chromatography and ozonolysis, differed markedly from the fatty acid composition of the corresponding plasma triglycerides. The papillary triglycerides were characterized by higher concentrations of stearic acid, arachidic acid, and polyunsaturated acids with 20 or more carbon atoms. Particularly interesting was the presence in the lipid droplets of docosa-7,10,13,16-tetraenoic acid. This acid has been shown to be a major component in the cholesterol ester fraction of rat and canine adrenal lipids. In the papillary triglycerides, this acid accounted for 7%, 15%, and more than 20% of the total fatty acids in the dog, rat, and rabbit, respectively. The pig differs from these three species in having only ca. 1% of this acid. These observations suggest that the interstitial cells produce these triglycerides. This production could occur either by a transacylation from phospholipids and cholesterol esters and by a de novo synthesis from locally produced fatty acids. The possibility that the triglyceride production may be involved in a control of the prostaglandin production of the renal medulla is discussed.

INTRODUCTION

Characteristic interstitial cells with numerous lipid droplets in the cytoplasm have been described in the renal papilla of various mammals (1-5).

In a preceding article (6) describing the isolation of lipid droplets from the renal papillae of rats, a preliminary qualitative analysis was presented. It indicated that the isolated lipid droplets consisted mainly of triglycerides, free long chain fatty acids, and cholesterol esters. The characterization of the small amounts of the different components was based upon R_f values and the quantification on the spot size in thin layer chromatography (TLC). The composition of the long chain fatty acids in triglycerides was shown to differ from that of the plasma lipids and depot fat and in particular by the large proportion of polyunsaturated fatty acids.

The major purpose of the present investigation was to carry out a more exact analysis of the droplet lipids from various mammals and, thereby, obtain further information about the lipid metabolism of the interstitial cells.

MATERIALS

Standard triglycerides, as well as methyl esters of fatty acids, were obtained from Applied Science Laboratories, State College, Pa.

Standard phospholipids, cholesterol oleate, petroselic acid, and behenic acid were purchased from Sigma, St. Louis, Mo.

$^{14}CH_3OH$ (specific activity, 12.8 mCi/mmole) was obtained from the Radiochemical Center, Amersham, England.

Super dry methanol for transesterification and quantitative glycerol determinations was prepared as follows: 100 g methanol (E. Merck, Darmstadt, Germany) was allowed to react with 1 g $NaBH_4$ for ca. 20 min and then distilled under anhydrous conditions. Toluene (Merck) was refluxed over metallic sodium and distilled. All other chemicals were analytical grade from Merck and were used without further purification.

METHODS

Tissue Specimens

Animals used for the experiments were 5 pigs, 5 rabbits, 2 dogs, and rats (eight groups of five rats). Five rats were required to obtain sufficient amount of lipid droplets. No attempts were made to standardize the kidney

function of the animals. Both young and matured animals of both sexes were used. To ensure that the diet had no influence on the composition of the papillary lipids, 8 groups of rats were fed through stomach tubes 3 times a day with a meal consisted of 20% casein, 40% sucrose, 20% wheat flour, and 20% soya oil (15 g/rat/day). To ensure a satisfactory development, a daily supplement of calcium, phosphate, magnesium, vitamins, and choline chloride was given as 172 mg $CaHPO_4$, $2H_2O$, 89 mg $Mg(OOCCH_3)_2$, $4H_2O$, 5 drops of adetamin, 1 mg choline chloride, and 1 mg tocopherol. One group of rats was kept on the same diet, but with sucrose replacing the soya oil. Five groups were kept on normal Rostock rat pellets.

Blood samples and the kidneys from pigs were obtained from freshly slaughtered animals at the Royal Danish Veterinary and Agricultural High School. The dogs were shot unanaesthetized. The kidneys from these animals were perfused with 0.9% saline solution to remove most of the blood from the tissue. Unanaesthetized rats and rabbits were decapitated and shot, respectively. The renal papillae obtained were pale and without visible blood.

Isolation of Lipid Droplets and Extraction

Isolation of the lipid droplets by ultracentrifugation and extraction was carried out as described earlier (6). The plasma samples were extracted 3 times with double the volume of a chloroform-methanol mixture (2:1 by volume).

Separation of Lipids by TLC

The lipids were separated into classes by the method of Skipski, et al. (7). Triglycerides were separated further on TLC plates impregnated with 2% $AgNO_3$. To prevent oxidation of the highly unsaturated triglycerides, it was necessary to furnish the plates with a band of 3 cm kieselgel without $AgNO_3$ for application. The plates were developed in diisopropylether: hexane (65:35 by volume).

2,7-Dichlorfluorescein spray was used for spot detection and a quantitative extraction of the triglycerides was accomplished with 3 x 0.5 ml 10% diethyl ether in hexane after all silver ions had been complexed with KCN.

Transesterification Procedure

The method of Stoffel, et al., (8) was taken down to a 500 μliter scale (0.55 ml reaction medium). The silica gel was scraped directly into the reaction tubes and dried for 10 min, while shaking at 80 C and 16 mm Hg. Dry toluene (0.5 ml, 10%) in super dried methanol then was added to the tubes filled with dry nitrogen. Finally, gaseous HCl was introduced in the calculated amount (5%) from a syringe. Transesterification was carried out by refluxing for 2 hr at 80 C, and the methyl esters were extracted with hexane after the addition of 750 μliter water.

Analyses of Lipids by Gas Liquid Chromatography (GLC)

A Pye gas chromatograph, series 104, with a dual hydrogen flame ionization detector was used for all analyses. Triglycerides were analyzed on steel columns (1 ft x 4 mm inside diameter) packed with 3% OV-1 on 100-200 mesh. Gas Chrom Q and helium carrier gas 100 ml/min was used, as was a column temperature, 310 C; detector, 370 C; and injection heater, 350 C. Methyl esters were analyzed on the same columns with other conditions, namely argon carrier gas, 60 ml/min; column temperature, 155 C; detector heater, 175 C; and injection heater, 175 C. Additional analyses of methyl esters were carried out on Pyrex glass columns (5 ft x 4 mm inside diameter) packed with 5% polyethylenglycoladipate on 80-100 mesh, celite JJ CQ. Argon carrier gas was 30 ml/min; column temperature, 180 C; detector heater, 200 C; and injection heater, 200 C. Saturated acids were identified by comparison with known standards and the unsaturated acids by hydrogenation to determine the chain length and by pyrolytic ozonolysis to determine the position of the double bonds. The resulting aldehyde esters and aldehydes were chromatographed on Pyrex glass columns (3 ft x 4 mm inside diameter) packed with 15% diethylenglycoladipate on 80-100 mesh. Chromosorb W, argon carrier gas, 60 ml/min was used, as was column temperature, 170 C; and 60 C, respectively; detection heater, 180 C; and injection heater, 230 C. Since standard samples of aldehyde esters were not available, methyl esters of known structure (oleic acid [18:1ω9], petroselic acid [18:1ω12], linoleic acid [18:2ω6], and arachidonic acid [20:4ω6]) were subjected to ozonolysis and GLC under the same conditions employed for the unknown fatty acids. A plot of carbon number against log retention time for the standards then was applied for the identification of the unknown aldehyde esters and aldehydes not identical with the standards.

Quantitative Analyses

Standard lipids (dipalmitoyl-phosphatidyl choline, dipalmitoyl-phosphatidyl ethanolamine, cholesterol oleate and glyceryl trioleate) were analyzed parallel with the papillary lipids to correct for losses through the procedure.

TABLE I

Amount of Lipids Extracted from Floating Layer of Papillary Homogenates and Triglyceride Fraction in These Extracts

Papillary tissue (mg)	Isolated lipids (μg)	Percent triglyceride in isolated lipids (%)	Amount of floating lipid droplets/wt papillary tissue (μg/100 mg)
	Dog		
1786	120	92	
1097	105	92	
	Pig		
1061	308	87	
1009	219	97	
----	235	92	
737	213	95	
----	118	96	
	Rabbit		
528	207	80	
487	148	85	
747	149	90	
532	195	98	
591	190	97	
	Rat		
242	145	96	60
276	183	96	66
248	111	96	45
126	277	98	218
333	166	95	50
326	407	96	124
201	169	95	84
235	136	97	58

Phospholipids and triglycerides were determined by transesterification of the compounds followed by an analysis of phosphorus and free glycerol in the water phase, according to Bartlett (9) and Hanahan and Olley (10). The liberated cholesterol (80%) from cholesterol esters was found in the hexane phase together with the methyl esters. The two components could be separated easily by TLC and cholesterol measured by the Liebermann-Burchart reaction. The amount of papillary lipids is calculated using the esters of oleic acid as references, since the mol wt of these esters represent suitable means of the esters found.

Free fatty acids in small amounts were quantified by methylation with $^{14}CH_3OH$ (specific activity 1μCi/mmole) and counted in a Packard Tri Carb liquid scintillation counter after chromatographic isolation.

Ozonolysis

The ozonides were prepared according to Privett and Nickell (11) by a modified procedure described previously (12). The ozonides were split by a pyrolysis procedure described by Davison and Dutton (13).

RESULTS AND COMMENTS

Quantitative Determinations

The total amount of extracted lipids from the isolated floating layer of papillary homogenates is given in Table I. Under these conditions, the droplets were not recovered quantitatively. Extraction of lipid from the remaining part of the homogenates and subsequent analysis showed that the composition of these residual triglycerides was indistinguishable from that of the floating layer. In the case of rats, these unreleased triglycerides amounted to less than 15% of the total, and the data of Table I, thus, demonstrate a great variation in lipid droplets content of the papillary tissue in the different groups of rats (factor ca. 5). Papillary tissue in the other three species, in particular the dog, was more resistant to homogenization. Since the proportion of unreleased triglycerides was high in these species (80% in one case), a meaningful calculation of lipid droplet content is excluded.

Table II shows the results of quantitative determinations of the different lipid classes of the floating layer of renal papillary homoge-

TABLE II

Quantitation of Lipid Classes of Floating Layer of Renal Papillae Homogenates[a]

Triglycerides (glycerol determination)	Free fatty acids	Phosphatidyl choline (phosphate determination)	Phosphatidyl ethanolamine (phosphate determination)	Cholesterol esters (cholesterol determination)
		Dog		
110	not detected[b]	10	not detected[b]	----
97	4	not detected[b]	not detected	4
		Pig		
267	not detected	24	17	< 2[c]
202	not detected	17	not detected	< 2
216	8	11	not detected	< 2
196	not detected	10	7	< 2
113	5	not detected	not detected	< 2
		Rabbit		
159	4	22	5	17
126	17	5	not detected	< 2
134	not detected	15	not detected	< 2
190	---	5	not detected	< 2
184	not detected	6	not detected	< 2
		Rat		
139	not detected	6	not detected	< 2
175	6	----	---	2
104	4	not detected	not detected	2,5
268	7	not detected	not detected	2
158	8	not detected	not detected	< 2
387	7	7	2	4
161	not detected	5,5	2,5	< 2
132	not detected	4	not detected	< 2

[a]Table given in μg.

[b]Detection limit on thin layer plates for free fatty acid using the 2,7-dichlorfluorescein spray is 2.5 μg. Detection limit on thin layer plates for phospholipids using the phospholipid spray (14) is 1 μg.

[c]Sensitivity of the cholesterol determination was ca. 2 μg.

TABLE III

Long Chain Fatty Acid Composition of Papillary Triglycerides from Various Species[a]

Number of carbon atoms: number of double bonds	Pig (5) Percent ± standard error of mean	Dog I	Dog II	Rabbit (5) Percent ± standard error of mean	Rat (8)[b] Percent ± standard error of mean
14:0	trace[c]	1.2	2.4	trace	trace
16:0	29.3 ± 0.6	24.6	20.8	11.8 ± 0.5	14.7 ± 1.7
16:1Δ9	3.0 ± 0.5	3.6	3.9	trace	2.4 ± 0.5
18:0	19.6 ± 1.9	16.1	14.5	10.7 ± 0.7	13.3 ± 1.1
18:1Δ9	27.1 ± 1.7	28.0	31.2	19.8 ± 2.4	14.9 ± 1.3
18:2Δ9,12	11.8 ± 0.6	13.8	6.5	16.5 ± 1.3	14.4 ± 1.0
18:3	--	trace	--	trace	1.4 ± 0.8
20:0	3.7 ± 0.6	2.1	--	trace	2.1 ± 0.6
20:1	1.3 ± 0.3	1.9	1.2	1.6 ± 0.3	1.0 ± 0.2
20:2	trace	--	1.2	1.9 ± 0.5	trace
20:3	(2,8)[d]	1.4	1.2	1.9 ± 0.4	4.6 ± 0.5
20:4Δ5,8,11,14	2.0 ± 0.2	3.9	6.0	6.4 ± 0.4	14.3 ± 1.1
22:4Δ7,10,13,16	trace (0.8 ± 0.3)	2.5	11.0	22.4 ± 3.9	12.6 ± 1.2
(22:5)[e]	trace (0.9 ± 0.3)	*[f]	*	4.1 ± 1.2	3.4 ± 0.7
(22:6)[e]	--	*	*	1.2 ± 0.3	1.2 ± 0.6

[a]Number of animals used is given in parentheses. In rat, eight groups of five animals.

[b]Papillae from rats fed on a diet containing 10% linoleic acid (see text).

[c]Trace indicates less than 1%.

[d]2.8% only in one case.

[e]According to argentation thin layer chromatography more unsaturated than 22:4.

[f]* = The recording was stopped after elution of 22:4.

TABLE IV

Long Chain Fatty Acid Composition of Plasma Triglycerides from Various Species[a]

Number of carbon atoms: number of double bonds	Pig (5) Percent ± standard error of mean	Dog I	Dog II	Rabbit (5) Percent ± standard error of mean	Rat (8)[b] Percent ± standard error of mean
14:0	trace[c]	1.0	2.0	3.5 ± 0.4	2.5 ± 1.1
16:0	24.6 ± 1.9	20.0	24.2	32.9 ± 1.0	25.9 ± 1.1
16:1Δ9	--	4.6	7.0	7.7 ± 1.0	trace
18:0	7.1 ± 2.3	9.5	9.9	4.6 ± 0.6	4.1 ± 0.3
18:1Δ9	42.4 ± 3.7	37.5	39.2	36.5 ± 1.3	25.5 ± 1.9
18:2Δ9,12	21.4 ± 2.8	21.8	11.2	12.0 ± 0.8	35.6 ± 0.9
18:3	1.9 ± 1.0	trace	trace	3.0 ± 0.9	3.1 ± 1.1
20:0	--	--	trace	--	--
20:1	--	--	trace	--	--
20:3	trace	1.3	3.7	--	--
20:4Δ5,8,11,14	(1.3)[d]	1.6	1.5	--	trace
22:4Δ7,10,13,16	--	--	--	--	--

[a]Number of animals used is given in parentheses. In rat, eight groups of five animals.

[b]Plasma from rats fed on a diet containing 10% linoleic acid (see text).

[c]Trace indicates less than 1%.

[d]1.3% only in one case.

nates. Obviously, the preliminary estimations of the chemical composition of the lipid droplets with respect to the relative contribution of cholesterol esters (6) were entirely erroneous. In one case (rabbit 1), 17 μg was detected, but the fatty acid composition of this cholesterol ester was indistinguishable from that of the corresponding plasma ester. The phospholipid composition seems to be fairly similar in the floating material. The amounts of phosphatidyl ethanolamine found were seldom significant, whereas phosphatidyl choline is present in a variable amount from 1-10%. Regarding fatty acids, which also were present in variable amounts, the found variations may reflect differences in the amount of fatty acids liberated during homogenization. Anyway, the actual content of free fatty acids in the floating layer is determined by phase distribution of fatty acids in the homogenate.

TABLE V

Fatty Acid Composition of Triglycerides from Renal Papillae and Plasma of Rats Fed Different Diets

	Percent ± standard error of mean					
	Special diet[a] without soyaoil (n=1) 0.05% linoleic acid		Special diet with soyaoil (n=8) 10% linoleic acid		Normal diet Rostock pellets (n=5) 1% linoleic acid	
Number of carbon atoms: number of double bonds	Papil TG[b]	Plasma TG	Papil TG	Plasma TG	Papil TG	Plasma TG
14:0	trace[c]	trace	trace	2.5 ± 1.1	trace	trace
16:0	12.7	29.2	14.7 ± 1.7	25.9 ± 1.1	11.0 ± 0.7	18:8 ± 0.3
16:1Δ9	2.6	16.9	2.4 ±0.5	trace	1.4 ± 0.4	2.7 ± 0.5
18:0	8.7	2.6	13.3 ± 1.1	4.1 ± 0.3	11.7 ± 1.3	1.5 ± 0.2
18:1Δ9	16.5	45.5	14.9 ± 1.3	25.5 ± 1.9	12.3 ± 1.5	23.2 ± 0.5
18:2Δ9,12	9.1	5.1	14.4 ± 1.0	35.6 ± 0.9	12.7 ± 1.2	28.4 ± 0.8
18:3	--	--	1.4 ± 0.8	3.1 ± 1.1	--	--
20:0	1.1	--	2.1 ± 0.6	--	1.6 ± 0.2	--
20:1	2.2	--	1.0 ± 0.2	--	1.3 ± 0.3	--
20:2	1.6	--	trace	--	1.7 ± 0.1	--
20:3	5.2	--	4.6 ± 0.5	--	3.8 ± 0.6	--
20:4Δ5,8,11,14	16.6	trace	14.3 ± 1.1	trace	16.6 ± 0.8	4.1 ± 0.4
?[d]	--	--	--	--	--	6.2 ± 0.9
22:4Δ7,10,13,16	17.6	--	12.6 ± 1.2	--	15.8 ± 0.6	--
(22:5)[e]	3.2	--	3.4 ± 0.7	--	4.9 ± 0.9	--
(22:6)[e]	2.5	--	1.2 ± 0.6	--	4.2 ± 0.9	--
?[d]	--	--	--	--	--	12.9 ± 0.9

[a]The special diet is described in the text.

[b]TG = triglycerides.

[c]Trace indicates less than 1%.

[d]Acids found to constitute a high percentage of pellet rat food.

[e]According to argentation thin layer chromatography more unsaturated than 22:4.

Recovery and Reproducibility of Different Procedures

The recovery of glyceryl trioleate was 95.5% ± 2.1 (mean ± standard error of mean). Super dry methanol for transesterification cannot be prepared in the normal way via the alcoholate, as the content of formaldehyde, which interferes with the reaction for glycerol, becomes too high. It results in a variable and high blank of at least 3 μg. Distillation from $NaBH_4$ reduces the blank to a constant value of 1.5 μg.

Recovery of dipalmitoyl-phosphatidyl choline was 51.8% ± 2.1 and that of dipalmitoyl-phosphatidyl ethanolamine 49.6% ± 4.7.

Cholesterol oleate recovery was 46.8% ± 2.1.

Qualitative Determinations

The composition of the long chain fatty acids of the major constituent in the lipid droplets, the triglycerides, is given in Table III, while Table IV shows the composition of the corresponding plasma triglycerides.

The effect of diet upon the fatty acid spectrum in rat plasma triglycerides is presented in Table V. A similar change of the spectrum could not be demonstrated in papillary triglycerides.

Table VI shows an example of the separation of triglycerides extracted from rabbit lipid droplets. Column I shows fractionation of the triglycerides according to their degree of unsaturation. In conjunction with gas chromatography, each band appeared to consist of several triglycerides (Column 2-7). Relatively heavy triglycerides can be seen to dominate the composition.

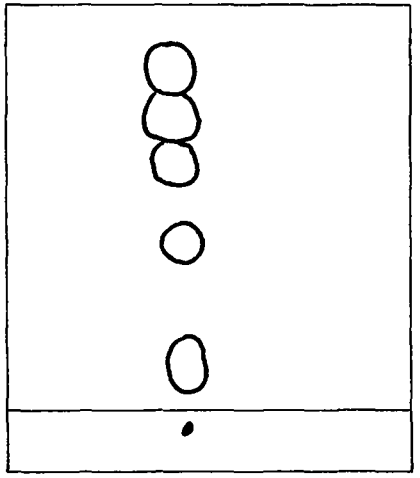
DISCUSSION

The presence of cytoplasmic lipid droplets has been observed in other normal mammalian tissues, such as adrenals, testes, ovaries, and hepatocytes. However, only in the case of hepatocytes have such droplets been isolated for chemical analysis (15). In the other cases where analyses have been carried out on the lipid extracts of the tissue, only indirect information regarding the lipid composition of droplets has been made available.

In this study on renal papillae, the major lipid component (80-98%) extracted from the floating layer was triglyceride for all 4 species. These triglycerides, separated in groups according to saturation, revealed a wide spectrum of mol wt within each group, and no particular

TABLE VI

Percentage of Individual Triglycerides from Rabbit Lipid Droplets after Separation on Argentation Thin Layer Chromatography and Gas Liquid Chromatography

Thin layer chromatogram impregnated with $AgNO_3$ (except for the first 3 cm)	Number of carbon atoms in the fatty acid moiety of the triglycerides (TG)							
	46	48	50	52	54	56	58	
	5.7	24.4	45.2	20.2	4.5	--	--	In % of total TG 5
	--	8.4	30.2	48.5	12.9	--	--	In % of total TG 4
	--	8.3	36.8	36.0	18.9	--	--	In % of total TG 3
	--	3.7	18:0	47.4	31.0	--	-	In % of total TG 2
	-	2.6	5.9	23.5	36.3	21.8	9.8	In % of total TG 1
In % of total triglycerides calculated on basis of the peak areas from gas chromatogram	1.7	8.5	23.2	33.5	24.8	5.3	3.0	
In % of total triglycerides calculated on basis of the quantitative determinations	0.9	7.5	20.2	31.0	25.9	10.2	4.6	

triglyceride is represented predominantly in the droplets. The number of carbon atoms in the fatty acid moiety varies from 46-58, the heavy triglycerides also being the most unsaturated (Table VI). Anggård, et al., (16) isolated the lipid droplet fraction from rabbit renal papillae and also found 84% of nonpolar lipids in this fraction.

The minor components (2-20%) were phospholipids and free fatty acids. The great variability suggests a variable contamination with adherent endoplasmic reticulum (cluster of droplets often are seen in the microscope). The phospholipid fraction found is, therefore, a maximal value. Anggård, et al., (16) found 17% polar lipids (phospholipids, sphingolipids, and ceramides) in the washed lipid droplet fraction from rabbit compared with 2.6-14.0% phospholipids in the present investigation. For rats, this percentage is lower (0-5%), which might be correlated with the above mentioned clusters of droplets, since the rat papilla is much less resistant to homogenization. Whether the lipid droplets actually contain phospholipids could not be demonstrated conclusively. Electron microscopic investigations have shown that the lipid droplets in the renal medulla do not have any visible membrane (3). However, the presence of a monomolecular layer of phospholipids covering the droplets would account for the amount found in droplet preparations from rats, according to calculations based upon the phospholipid monolayer investigations of Colacicco (17). This author calculated a minimal surface concentration of 21 μg/180 cm^2. An average droplet diameter of 0.6 μ (6) gives a phospholipid to total lipid ratio of 0.012 to compare with an analytical ratio between 0-0.047 (Table II).

The presence of free fatty acids in some preparations is (as mentioned above) most likely due to phase distribution in the homogenate and would be expected to vary with free fatty acid in the medium and with the pH.

The lipid droplets isolated from rat hepatocytes and analyzed by Diaugustine, et al., (15) consisted mainly of triglycerides like the droplets from rat renal papilla, but the long chain fatty acid composition was markedly different. Only linoleic acid, oleic acid, and palmitic acid were found in hepatocyte triglycerides, whereas papillary triglycerides from rats contained heavy polyunsaturated acids with high percentage of docosa-7,10,13,16-tetraenoic acid. This compound, being the major fatty acid in the rabbit papillary triglyceride, is identified by Anggård, et al., (16) as a docosapentaenoic acid. However, in a paper to be published, it is proved conclusively that this compound (previously called adrenic acid) is biosynthesized from arachidonic acid by a chain elongation. The occurrence of such a high proportion of this acid in glycerol esters has not been reported previously in lipids isolated from any other mammalian tissue. Only small amounts have been found in triglycerides extracted from testes (18) and ovaries (19) known to be rich in polyunsaturated fatty acids. Of most interest are the rat adrenal droplets, which contain 70-80% of the total cholesterol (20) and, according to Gidez and Feller (21), esterified with the same spectrum of long chain fatty acids as found in the rat papillary triglyceride fraction. Also, canine adrenal gland cholesterol esters showed this spectrum according to Lo Chang and Sweeley (22) in contrast to the triglycerides of this organ. The observation (Table III) that the fatty acid spectrum of the droplet triglycerides varies greatly from species to species with pig and rabbit showing the greatest differences may be explained either as a true species characteristic or a result of different diets. Failure to change the fatty acid composition in rat papillary triglycerides by changing the food for 3-4 days (Table V), in contrast to a marked effect upon plasma triglycerides, leaves a species characteristic as the most probably factor. However, this result does not exclude a very long term diet effect (subject to further investigations) caused by a very slow turnover of droplet lipids.

Regardless of the species differences, a marked difference in papillary triglycerdies compared with plasma triglycerides is characteristic for all species, which suggest a local fatty acid synthesis.

The significance of the lipid droplets is still unknown. Speculations about the importance of the droplets have been presented by several authors in relation to a possible excretion process. In their morphology and high content of triglycerides, they resemble chylomicrons. The possibility exists that the lipid droplets are locally synthesized chylomicrons destined for export. An alternative explanation is that the lipid droplets serve as a store of long chain fatty acids in continuous equilibrium with membrane phospholipids.

Previously, it has been suggested that the high content of arachidonic acid in the rat renal papillae triglycerides might serve as a prostaglandin E_2 precursor. Lands and Samuelsson (23) and Vonkeman and Van Dorph (24) have demonstrated an activation of a phospholipase A as the rate limiting step in the prostaglandin biosynthesis. Assuming an exchange between triglycerides and phospholipids in the renal papilla similar to the phenomenon found by Elsbach and Farrow (25) in granulocytes,

combined with the specific retroconversion of docosa-7,10,13,16-tetraenoic acid to arachidonic acid found by Stoffel, et al., (26) in liver, the system could serve as a control mechanism of a prostaglandin biosynthesis. It is known that rabbit and dog kidneys release prostaglandins, particularly under conditions with reduced blood supply (27-29). On the basis of the present findings, this phenomenon presumably will not be found to the same extent in experiments with pigs.

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Changes in In Vivo Metabolism of Bile Acids in Rat after Treatment with Phenobarbital

TOMAS CRONHOLM, Department of Chemistry, **KURT EINARSSON**, Department of Medicine, and **JAN-ÅKE GUSTAFSSON**, Department of Chemistry and Department of Germfree Research, Karolinska Institutet, Serafimerlasarettet, Stockholm, Sweden

ABSTRACT

The influence of phenobarbital on pool size and turnover of bile acids in rats have been investigated by administration of [24-^{14}C] cholic acid and tritium labeled chenodeoxycholic acid. Phenobarbital treated rats had a smaller cholic acid pool compared to control rats (6.08 ± 2.09 mg and 23.60 ± 7.66 mg, respectively). The pool size of chenodeoxycholic acid, plus its metabolites (α- and β-muricholic acids), was of the same magnitude in the two groups of animals. Also the daily production of cholic acid was decreased in phenobarbital treated rats compared to control rats (2.12 ± 0.46 mg and 7.24 ± 1.66 mg, respectively). No significant difference was observed between the synthesis of chenodeoxycholic acid in the two groups of animals.

INTRODUCTION

During the past few years, several studies on the effect of phenobarbital upon the formation and metabolism of bile acids in rat liver homogenates have been published (1-8). A great deal of interest has been attributed to the 7α-hydroxylation of cholesterol which is the rate limiting step in the biosynthesis of bile acids. It has been shown that the 7α-hydroxylase is stimulated by phenobarbital in rats of the Wistar strain (2,7) and slightly inhibited in Sprague-Dawley rats (1,6,7). Similarly the 12α-hydroxylase is depressed by phenobarbital in Sprague-Dawley rats (1). No information is available on the effects of phenobarbital upon bile acid formation in vivo. Some articles have appeared dealing with the influence of phenobarbital on bile flow and bile acid excretion in rats during the first hr after bile duct cannulation, but these studies reflect only the emptying of the bile acid pool (9-11).

Previous work has shown that phenobarbital stimulates the synthesis of bile acids in monkey (12) and in man (13) to an extent of 25-50%. In man preferentially, the formation of cholic acid was increased. It was, therefore, considered of interest to study the influence of phenobarbital on the turnover of bile acids in rats, in which the 7α-, as well as the 12α-hydroxylases are inhibited by phenobarbital, to evaluate whether in vitro data correspond to conditions in vivo.

This article discusses the turnover of ^{14}C-cholic and ^{3}H-chenodeoxycholic acids in control rats and rats treated with phenobarbital.

In this article, systematic names used are as follows: cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; hyodeoxycholic acid, 3α,6α-dihydroxy-5β-cholanoic acid; lithocholic acid, 3α-hydroxy-5β-cholanoic acid; β-muricholic acid, 3α,6β,7β-trihydroxy-5β-cholanoic acid; and α-muricholic acid, 3α,6β,7α-trihydroxy-5β-cholanoic acid.

MATERIALS AND METHODS

Radioactive steroids: [24-^{14}C]Cholic acid (specific radioactivity, 45.5 μCi/mg) was obtained from New England Nuclear Corp., Boston, Mass. Randomly tritium labeled chenodeoxycholic acid (specific radioactivity, 53.3 μCi/mg) was prepared by the method according to Wilzbach (14).

Animals: The study included 2 groups, each consisting of 3 male white rats of the Sprague-Dawley strain, weighing ca. 200 g. They had free access to water and were fed a commercial pellet diet ad libitum. One group of animals was treated throughout the experimental period with daily intraperitoneal injections of 1 ml saline solution of phenobarbital in a concentration corresponding to 100 mg phenobarbital/kg body wt. The other group of rats received the same volume of saline. After 10 days of treatment, 0.1 mg [24-^{14}C]cholic acid and 0.5 mg tritium labeled chenodeoxycholic acid as sodium salts and dissolved in saline were given intraperitoneally. Faeces then were collected in 24 hr fractions for 10 days. After this collection period, bile fistulas were prepared by introducing a polyethylene cannula into the common bile duct, and bile was collected for 12 hr.

Analysis of bile acids in bile: Bile was hydrolyzed with 1M KOH in 50% aqueous ethanol for 12 hr at 110 C. This method to cleave bile acid conjugates generally is used and usually results in good recoveries, although ketonic bile acids sometimes may be degraded partially (15). The saponification mixture was acidified with hydrochloric acid and extracted with ether. The ether extract was washed with water until neutral, and the solvent was evaporated. The residue of the ether extract was methylated with diazomethane, (trimethyl) silylated, and analyzed by gas liquid chromatography (GLC) and gas chromatography-mass spectrometry (GLC-MS).

The gas chromatographic analyses were carried out using 0.5% cyclohexanedimethanol succinate (CHDMS), 1.5% SE-30 and 3% QF-1 (Supelco, Bellefonte, Pa.) as stationary phases (16,17). Chromatography on CHDMS separated all major bile acids, except cholic and α-muricholic acids. α-Muricholic acid was separated from the other bile acids by chromatography on QF-1. Analysis on SE-30 permitted quantitation of hyodeoxycholic acid.

GLC-MS was carried out on an LKB 9000 instrument using 0.5% CHDMS as the stationary phase. Mass spectra were recorded on magnetic tape using the incremental mode of operation and were then treated in an IBM 1800 computer (18). A compound was considered identified if it had the same mass spectrum and GLC behavior as the reference compound.

Analysis of radioactivity in faeces: Faeces were disintegrated in 20 ml water. Ca. 40 ml ethanol was added, and the mixture was refluxed for 2 hr. After filtration, the residue was refluxed once more for 2 hr with 40 ml ethanol. The extracts were combined, and aliquots (1/100) were transferred into counting vials and evaporated. The residue was dissolved in 0.5 ml ethanol, and 15 ml scintillation liquid was added. Samples were counted in a Packard scintillation spectrometer, model 3003. Quenching corrections were made with samples after addition of [24-^{14}C]cholic acid and tritium labeled chenodeoxycholic acid, respectively, as internal standards.

Determination of bile acid turnover: The pool sizes of cholic acid and chenodeoxycholic acid were calculated approximately to be the amount of bile acids excreted during the first 12 hr after bile duct cannulation (19,20).

The bile acids are modified extensively by microbial enzymes during the intestinal passage and practically no unchanged cholic or chenodeoxycholic acids can be found in the faeces (21,22). Thus, it is not possible to isolate and determine the specific activities of the bile acids in faeces. We have, therefore, determined the total ^{14}C- and ^{3}H-radioactivities excreted in faeces/day during a 10 day period. The logarithm of the fraction of the injected dose that had not appeared in the faeces after a time was plotted vs this time, and a straight line was fitted. The half-life obtained (23) represents both processes of biodegradation and excretion of the corresponding bile acids, and the term half-excretion time is used to indicate the difference to the half-life obtained from studies of the decrease in specific activity. The daily production of cholic acid was calculated from the pool of cholic acid, its metabolite deoxycholic acid, and the half-excretion time of [24-^{14}C]cholic acid, and the daily production of chenodeoxycholic acid was calculated from the pool of chenodeoxycholic acid, α-muricholic and β-muricholic acids, and the half-excretion time of tritium labeled chenodeoxycholic acid. Thus, the pool of the bile acid and its metabolites times ln 2 was divided with the half-excretion time to obtain the daily excretion (23).

RESULTS

Identification of bile acids in bile: The pattern of bile acids in bile from control and phenobarbital treated rats is shown in Figure 1. Peaks 1-5 were shown by GLC-MS to represent α-muricholic acid, cholic acid, deoxycholic acid, chenodeoxycholic acid, and β-muricholic acid, respectively. Peak 6 contained small amounts of hyodeoxycholic acid, but the major constituent of this peak was of unknown structure, possibly an unsaturated derivative of a trihydroxylated bile acid (24).

Pool size: Table I summarizes the results. In control rats, cholic acid constituted ca. 65% of the total bile acid pool. Only minor amounts of deoxycholic acid (2%) occurred. Chenodeoxycholic acid constituted ca. 5% and the sum of chenodeoxycholic acid, plus its metabolites (α- and β-muricholic acid) ca. 35% of the total bile acid pool.

Phenobarbital treatment caused a reduction of the cholic acid pool by ca. 75%. Phenobarbital treated rats had an increased pool of α-muricholic acid compared to control rats, but the sum of the pool sizes of chenodeoxycholic acid and its metabolites was of the same magnitude for the two groups of animals. The ratio between the pool size of cholic acid and of chenodeoxycholic acid, plus its metabolites, was changed from ca. 2.0 in control rats to ca. 0.5 in phenobarbital treated animals.

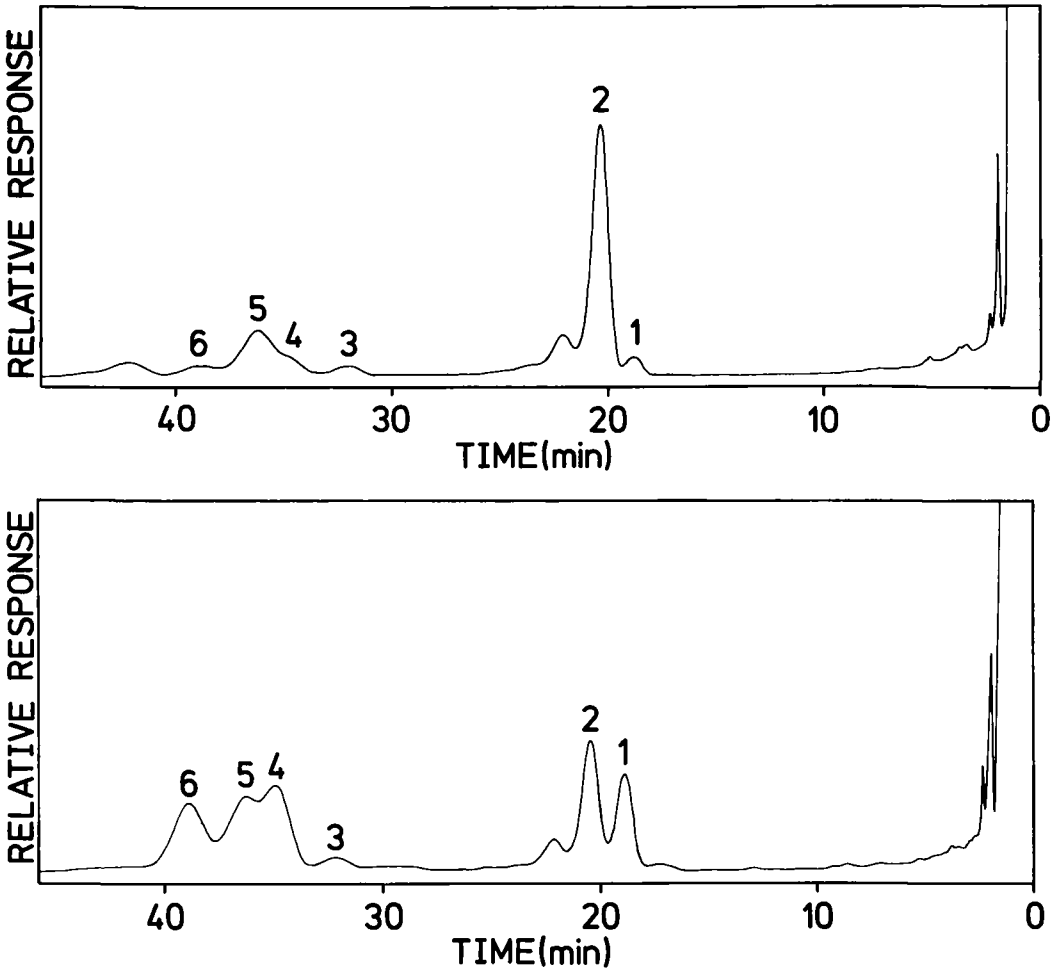

FIG. 1. Gas chromatographic analysis on 0.5% cyclohexanedimethanol succinate of 4/5000 of the bile acid pool in male control rats (upper panel) and in male rats treated with penobarbital (lower panel). The bile acids were analyzed as methyl esters and trimethylsilyl ethers. The following compounds were identified: peak 1, α-muricholic acid; peak 2, cholic acid; peak 3, deoxycholic acid; peak 4, chenodeoxycholic acid; and peak 5, β-muricholic acid. Peak 6 contained small amounts of hyodeoxycholic acid, but the major constituent of this peak was of unknown structure (see text).

Half-excretion time: The recovery of ^{14}C-radioactivity in faeces was ca. 95-100%. The ^{3}H-radioactivity was excreted rapidly during the first days of the collection period but the total recovery amounted only to ca. 40-60%. The low recovery of the ^{3}H-radioactivity might be explained by the fact that chenodeoxycholic acid was tritiated randomly, and loss of radioactivity might have occurred by hydroxylations, dehydroxylations, oxido-reductions, and conjugation reactions in the liver and during the intestinal passage. However, the semilogarithmic plots of the elimination of both chenodeoxycholic acid and cholic acid showed a straight line relationship with time. In control rats, the half-excretion time of cholic acid and chenodeoxycholic acid was found to be ca. 2.3 and 1.2 days, respectively (Table II). In phenobarbital treated rats, the half-excretion time was slightly shorter.

Production rate: Table II summarizes the results. The daily synthesis of cholic acid and of chenodeoxycholic acid in control rats was ca. 7.2 and 7.5 mg, respectively. In phenobarbital treated rats, the formation of cholic acid was reduced by ca. 70% compared with control rats. The synthesis of chenodeoxycholic acid was not influenced significantly by phenobarbital treatment. Neither was the total formation of bile acids significantly influenced by phenobarbital. The ratio between the synthesis of cholic and chenodeoxycholic acids was decreased

from ca. 1.0 to ca. 0.2.

DISCUSSION

Cholic acid and chenodeoxycholic acid are formed from cholesterol in the liver and are excreted with bile into the intestine (25). During the intestinal passage, they are partly 7α-dehydroxylated to yield deoxycholic acid and lithocholic acid, respectively. Lithocholic acid is reabsorbed poorly, but the other bile acids are reabsorbed almost quantitatively and reach the liver via the portal vein. A minor part of the bile acids escapes reabsorption and is excreted in faeces. They further are modified by microbial enzymes, and, therefore, faeces contain a complex mixture of various bile acids. In the liver, deoxycholic acid is 7α-hydroxylated to cholic acid and, therefore, rat bile contains only minor amounts of deoxycholic acid. Chenodeoxycholic acid is 6β-hydroxylated to α-muricholic acid which is transformed further into β-muricholic acid via the intermediate formation of 3α, 6β-dihydroxy-7-keto-5β-cholanoic acid. Thus, rat bile contains a complex mixture of bile acids.

The formation and metabolism of bile acids include hydroxylations at positions 7α, 12α, 26, and 6β. Recent in vitro studies have indicated that the 6β-hydroxylation is catalyzed by the common drug-metabolizing enzyme system which is stimulated severalfold by phenobarbital (3,5). The 7α-hydroxylation of cholesterol, which is the rate limiting step in the biosynthesis of bile acids, has been reported to be stimulated by phenobarbital in rats of the Wistar strain but is slightly inhibited in rats of the Sprague-Dawley strain (1,2,6,7). The 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one also seems to be a more specific hydroxylation reaction and is inhibited by phenobarbital (1). Conflicting reports have appeared concerning the 26-hydroxylase. Cronholm and Johansson (4) found that the 26-hydroxylation was slightly stimulated by phenobarbital treatment, whereas Björkhem and Gustafsson (8) recently have shown that this hydroxylation is inhibited by phenobarbital.

Treatment of rats with phenobarbital results in an increase in biliary flow, but recent studies have indicated that this increase in bile flow is not due to an enhanced excretion of bile salts into the bile neither in rats of the Wistar strain nor in rats of the Sprague-Dawley strain (9-11). Klaassen (10) found a significantly smaller excretion of taurocholic acid in phenobarbital treated rats compared to control rats during the first 9 hrs after bile duct cannulation. No difference in the biliary excretion of tauro-

TABLE 1

Influence of Phenobarbital Treatment on Pool Size of Bile Acids in Rat[a]

Rat	Cholic acid	Deoxycholic acid	Chenodeoxycholic acid	α-Muricholic acid	β-Muricholic acid	Cholic + deoxycholic acid	Chenodeoxycholic + α- and β-muricholic acid	Total bile acids
C1	22.35	0.26	1.32	1.45	13.60	22.61	16.37	38.98
C2	31.81	1.68	3.44	2.39	8.55	33.49	14.38	47.87
C3	16.65	0.44	1.24	1.25	4.94	17.09	7.43	24.52
Mean	23.60 ± 7.66	0.79 ± 0.77	2.00 ± 1.25	1.70 ± 0.61	9.03 ± 4.35	24.40 ± 8.34	12.73 ± 4.69	37.12 ± 11.79
PB1	8.24	0.29	3.59	4.06	11.01	8.53	18.66	27.19
PB2	4.07	0.35	4.15	3.52	3.32	4.42	10.99	15.41
PB3	5.94	0.71	4.91	3.29	4.67	6.64	12.87	19.51
Mean	6.08 ± 2.09	0.45 ± 0.23	4.22 ± 0.66	3.62 ± 0.40	6.33 ± 4.11	6.53 ± 2.06	14.17 ± 4.00	20.70 ± 5.98
pb	<0.02	NS	NS	<0.02	NS	<0.025	NS	NS

[a]C = control rat, PB = phenobarbital treated rat, and NS = no significance. Table is in mg.
[b] According to Student's t-test.

TABLE II

Influence of Phenobarbital Treatment on Turnover of Cholic Acid and Chenodeoxycholic Acid in Rat[a]

Rat	Cholic acid		Chenodeoxycholic acid		Total bile acid turnover mg/day
	Half-excretion time days	Excretion mg/day	Half-excretion time days	Excretion mg/day	
C1	2.20	7.15	1.20	9.45	16.60
C2	2.60	8.94	1.10	9.06	18.00
C3	2.10	5.63	1.25	4.12	9.75
Mean	2.30 ± 0.26	7.24 ± 1.66	1.18 ± 0.08	7.54 ± 2.97	14.78 ± 4.41
PB1	2.30	2.57	1.05	12.30	14.87
PB2	1.85	1.66	0.95	8.02	9.68
PB3	2.15	2.14	1.00	8.91	11.05
Mean	2.10 ± 0.23	2.12 ± 0.46	1.00 ± 0.05	9.74 ± 2.25	11.87 ± 2.69
p[b]	NS	<0.01	<0.05	NS	NS

[a]C = control rat, PB = phenobarbital treated rat, and NS = no significance.

[b]According to Student's t-test.

chenodeoxycholic acid was observed. Our results agree relatively well with the latter study. Thus, the amount of cholic acid excreted during the first 12 hr after bile duct cannulation, which ca. represents the pool size of the acid, was decreased with ca. 75% by phenobarbital treatment. On the other hand, was the pool size of chenodeoxycholic acid, plus its metabolites, not significantly influenced by phenobarbital.

The total formation of bile acids was of the same magnitude in the two groups of animals, but the synthesis of cholic acid was reduced significantly by phenobarbital treatment. These results give support to above mentioned in vitro studies which have shown that phenobarbital does not induce hydroxylases involved in the biosynthesis of bile acids in Sprague-Dawley rats (1, 6-8). The low formation of cholic acid in treated animals agrees with a reduced activity of the 12α-hydroxylase (1). In contrast to rat, man and monkey increase the synthesis of bile acids during treatment with phenobarbital (12,13). Especially the formation of cholic acid is stimulated in man (13). Hence, the present work gives further support to the view that various species and strains of mammals show differences in their ability to induce liver enzyme activities during drug treatment.

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Fatty Acid Biosynthesis by Avocado Pear

J.L. HARWOOD, Department of Biochemistry, University College, Cardiff, United Kingdom

ABSTRACT

Fatty acid synthesis in the avocado (*Persea americana*) mesocarp is due to three immunologically distinct enzyme systems. The plastid membranes synthesize a mixture of palmitic, stearic, and oleic acids. The plastid stroma, on the other hand, contains only palmitate elongase and cytoplasm only fatty acid synthetase. The elongation of palmitate requires malonyl CoA and is sensitive to arsenite inhibition.

INTRODUCTION

Several workers have studied fatty acid biosynthesis in the avocado pear (*Persea americana*). Stumpf and Barber (1) used a particulate fraction from the mesocarp which synthesized palmitic and oleic acids from acetate. Later, a soluble preparation was made (2) which would catalyze the incorporation of malonyl-coenzyme A into long chain fatty acids. Overath and Stumpf (3) demonstrated that acyl carrier protein was required for optimal synthesis by the supernatant fraction and Yang and Stumpf (4) found that two sites of fatty acid biosynthesis were present—a particulate fraction which could use acetate but not malonate and a soluble fraction which could use malonate. Weaire and Kekwick (5) concluded that the particulate activity was due to the intact chloroplast and the soluble activity to leakage of the stromal proteins. Using a comprehensive subcellular fractionating scheme, Harwood and Stumpf (6) were able to show that the plastid fraction contained two immunologically distinct synthesizing systems and that the soluble fraction contained enzymes derived both from the stroma and cytoplasm.

The soluble fraction contains fatty acid synthetase and a palmitate elongase (7). The plastid stroma, on the other hand, only synthesises stearate (8), as do other soluble preparations from chloroplasts (9). The present work demonstrates that there are three separate fatty acid synthesizing systems in avocado mesocarp: (A) one which synthesizes predominantly palmitate, stearate, and oleate is localized in plastid membranes; (B) palmitate elongase in the plastid stroma; and (C) fatty acid synthetase in the cytoplasm.

EXPERIMENTAL PROCEDURES

Materials

Avocado (*Persea americana* var. Fuerte) was purchased from the local supermarket. Acetate-1-^{14}C(58 mCi/mMole) and malonate-1-^{14}C(6.9 mCi/mMole) was obtained from the Radiochemical Centre, Amersham, England. Malonyl CoA-1,3,-^{14}C(10 μCi/0.24 mg) was purchased from New England Nuclear Corp., Boston, Mass.

Acetyl CoA-1-^{14}C was prepared by the method of Simon and Shemin (10) and acyl carrier protein by the method of Sauer, et al. (11).

Silica gel for chromatography was from E. Merck, Darmstadt, Germany, and silicone oil MS 200/200 from British Drug Houses (BDH), Poole, Dorset, U.K.

Nicotinamide adenine dinucleotide, reduced form (NADH), nicotinamide adenine dinucleotide phosphate, oxidized form (NADP), glucose-6-phosphate (Glu-6-P), adenosine 5′-triphosphate (ATP), and glucose-6-phosphate dehydrogenase were from Boehringer, London, England, CoA, avidin, malonyl CoA, acetyl CoA, and fatty acid standards from Sigma, St. Louis, Mo.; dithiothreitol from Koch-Light, Colnbrook, England, and reduced glutathione from BDH.

Chlorophyll and Protein Estimations

The methods of Arnon (12) and Lowry, et al., (13) respectively, were used.

Fatty Acid Biosynthesis

Incubation conditions and lipid extraction were carried out, as described previously (14). Total fatty acid methyl esters were counted in a scintillant containing 0.5% diphenyloxazole (PPO) and 0.03% 1,4-di 2(4-methyl-5 phenyloxazolyl)benzene (dimethyl POPOP) in toluene. Individual fatty acids were separated by gas liquid chromatography (GLC) on a Perkin-Elmer F.11 chromatogram fitted with a Perkin-Elmer 170 radioactivity detector using a 20% diethylene glycol succinate on Chromosorb W HMDS column (Perkin-Elmer). Isothermal runs at 170 C generally were used.

Fatty acids also were separated by reversed phase thin layer chromatography (TLC) using 5% silicone oil MS 200/200 on Silica Gel G plates. The silicone oil was added by means of a prerun with petroleum ether (60/80) solvent.

TABLE I

Fatty Acids Synthesized by Avocado Mesocarp Subcellular Fractions[a]

Fraction	Fatty acid synthesis pmoles/min/mg protein	Fatty acids (% total)[b] 16:0	18:0	18:1	Others
Plastid lamellar	196	22	26	37	15
Plastid stromal	123	3	97	ND	Tr
Supernatant	112	46	51	ND	3

[a]Figures are average of three experiments. The assay system contained: adenosine 5'-triphosphate, 2 μmoles; nicotinamide adenine dinucleotide, reduced form, 0.5 μmole; nicotinamide adenine dinucleotide phosphate, reduced form, 0.5 μmole (or nicotinamide adenine dinucleotide phosphate, oxidized form, 0.5 μmole, Glucose 6 phosphate 4 μmole, glucose 6 phosphate dehydrogenase, 0.5 units); Glu-6-phosphate, 8 μmoles; *Escherichia coli* acyl carrier protein 0.5 mg and malonyl CoA-1, 3-^{14}C, 0.1 μmole containing about 100,000 cpm. The total incubation volume was 1.0 ml. Subcellular fractions containing 0.8-2.0 mg protein were incubated for 1 hr at 25 C. The reaction was stopped by the addition of 0.1 ml 60% KOH and lipid extraction and fatty acid analysis carried out, as described previously (14).

[b]ND = not detected and Tr = trace.

Fatty acids were separated using methanol solvent and the spots visualized by spraying plates with 0.04% Bromocresol green (BDH) in ethanol. Plates were scanned using a Packard model 7201 radiochromatogram scanner.

Subcellular Fractionation

This was carried out, as previously described (6), and purified lamellar and stromal fractions prepared from plastids by the method of Leech (15).

Antibodies

Purified lamellar or stromal fractions were injected intramuscularly into a rabbit in a 1:1 emulsion with Fruends complete adjuvant. Ca. 5 mg protein was used each time. Three injections at intervals of 3-4 weeks were used to raise high activity antibodies.

Preparation of Protein-bound Palmitate

^{14}C-Palmitate bound to protein was prepared as follows. An incubation was performed using the supernatant fraction as enzyme source in the presence of 3 mM arsenite. The reaction was terminated by the addition of sulphuric acid to a final pH of 3. The system then was spun at 105,000 g x 60 min and the sediment resuspended in dilute sulphuric acid (pH 3). It was filtered through Whatman GF/A glass fiber paper and the precipitate washed with several changes of acid. Extensive washing was found necessary to remove all traces of unreacted ^{14}C-malonyl CoA. The protein-bound-^{14}C-palmitate then could be redissolved in 0.1 M phosphate buffer pH 7.4-1 mM dithiothreitol.

Degradation of Products

The separated fatty acids were analyzed by permanganate α-oxidation (16) and by Schmidt decarboxylation (17).

Sephadex Gel Filtration of Reaction Products

Filtration of reaction products was carried out at 4 C on 25 x 1.5 cm Sephadex G-75 columns. Elution was with 0.1 M potassium phosphate buffer pH 7.4-5x10^{-4}M dithiothreitol at a flow rate of 25-30 ml/hr. Fraction size was 1.2 ml. Fractions were analyzed for fatty acids by hydrolysis and methylation and for protein by optical density measurement at 280 nm.

RESULTS AND DISCUSSION

Fatty Acid Products from Different Fractions

Harwood and Stumpf (6) showed that, with acetate as substrate, most fatty acid synthesis by avocado took place in the plastid fraction, but, when malonyl CoA was used, there was much soluble activity. Acetate was an ineffective substrate in the supernatant due to an inhibitor of acetyl CoA carboxylase (6) which probably derived from the stroma (18). Analysis of the fatty acid patterns made by the plastid membrane and stromal fractions and the supernatant are shown in Table I. It will be seen that the two plastid subfractions synthesize a completely different pattern of acids. The formation of a complex mixture of acids by plastid membranes is in keeping with previous observations (4, 6, 8) and indicates a tightly coupled system of enzymes. Added acyl carrier protein caused little stimulation of incorporation by this fraction. This is similar to chloroplast systems which do not need added acyl carrier protein (19) but in contrast to soluble plant preparations (6, 7, 20).

TABLE II

Inhibition of Fatty Acid Biosynthesis by Antisera[a]

Fraction	Fatty acid synthesis (% control)		
	+ Normal serum	+ Antilamellar serum	+ Antistromal serum
Plastid lamellar	104 ± 6	25 ± 1[b]	114 ± 16
Plastid stromal	103 ± 15	98 ± 8	0
Particle free supernatant	105 ± 6	102 ± 7	52 ± 10

[a]Figures represent means ± standard deviation. (Three experiments). Control values were obtained in the absence of any serum.

[b]Concentration curves were recorded for each antiserum, and the figures represent maximal inhibition obtained. With the exception of the assay of lamellar activity in the presence of antilamellar serum, a plateau in the inhibition curve had been reached. In this case, high concentrations of antiserum had to be added causing difficulties in assay and extraction. The figure of 25 ± 1 must, therefore, represent a minimal value of 75% inhibition.

TABLE III

Effect of Arsenite upon Fatty Acid Synthesis by Avocado Subcellular Fractions[a]

Fraction	Arsenite (mM)	Fatty acids (% control)	Fatty acids (% total)		
			16:0	18:0	Others
Plastid stroma	0	100 ± 9	3	97	Tr
	3	36 ± 6	5	95	Tr
Supernatant	0	100 ± 6	46	51	3
	3	64 ± 4	84	10	6

[a]Figures are mean (± standard deviation) from three experiments. Incubations were carried out as detailed in Table I in the absence or presence of arsenite.

[b]Tr = trace.

Since the supernatant fraction synthesises almost entirely palmitate and stearate (7) and the percentage of each varies from fractionation to fractionation, it seemed logical to attribute the stearate synthesis to stroma gained from plastid damage during fractionation. Harwood and Stumpf (6) suggested that the cytoplasm itself could synthesize fatty acids as well, so experiments were conducted to see if palmitate formation was from that source.

In Table II, the effect of antibodies raised to lamellar or stromal fractions is shown. Plateaus on the inhibitor curves for antisera were obtained with the exception of the lamellar fatty acid synthesis and antilamellar serum. The figure of 25% of control activity in this case, therefore, represents an elevated value. It is also difficult to obtain complete inhibition of membrane-bound enzymes with antibodies due to accessibility problems. It can be seen that there are three immunologically distinct systems present and, moreover, the antistromal serum only inhibits stearate biosynthesis. Thus, it results in complete inhibition of the stromal synthesis and reduces that of the supernatant by ca. half. The lack of effect of antistromal serum on lamellar synthesis (even though the latter produces stearic acid) may be due either to the presence of isoenzymes or because the lamellar enzymes, being membrane bound, are not readily accessible to antistromal serum in the absence of a detergent.

Studies with a number of plant systems (7, 14) revealed that arsenite will inhibit palmitate elongase but is without effect upon chloroplast fractions (J.L. Harwood, unpublished observations) or fatty acid synthetase (7, 20). The effect of its addition to the different incubation systems is shown in Table III. A close parallel is seen between the inhibition by antistromal serum and by arsenite. Both prevent stearate formation and, therefore, cause high inhibition of the stromal enzyme and less for the supernatant. These results indicate that the cytoplasm is the source of fatty acid synthetase.

Properties of Stromal System

The fatty acid products were subjected to chemical degradation to determine their manner of synthesis. The results for decarboxylation are shown in Table IV and for α-oxidation in Table V. These indicated that stearate is

TABLE IV

Decarboxylation of Reaction Products[a]

Fraction used	Fatty acid	CPM		% Released
		Total	CO_2	
Plastid stroma	Stearic	8030	5701	71
Supernatant	Palmitic	3140	439	14
Supernatant	Stearic	4070	2279	56

[a]Recoveries for 1-^{14}C-palmitate decarboxylated simultaneously were 89-96%. For details see text.

made by elongation and palmitate de novo, in agreement with previous observations using various plant systems (7, 21).

The fact that the stromal enzyme can be more accurately described as palmitate elongase, raises the question of the nature of the two carbon unit added. Harwood and Stumpf (7) using acetone powder preparations, presented evidence that palmityl acyl carrier proteins, but not palmityl CoA or free palmitate, could be elongated by malonyl CoA. Weaire and Kekwick (8) also suggest that malonyl CoA is the donor. Two types of experiment were conducted to resolve the problem conclusively. In the first, elongation of protein-bound palmitate was measured directly using acetyl CoA + avidin or malonyl CoA (Table VI). Elongation was much greater with malonyl CoA.

Secondly, dilution of any ^{14}C-acetyl CoA formed by decarboxylation of ^{14}C-malonyl CoA was made using a 10-fold excess of unlabelled acetyl CoA. This had no significant effect upon the rate of elongation to stearate: thus, acetyl CoA is not used by the stromal enzyme. Carboxylation of acetyl CoA, even in the absence of avidin, is low due to the presence of an inhibitor (6, 18). Jaworski, et al., (22) recently showed that a palmityl elongase from safflower also uses palmityl-acyl carrier protein and malonyl CoA.

The products of the stromal elongase and the supernatant enzymes were fractionated by Sephadex gel filtration. Figure 1 shows a typical separation. Interestingly, it will be seen that they are similar, even though fatty acid synthetase is absent from the stromal preparation. The first, and largest, fatty acid peak contains very little in the form of sulphur esters, but the second protein peak contains significant amounts as sulphur esters. The latter peak has been shown to contain fatty acid synthetase but elongation of palmityl ACP could not be obtained with the supernatant fraction or with either of the peaks (7). The elution position of the palmitate elongase is, therefore, not known, but it is possible that, since stearyl-sulphur esters are present there, it is also in the second protein peak but is

TABLE V

α-Oxidation of Stearic Acid Synthesized by Plastid Stroma[a]

Fragment size	Counts/min	Ratio (radioactivity/mass)
C 18	5736	6.48
C 17	598	1.05
C 16	460	1.00
C 15	221	0.87
C 14	57	0.92
C 13	ND	---

[a]Incubation was carried out as described in Table I and in the text with 1,3,-^{14}C-malonyl CoA substrate. The stearic acid was separated and permanganate oxidation carried out (16). Ratio of mass to radioactivity for palmitate was taken as unity.

[b]ND = not detected.

TABLE VI

Elongation of Protein-Bound Palmitate by Plastid Stromal Fraction[a]

	Fatty acid products (cpm)		
	16:0	18:0	% Elongation
Zero time control	4503	102	---
+ Acetyl CoA	3887	667	12
+ Malonyl CoA	1431	3213	70

[a]Incubations and synthesis of protein-bound palmitate were carried out as described in the text. Unlabeled acetyl CoA or malonyl CoA was added to a final concentration of 100 μg/ml. Avidin was present at 1 unit/ml.

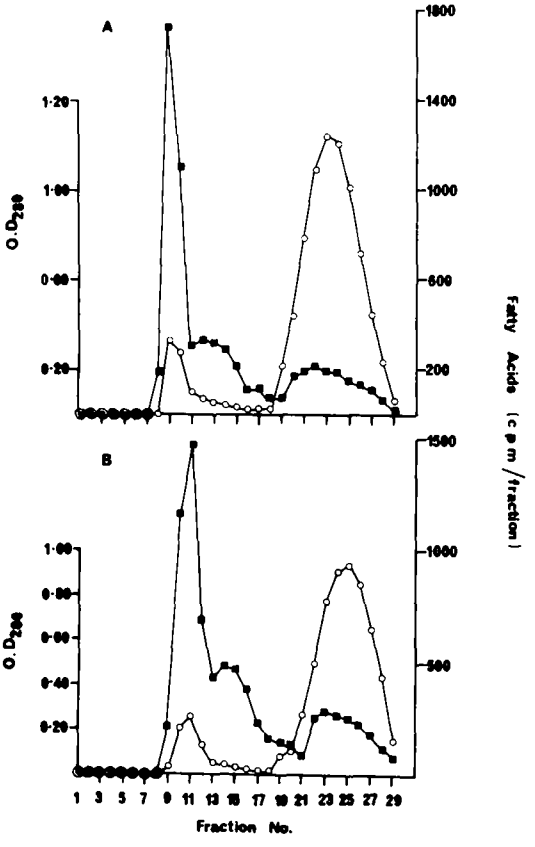

FIG. 1. Separation of reaction products by Sephadex G-75 gel filtration. Full experimental details are given in the text. (A) shows the separation for the supernatant fraction and (B) for the stroma. o ——— o Represents protein, and x ——— x represents fatty acids.

inactivated easily. On the other hand, recoveries of the products in the first peaks from the column were 60-80%, so that palmitate elongase may represent a lower mol wt protein.

The results presented here show that the fatty acid synthesis observed in fractions from the outer mesocarp of the avocado can be accounted for by three distinct enzyme systems. These are located in the plastid membrane and stromal fractions and the cytoplasm. Soluble fractions from most plants synthesize palmitic and stearic acids, the proportion of the latter being high in photosynthetic tissues (9, 22). Membrane fractions from plants generally synthesize a mixture of acids, including unsaturated (6). Thus, fatty acid synthesis in avocado is typical of plant tissues, and the presence of a cytoplasmic fatty acid synthetase and a stromal palmitate elongase may be general.

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Effect of Long and Medium Chain Length Lipids upon Aqueous Solubility of α-Tocopherol

Y.I. TAKAHASHI, Institute of Human Nutrition, College of Physicians and Surgeons, Columbia University, New York, New York 10032, and **BARBARA A. UNDERWOOD**[1], Nutrition Program, Division of Biological Health, College of Human Development, The Pennsylvania State University, University Park, Pennsylvania 16802

ABSTRACT

The efficiency by which α-tocopherol is solubilized in vitro into mixed bile salt micelles containing different lipids was studied. Alterations in solubility due to addition to the incubation media of triglyceride, free fatty acid, monoglyceride, and lecithin of either long or medium chain length were examined. Results are expressed as a partition ratio between a micellar and an oil phase. The triglyceride of both long and medium chain length fatty acids greatly decreased the solubility of α-tocopherol in bile salt solutions. When added singly, monoglyceride and lecithin of long chain length fatty acids increased the α-tocopherol solubilized four- to fivefold; fatty acids of either chain length and medium chain monoglyceride when added singly had no significant effect upon the tocopherol solubilized. An additive effect was observed when a combination of long chain monoglyceride and lecithin was added. Addition of fatty acid to this combination, however, significantly decreased the α-tocopherol solubility into the micellar phase. Although the solubility of α-tocopherol was increased by all combinations of medium chain length polar lipids, except the fatty acid-monoglyceride pair, the effect was three to seven times less than for the corresponding long chain mixture.

INTRODUCTION

Medium chain triglyceride (MCT) is being used with increasing frequency as a caloric adjunct in the treatment of malabsorption syndromes. MCT and its hydrolytic products have several chemical and physical characteristics quite different from those of long chain triglyceride (LCT) which make them particularly useful for this purpose. For example, hydrolysis and micellar solubilization is not obligatory to absorption (1,2), and absorption occurs primarily by way of the portal, rather than the lymphatic, system (3-5). These attributes, though favorable to absorption of MCT could have adverse effects upon the absorption of relatively nonpolar lipids, such as the fat-soluble vitamin tocopherol. Tocopherol, like other relatively nonpolar lipids, probably requires solubilization into micelles prior to uptake by the mucosa (6), and is absorbed primarily by way of the lymphatic circulation (7,8).

In vitro, the solubility of lipid molecules in water or in bile salt solution, increases with increased polarity and with decreased size of the hydrophobic region of the molecule (9-12). In bile salt solutions, the very polar lipids of long chain length, such as monoolein and lecithin, are not only soluble in bile salt solutions but, in addition, increase the micellar solubility of relatively nonpolar lipids, such as sterols (10, 13-17). Little precise information is available on how polar lipids of medium chain fatty acids, when present singly or in combinations, influence the solubility into micelles of relatively nonpolar lipids.

The in vitro study described herein was designed to determine the influence of the chain length of the fatty acid moiety when added singly or in combinations with monoglyceride and lecithin on the aqueous solubility of tocopherol. Conditions were maintained which resembled those occurring in the intestinal lumen with respect to temperature, bile salts, and sodium ion concentrations (Na^+) and pH.

EXPERIMENTAL PROCEDURES

Materials

Triolein, monoolein, oleic acid, trioctanoin and octanoic acid (all 99% pure by thin layer chromatography [TLC]) were obtained from Sigma Chemical Co., St. Louis, Mo.

A crude mixture of medium chain monoglyceride (MCM) was purified as follows: free fatty acids and diglycerides were separated from monoglyceride by elution from Florisil columns with ether-hexane mixtures as described by Carroll (18). The eluted monoglyceride containing fraction was purified by washing with a solution of 0.8 ml 5% ammoni-

[1]Author to whom correspondence should be directed.

TABLE I

Effect of Long and Medium Chain Length Lipids upon m/o[a] Ratio of α-Tocopherol

Experiment	Polar lipids added	Long chain length (Mean ± SD)[b]	p value[c]	Medium chain length (Mean ± SD)[b]	p value[c]
1	None[d]	0.14 ± 0.01		0.07 ± 0.01	
2	FA[e]	0.14 ± 0.00		0.08 ± 0.01	
3	MG[f]	0.62 ± 0.06	<0.001	0.08 ± 0.01	
4	PL[g]	0.76 ± 0.04	<0.001	0.11 ± 0.01	<0.01
5	FA + MG	0.60 ± 0.01	<0.001	0.07 ± 0.02	
6	FA + PL	0.85 ± 0.04	<0.001	0.25 ± 0.01	<0.05
7	MG + PL	1.42 ± 0.09	<0.001	0.21 ± 0.01	<0.05
8	MG + PL + FA	1.02 ± 0.04	<0.001	0.17 ± 0.03	<0.05

[a]The m/o ratio of tocopherol in each mixture used in the present study is calculated from the amount of tocopherol recovered in the aqueous phases, where m/o = M/(T-M), M = solute in the micellar phase and T = total solute in the mixture.

[b]Mean of at least 9 determinations. SD = standard deviation.

[c]Comparison of each value against experiment 1.

[d]Triglyceride concentration in the bile salt solution, 1.1 mM triolein or trioctanoin.

[e]Fatty acid (FA) concentration in the bile salt solution, 7.5 mM either oleic or octanoic acid.

[f]Monoglyceride (MG) concentration in the bile salt solution, 2.4 mM, either monoolein or medium chain monoglycerides.

[g]Phospholipids (PL) concentration in the bile salt solution, 0.7 mM, either dipalmitoyl lecithin or dioctanoyl lecithin.

um sulfate and 0.2 ml 5 N sulfuric acid. Ethanol (3 ml) was added and the monoglyceride extracted into benzene. The fatty acid composition of the isolated monoglyceride was determined by gas liquid chromatography (GLC) of the methyl esters on a column containing 15% diethylene glycol succinate on Chromasorb W. The composition was found to be 24% octanoic acid, 71% decanoic acid, 4% lauric acid, and 1% of various fatty acids of longer chain length.

β-γ-Dioctanoyl-L-α-lecithin and β-γ-dipalmitoyl-L-α-lecithin (A grade) were obtained from Supelco, Bellefonte, Pa., and Calbiochem, San Diego, Calif., respectively.

Unlabeled d-α-tocopherol was obtained from Eastman Kodak Co., Rochester, N.Y. Labeled d,l-α-tocopheryl-3,4-$^{14}C_2$-acetate, specific activity 13 μci/mg was generously given by Hoffmann-La Roche, Nutley, N.J. ^{14}C-Labeled tocopherol was obtained by hydrolyzing the ^{14}C-tocopheryl acetate in ethanolic potassium hydroxide (50%) at 75 C for 50 min under a constant flow of nitrogen. The labeled tocopherol was purified by TLC on plates coated with Silica Gel G and developed in the solvent system of petroleum ether (bp 30-60 C) and diethyl ether (9:1 v/v). Purification of the ^{14}C-α-tocopherol was repeated every 2 weeks or just prior to use to eliminate any decomposition products.

Sodium taurocholate (A grade, 98% pure by TLC) was obtained from Calbiochem.

Solvents were all spectro grade as purchased, except ethanol and petroleum ether. Ethanol was distilled after refluxing over aluminum and potassium hydroxide. Peroxide-free petroleum ether was prepared by refluxing 3 times for 2 hr each over concentrated sulfuric acid, then washing with distilled water until neutral to Congo red. The petroleum ether then was distilled over sodium hydroxide.

All materials, except MCM, labeled tocopherol, and the solvents noted were used without further purification.

Phosphate buffer (pH 6.3 and 0.15 M with respect to Na^+) and a stock solution of sodium taurocholate (24 mM and 0.15 M with respect to Na^+) were prepared and kept refrigerated. The bile salt stock solution was diluted 1:1 with phosphate buffer at the time of use. Individual fatty acids, their mono- and triglyceride esters were dissolved in chloroform to give concentrations of 75 mM, 24 mM, and 11 mM, respectively. Aliquots (10 ml) of each lipid solution were sealed under nitrogen and frozen until use. A 7 mM lecithin solution in benzene was made fresh for each experiment.

Tocopherol solutions were prepared freshly before each experiment. An appropriate amount of purified ^{14}C-α-tocopherol and unlabeled tocopherol were dissolved in hexane to give solutions with radioactivity of ca. 0.5 μci/mg α-tocopherol.

Preparation of Micellar Solutions

Two series of in vitro studies were done to

determine the effects of polar lipids singly or in combination upon the micellar solubilization of tocopherol. Polar lipids in the first series of studies were composed of fatty acids of long chain lengths (16-18 carbons) and in the second series of studies of fatty acids of medium chain length (8-12 carbons). Micellar solutions were prepared as follows: 1 mg tocopherol containing a known amount of radioactivity and 1 ml each lipid solution to be studied were placed in flasks protected from light. The combination of lipids studied are shown in Table I. After evaporation of the solvent at room temperature under nitrogen, 10 ml buffered bile salt solution was added. The final concentration of lipids in the incubation media was 1.1 mM triglyceride; 7.5 mM fatty acid, 2.4 mM monoglyceride, and 0.7 mM phospholipid. The flasks were stoppered with interconnecting tubing and all flasks were flushed with nitrogen for 5 min. The flasks then were sealed under nitrogen and shaken at 37-38 C for 22-24 hr. Preliminary studies had shown maximum solubilization of tocopherol had occurred by 22 hr of incubation under the conditions described. The concentrations of polar lipids (Table I), bile salts (12 mM), and Na^+ (0.15 M) and the pH (6.3) of the mixture were within the range reported to occur in vivo (19-21). Triglyceride was added in a constant concentration to provide a biphasic mixture. It was realized that, under normal physiological conditions, the quantity of triglyceride in the intestinal lumen is time dependent.

The mixtures after incubation were centrifuged at room temperature for 16 hr at 41,000 rpm (100,000 x G) in a Beckman model L ultracentrifuge. The micellar phase (usually 6 ± 0.5 ml) was drained into calibrated tubes. The aqueous phase (50 μliters) was mixed with 10 ml Bray's solution (22) in a counting vial, and radioactivity was counted in a liquid scintillation spectrometer model SL30 (Intertechnique Instrument, Dover, N.J.). The counting efficiency for ^{14}C with Bray's solution was found to be 90%. No significant quenching as judged by the internal-external standard ratio was observed.

A partition ratio (m/o ratio) was calculated as follows:

$$\text{m/o ratio} = M/(T\text{-}M),$$

where M is the amount of tocopherol in the micellar phase and T is the total tocopherol in the mixture.

RESULTS

The bile salt solution in the absence of triglyceride incorporated 87% of the tocopherol into the aqueous phase. Addition of unhydrolyzed lipid, thus providing a biphasic system, significantly decreased the solubilized tocopherol to 13% in the case of triolein and 7% in the case of trioctanoin. The difference between the amount of tocopherol in the aqueous phases when the mixture contained triolein or trioctanoin was small but statistically significant.

During fat absorption, lipids in the small intestine exist in a biphasic system, an oil phase, the quantity of which is time dependent, and an aqueous phase (21) between which polar and nonpolar lipids become partitioned. To simulate where possible these conditions in vitro, all subsequent studies (Table I, experiments 2-8) were done using this model and determining the effect of adding various polar lipids on the partitioning of tocopherol. The effectiveness of each polar lipid studied singly or in combinations on the partition ratio for tocopherol was evaluated against the biphasic system consisting of an oil and an aqueous phase (Table I, experiment 1).

Addition of oleic acid alone to mixtures containing triolein did not change the m/o ratio of tocopherol, whereas addition of either monoolein or dipalmitoyl lecithin significantly increased the ratio four- to fivefold. Addition of combinations of oleic acid and monoolein or of oleic acid and dipalmitoyl lecithin did not increase the m/o ratio of tocopherol above that which occurred following single addition of either monoolein or dipalmitoyl lecithin, respectively. The oleic acid-dipalmitoyl lecithin mixture, however, solubilized significantly ($P<.01$) more tocopherol than the oleic acid-monoolein mixture. When monoolein and dipalmitoyl lecithin were added together, the m/o ratio reached its maximum value which was significantly greater than that found for any of the other mixtures studied. In fact, addition of a third polar lipid, oleic acid, decreased the ratio.

Among singly added medium chain lipids, dioctanoyl lecithin was the only lipid which increased the m/o ratio of tocopherol significantly ($P<.01$). Of the combinations of medium chain lipids studied, all combinations, except the fatty acid-monoglyceride, increased significantly the m/o ratio of tocopherol ($P<.05$).

In all studies, the m/o ratios in mixtures containing long chain length lipids were consistently three-seven times greater than those in mixtures containing the corresponding polar lipids of medium chain length.

DISCUSSION

At a molar ratio of tocopherol to bile salt of

1:50, more than 85% of the tocopherol was solubilized into an aqueous phase when no triglyceride was present. The total amount of tocopherol solubilized, however, when based on wt, was less than 1.5% of the bile salt present. A similar amount of cholesterol is reported to be solubilized in a bile salt solution of comparable concentration (14, 15, 23). In a bulk water phase, both cholesterol and tocopherol have been classified as behaving similarly, as being an insoluble, nonswelling amphiphile (23). Small (23) suggested that this amount of aqueously dispersed, insoluble, nonswelling amphiphile may not be incorporated into a micelle per se but may be associated alternately with one molecule of bile salt or another in a type of monomolecular dispersion.

Adding triglyceride of either medium or long chain length dramatically decreased the tocopherol in the aqueous phase. The high affinity of tocopherol in a biphasic system for the oil, rather than the aqueous, phase, even in the presence of bile salts in concentrations well above their critical micellar concentration, suggests that if the vitamin does exist in the aqueous phase as part of a micelle, it is a very labile association.

In the absence of other polar lipids, the decrease in solubilized tocopherol was greater after addition of trioctanoin than after addition of triolein. This difference may be due to competition between tocopherol and trioctanoin for the limited space within the interior of the nonexpanded bile salt micelle. Studies using a variety of lipids indicate that the larger the hydrophobic region of the solubilizate the smaller the m/o ratio (24). Thus, one might anticipate that tocopherol which has a larger hydrophobic region has a small m/o ratio and that triolein would have a smaller m/o ratio than trioctanoin.

The m/o ratio for tocopherol was depressed following addition of oleic acid to the biphasic mixture containing triolein, monoolein, and dipalmitoyl lecithin. In biphasic systems not containing other polar lipids, Freeman (13) found the addition of oleic acid depressed the m/o ratio for stearic acid, although oleic acid enhanced the solubilization in a monophasic system. The effect on the m/o ratio suggests that oleic acid enhanced partitioning of the fatty acid into the oil phase. Cholesterol, another nonpolar lipid, also is solubilized more readily into an oil phase by the addition of oleic acid (25). Because tocopherol behaves as a nonpolar solute, the addition of oleic acid to the biphasic system might be expected to favor the depressed m/o ratio as observed.

The physiochemical reasons for the effect upon the system containing monoglyceride and phospholipid are not clear from our data. Addition of oleic acid to the mixed micellar biphasic system is likely to alter both phases. An effect upon the m/o ratio would occur only if one of the alterations is dominant. Any increased incorporation of fatty acid into the micellar phase could be offset by an enhanced solubilization of tocopherol in the oil. Such a mechanism in which the polar solute retinol competes with fatty acid for incorporation into micelles has been suggested by El-Gorab and Underwood (26). In the studies of El-Gorab and Underwood, however, addition of polar lipid retinol in the biphasic system containing monoglyceride and lecithin enhanced incorporation of the very nonpolar solute β-carotene. This suggested that these two solutes are incorporated into different regions of micelles. The polarity of tocopherol lies somewhere between that of retinol and β-carotene. The argument for competition for micellar incorporation favoring oleic acid over tocopherol might explain the depressed tocopherol m/o ratio. However, an expanded oily phase, by incorporation of the fatty acid which, in turn, would favor the partitioning of tocopherol in the oil phase, cannot be excluded.

Both monoolein and lecithin are known to increase the m/o ratio of a variety of relatively nonpolar solutes in monophasic bile salt solutions (13-17). One would anticipate that these lipids would act similarly, but less effectively, in biphasic systems. The ability of such amphiphiles, which are insoluble but swelling in water, to increase the solubility of nonpolar and insoluble, nonswelling amphiphiles, is due to an increase in the interior volume of the micelle (10, 16, 27, 28). Hence, tocopherol, another insoluble, nonswelling amphiphile presumably would be solubilized to a greater degree as the interior volume of the micelle expanded in the presence of monolein or dipalmitoyl lecithin. Support for this orientation of tocopherol within the micelle is found in the fact that the m/o ratio of tocopherol, when both monoolein and dipalmitoyl lecithin were present (1.42), was additive of that of the two mixtures containing one of these lipids (0.62 + 0.76) (Table I). This agreed with what was expected from findings reported by others (13).

The effectiveness of medium chain polar lipids on micellar solubilization of tocopherol was ca. one-third to one-sixth of that of long chain length lipids, except in the case of fatty acids. Two possible explanations for these observations were considered. First, due to their small molecular size, medium chain polar lipids may be unable to increase the size of micelles

sufficiently to increase the solubility of such a large molecule as tocopherol, unless the concentration of the polar lipids is increased. Furthermore, as the chain length of fatty acid decreases, the solubility of the fatty acid or of the fatty acid containing substance in bile salt solution increases (10, 13, 24). Lecithins, consisting of two short chain fatty acids or one short and one long chain length fatty acid, are reported to be soluble in buffer solution alone (29). Thus, to some extent, monomolecular solubilization of dioctanoyl lecithin (two medium chain length fatty acid) or monooctanoin might have occurred.

Alternately, medium chain polar lipids may contribute little to expansion of micellar size even when they are incorporated in the micelles due to their small hydrophobic region. The hydrophilic-hydrophobic balance determines how deeply a molecule will enter the micellar structure. The medium chain polar lipids would be expected to be more hydrophilically oriented, hence, more peripherally located than the corresponding polar lipids of long chain length. Therefore, most of the medium chain polar lipids solubilized in the micellar solutions would stay close to the surface of the micelle and contribute minimally to expansion of the internal volume.

Although the present study using tocopherol supports these explanations, opposite results have been reported for cholesterol. The addition of tri-, di-, and monoglycerides of 8 carbon fatty acids resulted in a significantly higher partition ratio of cholesterol than with the corresponding lipid of 18 carbon fatty acids (30). The discrepancy between the results using tocopherol as a relatively nonpolar solute and the work reported by others using cholesterol is not explained readily. The conditions employed in both studies were relatively similar, except for the bile salt concentration (12 mM for the present studies and 6 mM in previously reported studies), and the addition of lecithin in the present studies. The major difference, however, was the procedure for preparation of the micellar phase. In the present studies, the micellar phase was obtained after centrifuging, whereas in the studies reported earlier the micellar phase was prepared by equilibrating the aqueous and oily phases on both sides of a Millipore filter in a dialysis cell. Preliminary attempts to use the dialysis technique in our laboratory suggested that the bile salt concentration of the micellar solution was altered by preferential retention on the surface of the Millipore filter. This difference in technique might have contributed to the difference in results, but detailed comparative experiments of both techniques are needed to resolve the apparent discrepancy.

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Ratios of Polyunsaturated Fatty Acids to α-Tocopherol in Tissues of Rats Fed Corn or Soybean Oils

R. POUKKA EVARTS and **J.G. BIERI**, Laboratory of Nutrition and Endocrinology, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

ABSTRACT

This study was part of a larger experiment designed to assess the vitamin E adequacy of corn and soybean oils in relation to their polyunsaturated fatty acids (PUFA). Young male rats were fed a semipurified diet containing 20% corn or soybean oil and adequate selenium. After 8 and 12 weeks, animals were sacrificed, and 7 tissues analyzed for α- and γ-tocopherols and for fatty acids. Calculations were made of the molar ratios of total polyunsaturated fatty acids/α-tocopherol, and also of all polyunsaturated fatty acids, except linoleate, designated polyunsaturated fatty acids>18:2, to α-tocopherol. It is proposed that the latter ratio may have more significance, physiologically, than when linoleic acid also is considered. Tissues from rats fed corn oil had slightly more favorable (lower) ratios than did tissues from rats fed soybean oil. In both groups, the molar polyunsaturated fatty acids>18:2/α-tocopherol ratio was lowest for heart and lung, intermediate for muscle and testis, and highest for liver, kidney, and adipose tissue. Since both corn and soybean oils provide adequate vitamin E as determined by several biochemical and physiological parameters, adequate molar ratios of polyunsaturated fatty acids>18:2/α-tocopherol were: lung, 400; heart and leg muscles, 700; testis, 1100; liver and kidney, 1500-2000; and adipose tissue, 2000.

Animal studies clearly have shown that the vitamin E requirements of many species are affected by the amount of dietary polyunsaturated fatty acids (PUFA). Although interaction between tocopherols and unsaturated fatty acids may occur prior to intestinal absorption with loss of tocopherol, it is apparent that vitamin E adequacy at the tissue level is dependent upon a critical ratio of tocopherols to PUFA (1). Thus, it generally is acknowledged that a primary function of tocopherols is to stabilize cellular and subcellular membranes by preventing peroxidative damage to structural fatty acids (2,3).

Much information is in the literature on either the tocopherol or fatty acid content of tissues from animals fed various dietary regimes, but rarely have these two constituents been determined simultaneously in the same tissue. One of the first attempts to relate membrane PUFA and vitamin E was directed at the red cell membrane (4). It would be desirable to know the PUFA and vitamin E content of a variety of membrane systems but analytical difficulties have precluded efforts in this direction. As an alternative, analyses of whole tissues may be informative and can be rationalized on the basis that PUFA in tissues are predominantly in membrane phospholipids and that tocopherol in tissues is found predominantly in the membranous structures, mitochondria, and microsomes (5). In rat heart and skeletal muscles, phospholipids constitute ca. 50% of total lipid, while in liver, kidney, and spleen it is ca. 65-70% (6,7).

As part of a study to determine the vitamin E adequacy of the primary dietary vegetable oils in the U.S., we have analyzed tissues for α- and γ-tocopherols (8) and subsequently for fatty acids. This report gives the ratios of PUFA to α-tocopherol for eight tissues, and discusses the relevance of such ratios to estimating vitamin E status.

METHODS

Animals

Male, weanling Sprague-Dawley rats were caged individually and fed a vitamin E deficient diet containing 22% vitamin free casein, 6% salt mixture, 2% vitamin mixture, 4% cellulose, and 5% stripped lard (Eastman Organic Chemicals, Rochester, N.Y.). Selenium at 0.1 ppm was added as sodium selenite. After a week, when tissues partially were depleted of tocopherols (9), the animals were divided into two groups and fed the experimental diets ad libitum. These were prepared by replacing the lard and 15% of the sucrose with 20% of either corn oil (Mazola, Best Foods, CPC International, Englewood Cliffs, N.J.) or soybean oil (Crisco Oil, Proctor and Gamble, Cincinnati, Ohio). (Mazola contains propyl citrate and methyl silicone added by the manufacturer. 0.02% Butylated hydroxytoluene [BHT] was added as an addi-

TABLE I

Tocopherol and Polyunsaturated Fatty Acid Contents of Corn and Soybean Oils

Oil	Tocopherols, mg/100 g[a]		Fatty acids, %[b]	
	α	γ	18:2ω6	18:3ω3
Corn	20.3-22.8 (21.6)	65-85 (76)	57.6	1.1
Soybean	11.8-17.2 (14.5)	100-123 (108)	34.7	4.4

[a]Range of three samples, with average in parentheses.

[b]Average of two samples. Percent of total fatty acids.

tional precaution against dietary rancidity. Crisco oil has butylated hydroxyanisole [BHA], BHT, and methyl silicone added by the manufacturer.) Diets were prepared ca. every 14 days and were refrigerated. Food cups were filled Mondays, Wednesdays, and Fridays. Prior to sacrificing, the rats were fasted overnight and bled from the descending aorta while under ether anesthesia. Tissues were removed and frozen at -20 C until analyzed, usually within two weeks.

Tocopherol Analyses

Frozen tissue samples of 0.5-1 g were analyzed by a combination of saponification and thin layer chromatography (TLC), as previously described (10). Since this method originally was designed for determining only α-tocopherol, recovery experiments for both d, α- and d, γ-tocopherols were performed with each type of tissue. The tocopherols were obtained from Eastman Organic Chemicals, and the γ-tocopherol was purified by TLC prior to use. Amounts of tocopherols expected to occur in the experimental tissues were added to 0.5 - 1 g tissue from rats depleted of vitamin E for at least 8 weeks. Tissues from these rats had less than 1 μg α-tocopherol/g and no detectable γ-tocopherol. Because of much poorer recoveries from adipose tissue than from organs, a modified procedure was adopted. Fat tissue (0.3-0.5 g) and 15 ml absolute ethanol, containing 5% pyrogallol in a 50 ml erlenmeyer flask fitted with a reflex condenser, were brought to boiling. A stream of nitrogen was passed into the flask and 2 ml 60% KOH was added down the condenser. After 30 min, the hot plate was replaced by an ice bath and 15 ml each of water and hexane were added down the condenser. The condenser and nitrogen tube were removed, the flask quickly stoppered and shaken for 1 min. After the layers separated, the hexane was pipetted off and the extraction repeated twice. The hexane extract was washed with water and evaporated under nitrogen prior to the TLC separation.

TABLE II

Recovery of α- and γ-Tocopherols from Vitamin E Deficient Rat Tissues[a]

Tissue	Number of trials	Percent recovery[b]	
		α	γ
Liver	12	88.1±1.1	51.6±1.8
Heart	4	89.8±2.3	70.5±2.3
Testes	4	94.8±0.7	74.0±3.3
Lung	4	85.5±2.8	66.3±3.3
Spleen	4	90.5±3.3	73.5±1.8
Muscle	4	95.0±1.0	81.0±3.0
Kidney	4	86.5±2.0	64.8±3.5
Adipose	4	69.0±3.3	59.5±3.3

[a]Each tocopherol (15-25 μg) in ethanol was added to 0.5-1 g tissue prior to saponification. Control tissues gave values below 1 μg/g for each tocopherol.

[b]Mean ± standard error of the mean.

Total Lipid and Fatty Acid Analyses

Total lipids were extracted from 0.5 g tissue with chloroform-methanol (2:1). After removal of the methanol, the chloroform was evaporated and the residue extracted with hexane. After evaporation of an aliquot, the lipids were weighed. Another aliquot was saponified, acidified with HCl, and the fatty acids extracted into hexane. These were determined gravimetrically after evaporation of the solvent. Methylation of the fatty acids and gas liquid chromatographic (GLC) analysis were performed as previously described (11).

RESULTS AND DISCUSSION

In Table I are shown the PUFA and tocopherol contents of the samples of corn and soybean oils fed during the 12 week experiment. Corn oil had ca. 50% more α-tocopherol than did soybean oil, but the latter had considerably more γ-tocopherol than did corn oil. The content of PUFA was similar to those generally found in these oils in this country.

TABLE III

Polyunsaturated Fatty Acids in Tissues of Rats Fed Corn or Soybean Oils[a]

Tissue	18:2ω6	20:4ω6	22:6ω3	Other[b]	Total
		Corn oil			
Heart	30.4	17.0	2.8	6.1	56.3
Liver	27.3	20.8	2.8	4.0	54.9
Muscle[c]	35.7	9.4	2.3	4.5	51.9
Lung	35.2	6.0	0.2	5.0	46.4
Testis	10.1	14.3	0.5	22.4	47.3
Kidney	22.0	22.9	1.0	4.9	50.8
Adipose[d]	49.6	0.5	0.0	1.4	51.5
Adipose[e]	50.7	0.8	0.0	1.3	52.8
		Soybean oil			
Heart	25.1	18.6	6.5	4.6	54.8
Liver	24.4	18.2	3.9	4.1	50.6
Muscle[c]	25.2	7.4	3.7	4.9	41.2
Lung	25.1	5.9	0.4	4.8	36.2
Testis	12.4	12.3	0.8	18.5	44.0
Kidney	18.5	22.8	1.4	4.0	46.7
Adipose[d]	32.5	0.3	0.0	2.7	35.5
Adipose[e]	34.4	0.5	0.0	3.0	37.9

[a]Percent of total fatty acids determined by gas chromatography. Average of two rats, one after 8 and one after 12 weeks of feeding.

[b]Includes 18:3ω3, 20:2ω6, 20:3ω6, 20:5ω3, 22:3ω6, 22:4ω6, and 22:5ω6.

[c]Thigh muscle.

[d]Perirenal.

[e]Epididymal.

Table II shows the recovery of α- and γ-tocopherols from analyses of each tissue. Recovery of α-tocopherol was better than for γ-tocopherol with all tissues. Poorest recovery for α-tocopherol was obtained with adipose tissue, in spite of the special precautions taken to prevent oxidation.

Tissues from a rat in each group were analyzed after 6, 8, and 12 weeks for fatty acids and tocopherols. Since the fatty acid distribution changed very little between 8-12 weeks, the results from these 2 periods were averaged. In Table III is shown the percent distribution of PUFA in tissues of rats fed the two oils. For brevity, only the acids which predominated in most tissues are given, with the remaining acids being combined. Tissues of rats fed corn oil had more linoleic acid (except testis) and more total PUFA. This difference was largely accounted for by a higher oleic acid content in soybean oil rats. Adipose tissue content of linoleic acid accurately reflected the content of this fatty acid in the dietary oils, as often described. Lung tissue had the lowest PUFA content of all the tissues analyzed (except for adipose tissue of soybean oil rats).

The α-tocopherol values in Table IV are the average of animals killed after 8 and 12 weeks. Most tissues during this time interval either had a slight increase or remained essentially constant in their content of α-tocopherol (8). Adipose tissue, however, underwent a 50-100% increase during this 4 week period. Tissues from rats fed corn oil had higher concentrations of the vitamin than did those from rats fed soybean oil, reflecting the tocopherol content of the oils (renal adipose tissue was an exception). Conversely, tissues of rats fed soybean oil had higher amounts of γ-tocopherol (data not shown [8]). In both groups of rats, heart and lung had the highest concentrations of α-tocopherol, while muscle and testes were lowest.

Several studies have shown that, when membrane lipids undergo peroxidation, the fatty acids destroyed are predominantly those with four or more double bonds (12,13). In evaluating tissue tocopherol adequacy, it, thus, may be more meaningful to consider the ratio between tocopherol and the more highly PUFA, rather than between tocopherol and total PUFA. Thus, in Table IV are given both the total PUFA concentration in tissues and also the concentration of all PUFA with three or more double bonds (PUFA>18:2). This

TABLE IV

Tissue Concentrations and Ratios of α-Tocopherol and Polyunsaturated Fatty Acids in Rats Fed Corn or Soybean Oil

	Corn oil[a]			
	α-Tocopherol[b]		μmoles Total PUFA[c]	μmoles PUFA>18:2[d]
Tissue	μg/g	nmoles/g	μmoles α-T	μmoles α-T
Heart	22.6	52.5	810	350
Liver	13.9	32.4	2,620	1,200
Muscle	6.2	14.2	2,310	630
Lung	21.6	50.1	1,470	300
Testis	7.4	17.0	1,250	950
Kidney	8.6	20.0	2,390	1,260
Adipose[e]	11.0	25.3	55,000	980
Adipose[f]	15.2	35.3	43,000	1,270
			Soybean oil[a]	
Heart	14.0	32.4	1,300	670
Liver	10.5	24.3	3,160	1,510
Muscle	5.0	11.6	2,190	780
Lung	14.6	34.0	1,660	430
Testis	5.2	12.0	1,670	1,140
Kidney	5.4	12.5	3,440	1,990
Adipose[e]	15.1	35.0	27,000	1,770
Adipose[f]	18.2	42.1	25,000	2,000

[a]Average of two rats, one after 8 weeks and one after 12 weeks of feeding.

[b]Values are corrected for losses shown in Table II.

[c]Molar concentrations of polyunsaturated fatty acids (PUFA) were calculated using the mol wt of arachidonic acid as an average. α-T = α-tocopherol.

[d]Includes all acids from 18:3ω6 to 22:6ω3 (see Table III). Molar concentrations calculated as in footnote c.

[e]Renal.

[f]Epididymal.

comparison shows that linoleic acid comprised ca. one-half of the total PUFA in heart, liver, and kidney in both groups of animals. Muscle and lung have a higher proportion of linoleic acid, ca. two-thirds of the total PUFA, while testis had only ca. one-fourth of its PUFA as linoleate. Adipose tissue PUFA, as expected, was almost totally linoleate. As noted above, tissues of rats fed corn oil generally had higher concentrations of total PUFA than did tissues from the soybean oil rats; however, this difference was primarily due to increased linoleic acid in the tissues from corn oil rats. Consideration of the more highly unsaturated acids (PUFA>18:2) shows that the tissue concentrations in most tissues were similar in the two groups.

The molar ratios of total PUFA/α-tocopherol for the different tissues, excluding adipose, varied ca. threefold in both groups of rats (Table IV). Heart had the most favorable (lowest) ratio; lung and testis were similar with slightly higher ratios, and liver, muscle, and kidney had the highest ratios. The ratios in adipose tissue were very high, with those in corn oil fed rats being ca. double those for soybean oil fed rats. When linoleic acid is removed from consideration and only the PUFA>18:2/α-tocopherol ratios caluculated, the tissues take on a slightly different order. Heart and lung now have similarly low ratios, muscle and testes are intermediate and liver and kidney are highest. It is of interest that the ratios for adipose tissue are similar to those of the other tissues when linoleic acid is not considered. All ratios in tissues of the corn oil rats were lower than in those from soybean oil rats.

As reported previously (8), assessment of the vitamin E status of the rats in these experiments was made periodically, including a few of the animals maintained on the oils for 26 weeks. As determined from in vitro red cell hemolysis tests, plasma creatine phosphokinase levels and histological examination of the testes, these animals were normal in all respects. It would, thus, appear justified to state that the ratios of PUFA:α-tocopherol found in these tissues are biochemically and physiologically adequate. From the higher ratios in the rats fed soybean oil, it may be generalized that the following molar ratios of PUFA>18:2/α-to-

copherol are adequate: lung, 400; heart and leg muscles, 700; testis, 1100; liver and kidney 1500-2000; and adipose tissue, 2000.

The molar ratios of total PUFA/α-tocopherol found in these studies, ranging from ca. 1000-3000 for the various tissues, excluding adipose, may be compared with the ratio of 1100 required to prevent in vitro hemolysis of rat red blood cells (4). It should be noted that this ratio for red cells was obtained with young, rapidly growing rats supplemented with α-tocopherol for only 10 days. Subsequent studies with older animals given vitamin E and 0.1 ppm selenium continously for several months, as in the present study, have shown that a red cell PUFA/α-tocopherol ratio of ca. 2000 can protect the membrane (unpublished observations). In calculations of rat liver mitochondria from literature values, Gruger and Tappel (14) obtained a total PUFA/α-tocopherol ratio of 2100, presumably from rats fed stock diets.

There can be little doubt that the ratios found in our study are higher than the true ratios of PUFA>18:2 to total vitamin E activity. Tissues from rats fed both of these oils had significant concentrations of γ-tocopherol, as well as α-tocopherol (8). In the rats fed soybean oil, γ-tocopherol in adipose tissue was 50-90% of the α-tocopherol concentration, and, in the other tissues, the range was 30-60%. The biological activity of γ-tocopherol in the tissues is not known; dietary γ-tocopherol has a biopotency ca. 10% that of α-tocopherol (15,16). Bunnell, et al., (17) found similar activity for injected α- and γ-tocopherols in protecting chicks against encephalomalacia. Until the relative activity of γ-tocopherol present in membranes is known, the true vitamin E status of tissues of animals consuming a diet containing mixed tocopherols cannot be assessed with accuracy. In view of the high amount of γ-tocopherol now present in the U.S. food supply (8,18), these considerations have important implications in the assessment of human vitamin E status.

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Simplified Procedure for Preparation of ^{35}S-Labeled Brain Sulfatide

ARVAN L. FLUHARTY, MARSHA L. DAVIS, and **HAYATO KIHARA,** Neuropsychiatric Institute-Pacific State Hospital Research Group Pomona, California 91766, and **GENE KRITCHEVSKY,** Division of Neurosciences, City of Hope National Medical Center, Duarte, California 91010

ABSTRACT

A simplified procedure for the preparation of ^{35}S-labeled brain cerebroside sulfates has been developed. The labeled sulfatides are synthesized in vivo after intracerebral administration of inorganic ^{35}S-sulfate in developing rats. The animals are sacrificed three days later and brain homogenates extracted with chloroform-methanol. The extract is subjected to mild alkaline treatment and washed with water. The organic phase is chromatographed on triethylaminoethyl-cellulose from which sulfatides are eluted with chloroform-methanol containing potassium acetate. Radioactive fractions are pooled, concentrated, and potassium acetate removed by dialysis against water. Alternatively, salts are removed by passing radioactive fractions through Sephadex G-25. After evaporating to near dryness, the radioactive cerebroside sulfates are dissolved in a small volume of chloroform-methanol and stored at -20 C. The ^{35}S-sulfatides are essentially free of lipid contaminants, and 97% of the radioactivity corresponds with sulfatides on chromatographic analysis.

INTRODUCTION

Radioactively labeled sulfatides have facilitated studies on the cerebroside sulfate sulfohydrolase activity of arylsulfatase A and its deficiency in the genetic disorder metachromatic leukodystrophy (1-4). Labeled cerebroside sulfates are biosynthesized by administration of ^{35}S-sulfate to developing brain during the period of maximal myelination. We initially utilized developing rabbit brain in the manner of Mehl and Jatzkewitz (1) and isolated the radioactive sulfatides by a procedure designed for the concomitant isolation and analysis of several classes of lipids. The limited half-life (87 days) of the isotope made it necessary to repeat this time consuming and excessively involved preparation at periodic intervals. In addition, isotope administration necessitated surgical procedures, and a more convenient protocol was desirable. We attempted to develop a simple system specifically for the preparation of radioactive sulfatides of chemical and radioisotopic purity for enzymatic and metabolic studies requiring only materials and skills normally available in a biochemical laboratory.

A number of procedures have been described for the isolation of brain sulfatides (5). Many were developed for total lipid analysis and are quite extended. Some were designed specifically for sulfatides, e.g. the linked distributions procedure of Lees, et al. (6), but they have had inadequate capacity, recovery, or purity for our needs. The procedure described herein has overcome these problems and is particularly attractive because of speed.

Developing rat pups are utilized because the period of maximal myelination has been characterized fully (7) and because during a portion of this period the skull is thin enough to permit direct injection of isotope into the brain without surgery. Readily available equipment and reagents of commercial purity are utilized, and the preparation can be completed in 1-2 days after sacrifice of the animals. Sulfatide recovery is essentially quantitative, and chemical and radioisotopic purity is greater than 95%.

MATERIALS AND METHODS

Materials

Analytical grade solvents and reagents were used without redistillation or further purification, and no effort was made to purge working systems with nitrogen. Carrier-free $H_2{}^{35}SO_4$ (40-50 mCi in 0.5-1.0 ml H_2O) was obtained from New England Nuclear Corp., Boston, Mass. This was transferred to a container having a conical bottom, the water removed under a stream of nitrogen, and 0.1 ml water was added to redissolve the radioactive material.

Pregnant Sprague-Dawley rats were obtained from a local breeder. Litters were reduced to 10 pups/dam. When the animals were 10-14 days old, pups from a single litter were injected through the skull just to the right of midline between the ears with 10 μliter ^{35}S-sulfuric acid solution. The appropriate location and depth of injection for depositing the isotope near the center of the cerebral hemisphere was established by injecting dye into animals of the same age, sacrificing, and locating the dye. A 50

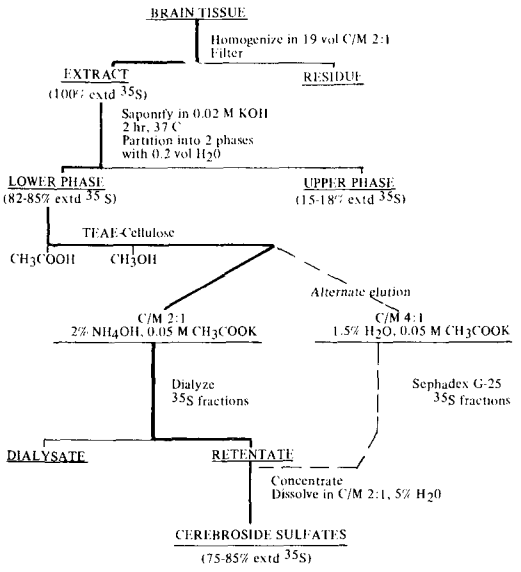

FIG. 1. Isolation of ^{35}S-cerebroside sulfates. Extd = extracted, TEAE = triethylaminoethyl, and C/M = chloroform-methanol.

μliter Hamilton syringe (no. 705N) with a fixed 2822 gauge needle provided a sufficiently rigid needle with relatively little dead space. After injection, the pups were returned to the dam. Three days after injection, the animals were sacrificed by decapitation and the brains removed.

Analytical Procedures

Radioactivity was estimated by liquid scintillation spectrometry. The colorimetric method of Kean (8) was used for sulfatide estimation with bovine brain sulfatides as the standard. Thin layer chromatography (TLC) was carried out on either E. Merck AG precoated silicic acid plates (without fluorescence indicator) or on plates prepared, as described previously (9). One dimensional plates were developed with chloroform-methanol-ammonium hydroxide (65:25:5, by volume); for two dimensional plates, the second solvent was chloroform-acetone-methanol-acetic acid-water (6:8:2:2:1, by volume). Radioautographs were prepared by exposing Kodak X-Omat X-ray film to dried plates. Radioactive zones were scraped and counted, or the plates were sprayed with sulfuric acid-formaldehyde and charred (9).

Preparation of Triethylaminoethyl (TEAE) Cellulose Column

A TEAE-cellulose column was prepared essentially as described by Rouser, et al. (10). Briefly, TEAE-cellulose (Selectacel, regular grade, Brown Co., Berlin, N.H.) was preconditioned by washing with three cycles of 1 N HCl and 0.1 N KOH, converted to the acetate form, washed with methanol, and dried in vacuo. A 10 g sample of TEAE-cellulose was dispersed in glacial acetic acid and allowed to equilibrate overnight. It then was packed into a 2.5 cm chromatographic column giving a packed ht of ca. 20 cm. The acetic acid was removed by washing with 450 ml methanol, and the adsorbent was converted to the hydroxyl form by washing with 300 ml 0.1 N KOH in methanol followed by 600 ml methanol, 300 ml chloroform-methanol 2:1 (C/M 2:1), and 300 ml C/M 9:1. The flow rate was maintained between 5-10 ml/min. Freshly prepared columns were used for each isolation, since residual radioactivity could not be removed.

Preparation of Sephadex G-25 Column

The Sephadex G-25 column used in an alternative procedure for removing potassium acetate was prepared by a procedure similar to that of Rouser, et al. (11). Sephadex G-25 (coarse, beaded, Pharmacia Fine Chemicals, New Market, N.J.), with fines removed and taken through several cycles of eluting and wash solvents, was equilibrated with methanol-water 4:1. This was packed to form a 2.5 x 12 cm column, and a 1 cm layer of sand was placed on top to stabilize the surface. The gel was washed with 300 ml methanol-water 4:1, then with an equal volume of C/M 4:1, containing 1.5% water at a flow rate of 5 ml/min. Preliminary testing revealed a small initial breakthrough of acetate when the TEAE eluant was applied. Otherwise, the capacity of the column for acetate removal was 10-fold greater than required. Contamination of sulfatides with acetate was circumvented by initial application of ca. 50 ml nonradioactive nonsulfatide containing forerun from the TEAE-cellulose column, before introduction of sulfatide containing fractions.

ISOLATION PROCEDURE

A flow chart of the isolation procedure is present in Figure 1. The pooled brains (ca. 10 g) were extracted with 19 volumes of C/M 2:1 by homogenization with two-thirds of the solvent in a Waring blender for 3 min. The solvent was decanted into the filter and the residue homogenized with the remaining solvent. The extract was gravity filtered through Whatman no. 1 filter paper. The blender and filter paper were rinsed with a small amount of C/M 2:1. The total volume at this state was ca. 180 ml.

TABLE I

Summary of ^{35}S-Sulfatide Preparations

Preparation	Animals (number)	Age (days)	^{35}S-SO_4 Injected (mCi)	Brain (g)	Sulfatide isolated (mg)	Sulfatide isolated (mCi)	Yield Sulfatide (mg/g)	Yield Isotope (%)	Specific activity (mCi/mg)
Rabbit 1	1	28	20	6.7	16.7	0.30	2.49	1.5	18.0
2	1	46	10	5.0	6.6	0.05	1.32	0.5	7.6
Rat 1	7	13	28	8.8	2.2	0.30	0.25	1.1	134
2	10	14	40	12.7	6.3	0.64	0.50	1.2	102
3	10	8	36.3	10.7	1.7	0.38	0.16	1.0	224
4	12	13	50	14.5	4.6	0.67	0.32	1.3	146
5	10	11	40	11.2	2.2	0.62	0.20	1.6	282
6	10	8	40	9.3	1.3	0.17	0.15	0.4	131
7	10	12	45	11.3	3.9	0.99	0.35	2.2	254
8	10	13	40	12.2	5.3	1.14	0.43	2.9	215

The extract was made alkaline to solvolyze phospholipids. Potassium hydroxide (224 mg) dissolved in 7 ml methanol was added to the filtered extract along with 14 ml chloroform, and the solution was incubated for 2 hr at 37 C. Water (40 ml, ca. 0.2 volumes) was added, the mixture shaken vigorously, and the phase separation facilitated by centrifugation at 600 x g for 20 min in 250 ml glass centrifuge bottles covered with aluminum foil. Upper phase was removed by aspiration without disturbing the white fluffy material at the interface. A small amount (ca. 10 ml) of equilibrated upper phase (6 ml $CHCl_3$, 96 ml CH_3OH, 94 ml H_2O) (12) was layered into the bottle without disturbing the interface, swirled gently, and aspirated. The surface washing was repeated three times. Sufficient C/M 2:1 was added to dissolve the residual aqueous phase and the interfacial fluff. Occasionally, some suspended solid material remained, but this contained no appreciable radioactivity and was removed by filtration.

The solution was applied to the TEAE-cellulose column and washed in with C/M 9:1 at a flow rate of ca. 5 ml/min. Neutral glycolipids, gangliosides, and weakly acidic lipids were removed by elution with 400 ml glacial acetic acid followed by 400 ml methanol. The strongly acidic lipids then were eluted with 400 ml C/M 2:1 containing 0.05 M potassium acetate and 0.3 N ammonium hydroxide (prepared by dissolving 2 g potassium acetate in 130 ml methanol then adding 260 ml chloroform and 8 ml concentrated ammonium hydroxide). The eluate was collected in 40-50 ml fractions which were monitored for radioactivity. After an initial 200-250 ml, the radioactivity appeared in 3-4 fractions. Fractions with more than 1% of the total radioactivity were pooled and concentrated in a rotary vacuum evaporator to near dryness. The concentrated material was transferred to a dialysis bag, and the vessel was rinsed a number of times with C/M 2:1 containing 5% water until the dialysis bag contained 10-20 ml. The bag then was tied very slack and dialyzed against 4 changes of 2 liters distilled water. Phase separation occurred almost immediately and an interfacial fluff formed. The phases were mixed periodically. The contents of the bag, both phases and fluff, were removed and evaporated to near dryness under a stream of nitrogen. The ^{35}S-sulfatide was taken up in 2-5 ml C/M 2:1 with 5% water and stored at -20 C.

Acetate was removed from the elution solvent by absorption to Sephadex G-25 in one preparation (Preparation rat 8, Table I). In this case, a less polar sulfatide eluant (C/M 4:1 containing 0.05 M potassium acetate and 1.5% water, prepared by dissolving 3 g potassium acetate in 120 ml methanol and 9 ml water, then adding 480 ml chloroform) was used in the TEAE chromatography. Sulfatide eluted as a sharp band after a forerun of ca. 500 ml. Starting with the one immediately preceeding the radioactive sulfatides, TEAE fractions were applied sequentially to the Sephadex G-25 column. Sulfatide containing eluate from the desalting column was pooled, concentrated, and stored as above.

RESULTS AND DISCUSSION

A summary of a number of rat brain sulfatide preparations is provided in Table I. Two rabbit brain preparations are included for comparison. In the last preparation, rat 8, acetate was removed with Sephadex G-25 and the isolation was completed in less than a day and a half. A trend in increasing isotope yield can be seen. We attribute this to experience and an increasing awareness of the parameters discussed below.

The effect of different routes of ^{35}S-sulfate administration upon isotope yield has been

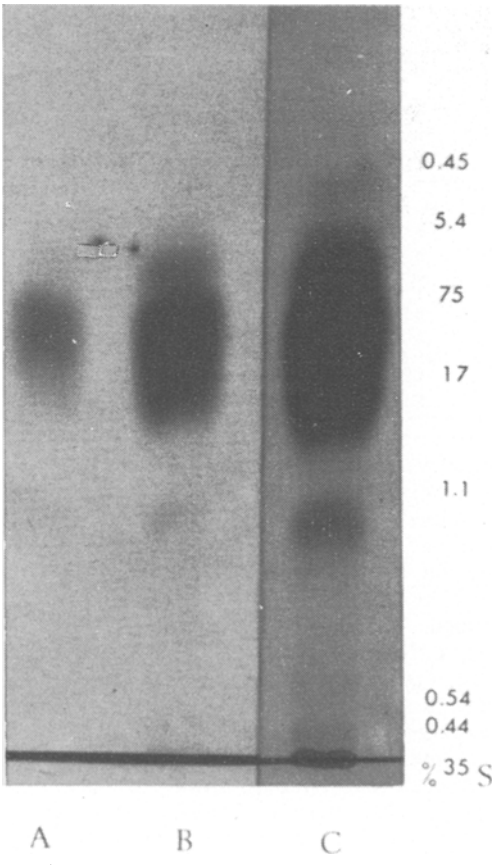

FIG. 2. Radioautograph of isolated ^{35}S-sulfatide subjected to thin layer chromatography. Aliquots of preparation rat 2 were chromatographed and radioautographed by exposure to X-ray film as follows: A-10 mμCi exposed for 24 hr, B-75 mμCi exposed for 24 hr; and C-75 mμCi exposed for 7 days. The areas indicated in the drawing were scraped from plate C, counted, and the distribution of the radioactivity is indicated. The solvent system for the chromatography was chloroform-methanol-ammonium hydroxide, 65:25:5.

evaluated with tracer doses. The variables tested included intraperitoneal, intravenous, subcutaneous, or intracranial (one or both hemispheres) injection, the same total dose injected as a single administration or divided into four daily administrations, and the $H_2{}^{35}SO_4$ prepared in Krebs-Ringer phosphate buffer, isotonic saline, or water. Brain sulfatides were labeled in all trials, but the intracranial injection gave the highest isotope yield. The yield was not increased by dividing the labeled precursor between hemispheres or into several daily doses, nor were there differences with the nature of the diluent. It was noted that too rapid an injection resulted in isotope loss by fluid forced out along the needle shaft which may have lowered yields in earlier preparations.

Isotope yield, sulfatide yield, and specific radioactivity are largely dependent upon the age of the animals used. The rat brain contains almost no sulfatide until ca. six days after birth. Subsequently, there is a period of rapid sulfatide synthesis which is maximal between the fifteenth and twenty-first day. About the twenty-first day, sulfatide content approaches adult levels, and the rate of synthesis begins to decline (7). For efficient utilization, radioactive sulfate should be administered during the period of enhanced synthesis. After the rats are two weeks old, their skulls have thickened such that injections with the fine needles we use becomes somewhat difficult. Highest specific activities should be achieved by administration of isotope early in myelination, while the endogenous level of sulfatide is low. When injections were made earlier than usual (rat 3 and 6), the specific activity of the isolated sulfatides was high in one preparation (rat 3), but the total sulfatide and radioactivity in both preparations were lower than normally obtained. We tend to favor injection of animals at 12-14 days, because high isotope yield, rather than specific activity, has precedence for current needs.

Rats were sacrificed three days after injection of ^{35}S-sulfate as a laboratory convenience, and this interval was not investigated as a variable. Green and Robinson (13) have reported that incorporation into sulfatides continues for ca. 2 days after administration of radioactive sulfate. Therefore, there may be a small increase in specific activity by sacrificing the animals earlier, while extension of this period would not be expected to lower the isotope yield noticeably because of the slow turnover of brain sulfatides.

The achievement of a high degree of isotopic purity of the isolated sulfatide was considered more important than chemical purity; however, reasonably high purity by both criteria was achieved. Early preparations contained phospholipid contaminants, but these were eliminated largely by the alkaline treatment. Several preparations have been examined by TLC and radioautography, and neither radioactive nor organic contaminants could be detected, unless the plates were loaded heavily. A radioautograph of such a plate is shown in Figure 2. Ca. 97% of the radioactivity was associated with the sulfatide areas. No contaminants could be seen on two dimensional plates spotted with 50 μg lipid and visualized by charring. More than 95% of the wt of material applied was recoverable from the sulfatide region. Aqueous, organic, or interfacial phases formed during dialy-

sis had identical sulfolipid compositions and were combined for the final product.

Over 80% of the radioactivity in the chloroform-methanol extract of the brain was recovered in the final product. Essentially all of the isotope loss occurred at the partition step. Material which distributed into the aqueous phase would not reextract into fresh organic phase which indicated hydrophilic compounds rather than sulfolipid.

Although this method relies on the relative homogeneity of brain sulfatides which contain little unsaturation, it should be useful for preliminary purification of sulfolipids from other sources. The near quantitative recovery of sulfatides also suggests its possible application for analytical purposes.

ACKNOWLEDGMENTS

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Lipid Composition of Normal and Hypertrophic Bovine Thyroids

A. LAGROU and **W. DIERICK**, RUCA Laboratory for Human Biochemistry, University of Antwerp, Antwerp, Belgium, and **A. CHRISTOPHE** and **G. VERDONK**, Laboratory of Gerontology, Dietetics, and Nutrition Research, State University of Ghent, Ghent, Belgium

ABSTRACT

The phospholipid content of bovine thyroid tissue amounts to 70% of total lipid. Triglycerides and cholesterol are the main neutral lipids. Only trace amounts of free fatty acid and esterified cholesterol are found, while two not yet identified components also are present. The distribution of lipid phosphorus in the different phospholipid classes is as follows: phosphatidyl choline, 43.0%; phosphatidyl ethanolamine, 25.2%; phosphatidyl serine, 5.6%; phosphatidyl inositol, 6.5%; sphingomyelin, 14.0%; cardiolipin, 2.8%; lysophosphatidyl choline, <1%; and phosphatidic acid, <1%. The phosphatidyl ethanolamines are rich in plasmalogens. The fatty acid patterns in the different lipid classes are reported. The essential differences between normal and hypertrophic bovine thyroid tissue are higher water content and lower triglyceride and sphingomyelin values for hypertrophic tissue.

INTRODUCTION

Phospholipids have been mentioned as possible mediators in iodide transport in thyroid tissue, unsaturated fatty acids being probable sites for iodide complexation (1-8). Lipids also have been implicated in thyroidal pathology. Hellwig and Wilkinson (9) did find much higher concentrations of total lipid and free fatty acid in cases of Hashimoto's disease than in non-inflammatory conditions. Levis, et al., (10) specifically reported striking differences in plasmalogen and lysophosphatidyl ethanolamine content between normal human thyroid tissue and cold nodules. From the foregoing, it is clear that thyroidal lipids are essential in normal thyroid function and probably also are involved in cases of dysfunction. Therefore, and because of the paucity of reports on thyroidal lipids in the literature (10-13), we decided to initiate the present study on the lipid composition of bovine thyroid. Most studies pay little or no attention to the neutral lipids. They are also imcomplete and sometimes conflicting with regard to the fatty acid composition of the different lipid classes (8, 10, 13).

MATERIAL AND METHODS

Thyroids from adult female bovine animals were transported to the laboratory chilled in crushed ice immediately after slaughtering. The thyroids available from the slaughterhouse were either normal or hypertrophic in nature. The hypertrophic thyroids collected from the town's slaughterhouse had a mean wt of ≅100 g, had a smooth appearance, and were relatively free of internal connective tissue. Histologically hyperplasia was noted. They also displayed a fivefold increase in acid phenyl phosphatase activity vs normal thyroid (mean wt ≅ 15 g). Adhering adipose and connective tissue was removed carefully. The remaining thyroid tissue was cut into small cubes and intensively washed with 0.14 M NaCl solution.

Lipids were extracted according to Rouser, et al. (14). However, it must be stressed that the first extraction step was performed by grinding a 5 g tissue sample in a Tenbroeck all glass homogenizer with 100 ml chloroform-methanol 2:1 (v/v) for 30 min (hollow pestle cooled with ice). The crude lipid extract was purified by Folch partition (0.2 vol 0.9% NaCl) (15).

The water content was calculated from wt differences (drying 0.1-0.2 g tissue in a vacuum dessicator to constant wt: ±72 h over potassium hydroxide at 40 C). Total lipids (TL) were estimated by gravimetric analysis with a Cahn electrobalance (16) and by the sulphuric acid-dichromate method using tripalmitin as a standard (17).

Neutral lipids were separated by thin layer chromatography (TLC) (Silica Gel H 0.3 mm/20 x 20 cm) using the following solvent systems: (A) petroleum benzine-acetone 80:20 (v/v) (bp range:60-80 C); (B) petroleum benzine-diethylether-acetic acid 90:10:1 (v/v/v); (C) petroleum benzine-diethylether-acetic acid 80:20:1 (v/v/v); (D) two step elution petroleum benzine-acetone 80:20 (v/v) + 5 g CO_2 ice/100 ml, 10 cm, and n-hexane, 15 cm; and (E) two step elution isopropylether-acetic acid 96:4 (v/v), ± 13-14 cm, and petroleum benzine-ethylether-acetic acid 90:10:1 (v/v/v), 20 cm. Triglycerides (TG) were assayed colorimetrically (18) and by gas liquid chromatography (GLC) after TLC using triheptadecanoate as internal standard (19). Cholesterol (CH) and cholesteryl esters (CHE) were determined by colorimetry

TABLE I

Chemical Composition of Normal and Hypertrophic Bovine Thyroid[a]

	Normal	Hypertrophic		Normal	Hypertrophic
Water	83.4 ± 1.0	87.1 ± 0.2		Percent of phospholipid P	
Total lipids	14.0 ± 1.5 (A)	15.9 ± 2.3 (A)	Phosphatidyl choline	43.0 ± 2.0	44.2 ± 2.1
	14.0 ± 2.1 (B)	14.6 ± 2.3 (B)	Phosphatidyl ethanolamine	28.2 ± 2.3	30.8 ± 2.8
Cholesterol	1.4 ± 0.2	1.6 ± 0.2	Nonplasmalogen PE	9.9 ± 0.7	12.4 ± 0.9
Cholesteryl esters	<0.05 (B)	<0.05 (B)	Plasmalogen PE	18.3 ± 1.4	18.4 ± 1.5
	0.04 ± 0.01 (C)	0.05 ± 0.01 (C)	Phosphatidyl serine	5.6 ± 0.8	5.7 ± 0.9
Triglycerides	4.1 ± 0.8 (B)	2.2 ± 0.3 (B)	Phosphatidyl inositol	6.5 ± 0.7	6.6 ± 0.7
	4.0 ± 1.0 (C)	2.1 ± 0.5 (C)	Sphingomyelin	14.0 ± 1.8	10.1 ± 1.4
Free fatty acids	<0.05 (B)	<0.05 (B)	Cardiolipin	2.8 ± 0.5	2.7 ± 0.4
	0.02 ± 0.004 (C)	0.02 ± 0.005 (C)	Phosphatidyl acid	<1%	<1%
Total phospholipids	9.2 ± 1.2	10.5 ± 0.9	Lysolecithin	<1%	<1%

[a]All values expressed as mg/g fresh wt, except water content (g/g%). All data are averages of at least 10 determinations performed on different extracts (5 g tissue). (A) gravimetric determination; (B) colorimetric determination; and (C) gaschromatographic determination.

after TLC separation (20-21). CHE also were estimated from their fatty acid content determined by GLC (internal standard: CH-heptadecanoate) (19). Free fatty acid (FFA) content was determined by colorimetry (22) and gas chromatography (internal standard: pentadecanoic acid) (19).

Phospholipids (PL) were assayed according to Rouser, et al. (23). Individual PL classes were estimated following two dimensional TLC in presence of the adsorbent (detection:iodine vapors). For two dimensional TLC separation of the PL, the following solvent mixtures were used: (A) Silica Gel H + 1% magnesium silicate 0.3 mm, chloroform-methanol-7N ammonia 90:54:11 (v/v/v), and chloroform-methanol-acetic acid-water 90:40:12:2 (v/v/v/v), (B) Silica Gel H + 10% magnesium silicate, chloroform-methanol-water 65:25:4 (v/v/v), and n-butanol-acetic acid-water 60:20:20 (v/v/v); (C) Silicia Gel H + 10% magnesium silicate, chloroform-methanol-28% ammonia 65:35:5 (v/v/v), and chloroform-acetone-methanol-acetic acid-water 5:2:1:1:05 (v/v/v/v/v). Plasmalogens were determined according to Broekhuyse (24). Possible interference by the breakdown of nonplasmalogen phospholipid was checked by using ^{14}C-labeled egg yellow phosphatidyl ethanolamine (PE) free of vinylether linkages (PE yield ∿99%).

The fatty acid patterns of the different lipid classes were determined after TLC, by GLC of the corresponding methyl esters. The spots were visualized with 0.05% Rhodamine 6G or 0.2% 2.7-dichlorofluoresceine and viewed under UV light. Each fraction was transmethylated in the presence of silica gel using 5 ml 5% sulphuric acid solution in methanol by heating at 65-70 C overnight (19). The different methyl ester peaks were identified by comparison of their equivalent chain length (ECL) on a polar (5% EGSS-X) and an apolar column (5% SE-30) with those of standards commercially available or prepared from brain lipids.

RESULTS

The results are summarized in Tables I and II. Table I shows TG and free CH to be the main neutral lipids. On TLC (Fig. 1), no mono- (MG) or diglycerides (DG) are detected. CHE and FFA are present in only minute amounts. However, TLC of the neutral lipids also reveals the presence of two unidentified compounds. The first one (X_1), migrating on TLC between CH and TG (R_f = 0.56/solvent system A) and staining intensively with iodine, cannot be identified as ubiquinone or α-tocopherol. It contains neither phosphorus nor fatty acids and does not stain with α-naphthol. The second one (X_2) runs closely behind the MG (R_f-value 0.06/solvent system A). It contains fatty acids with a distribution distinctly different from the TG fraction (Table II). It does not contain phosphorus and gives a negative α-naphthol staining. Running ahead of CHE (near the

TABLE II

Fatty Acid Composition of Lipids from Bovine Thyroid and Adhering Adipose Tissue[a]

Fatty acid designation[b]	Total fatty acids		Triglycerides		Free fatty acids		Total phospholipids		Cholesterol esters	X_2	Phosphatidyl choline	Phosphatidyl ethanolamine	Sphingomyelin	Phosphatidyl inositol	Phosphatidyl serine
	I[c]	II[d]	I[c]	II[d]	I[c]	II[d]	I[c]	II[d]	I[c]	I[c]	I[c]	I[c]	I[c]	I[c]	I[c]
14:0	Tr[e]	3.0	2.2	3.7	1.6	3.4			Tr				Tr		
A	2.7						2.3				Tr	11.8			
16:0	21.7	29.4	21.2	30.9	32.1	25.5	22.0	23.9	16.1	16.1	39.1	11.5	25.9	12.5	3.9
16:1	1.1	1.1	1.9	1.6	1.4	2.6	Tr	1.2	5.4	Tr	Tr	Tr	Tr	Tr	1.3
B	1.8						1.5				Tr	8.5			
18:0	14.3	31.5	17.5	28.1	18.2	18.3	13.1	27.4	6.7	15.4	7.5	12.7	12.1	33.7	35.7
18:1	33.7	33.5	55.6	34.6	30.4	48.1	28.2	30.9	19.2	27.3	36.2	25.3	1.5	12.3	37.1
18:2	6.8	1.3	1.6	1.2	5.2	2.2	10.6	2.6	40.6	9.2	12.4	6.1	1.1	5.5	2.8
18:3	Tr				1.6				11.7						
20:0	1.7						1.7	1.5					4.9	Tr	3.5
20:1	Tr						Tr								2.1
20:3	Tr						Tr							2.7	1.6
20:4	7.9				6.0		11.1	8.0		16.4	2.9	14.1		25.6	3.1
22:0	1.4						1.7	2.0					29.3		
22:1	Tr												0.9		
22:6	4.4				3.7		7.8	2.4		6.3	1.9	9.9		6.6	7.1
23:0	Tr												3.4		
24:0	2.6						Tr						18.0		
24:1	Tr									9.0 ?			2.7	1.1 ?	1.7 ?

[a]As percent of total fatty acids.

[b]A and B = aldehydes, hexadecanal and octadecanal.

[c]Bovine thyroid.

[d]Adhering adipose tissue.

[e]Tr = trace.

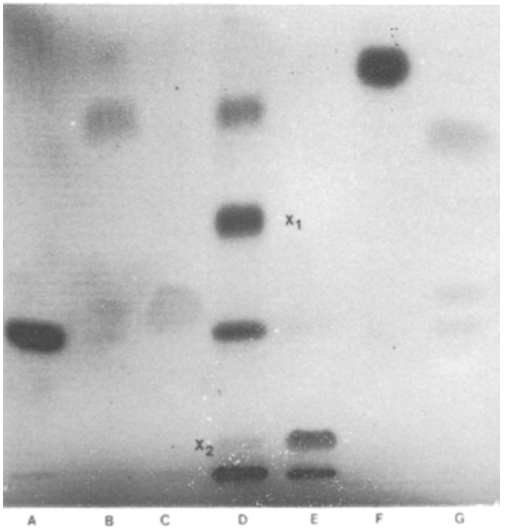

FIG. 1. Thin layer chromatographic pattern of total lipid extract. Experimental conditions: Silica Gel H, 0.3 mm; petroleum benzine-acetone, 80:20 (v/v); and iodine staining. Lanes: (A) cholesterol, (B) tripalmitin, (C) stearic acid, (D) thyroid lipid extract, (E) lecithin + monopalmitate, (F) cholesteryl stearate, and (G) methyl stearate + dipalmitate. X_1 and X_2 = unknowns.

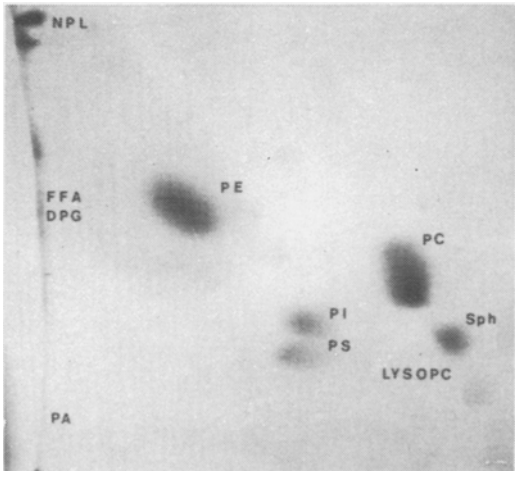

FIG. 2. Two dimensional thin layer chromatography of total lipid extract. Lysolecithin (LysoPC) added as internal standard. Experimental conditions: Silica Gel H + 1% alkaline magnesium silicate:solvent systems (A). NPL = nonpolar lipids, FFA = free fatty acids, PE = phosphatidyl ethanolamine, DPG = cardiolipin, PC = phosphatidyl choline, PI = phosphatidylinositol, Sph = sphingomyelin, and PS = phosphatidyl serine.

solvent front), a faint spot probably due to hydrocarbons is visualized.

PL represent the bulk (70%) of total thyroidal lipids. Two dimensional TLC (Fig. 2) shows phosphatidyl choline (PC) to be the main PL next to PE. PE has a remarkable high plasmalogen content. High values also are found for sphingomyelin (Sph). Phosphatidyl inositol (PI) and phosphatidyl serine (PS) are present in nearly equal amounts. Cardiolipin (DPG), phosphatidic acid (PA), and lysolecithin (LysoPC) are found in much lower concentrations.

$C_{16:0}$, $C_{18:0}$, and $C_{18:1}$ fatty acids are the main fatty acids in the TG fraction (Table II), $C_{14:0}$, $C_{16:1}$, and $C_{18:2}$ fatty acids being present in much smaller amounts. The fatty acid pattern of FFA is distinctly different, showing the presence of polyunsaturated fatty acids as $C_{20:4}$ and $C_{22:6}$. The fatty acid distribution in the CHE fraction is characterized by the presence of more than 10% $C_{18:3}$. The fatty acid pattern of X_2 is similar to the fatty acid distribution in PL. The total PL fraction shows predominance of $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{20:4}$, and $C_{22:6}$. The fatty acid pattern in the PC fraction is similar. PE is characterized by the presence of two additional peaks (A and B). In the fatty acid composition of PI and PS, one finds all fatty acids mentioned in the other fractions, however, in a quite different distribution pattern. Arachidonic acid amounts to 25% of the fatty acids present in PI, while in PS only a small percent of this fatty acid is found. PS also is characterized by the presence of small amounts of $C_{20:0}$ and $C_{20:1}$. The fatty acid distribution in Sph is, as in most tissues, characterized by the absence of polyunsaturated fatty acids and the presence of long chain fatty acid, $C_{22:0}$ being the most abundant fatty acid next to $C_{16:0}$, $C_{18:0}$, and $C_{24:0}$.

Finally, the fatty acid patterns in the different lipid classes (neutral and polar) are the same for both normal and hypertrophic tissues.

DISCUSSION

The data summarized in Tables I and II do not show clear-cut differences between normal and hypertrophic tissues. Water and TG contents, however, differ: the water content being higher, the TG content being lower, for hypertrophic tissue. A lower TG value for hypertrophic tissue could be due to a lesser degree of contamination with exogenous adipose tissue, as anatomical preparation of pure hypertrophic thyroid tissue is much easier to perform. However, comparison of the fatty acid patterns (Table II) for TG and FFA of exogenous fat vs thyroid tissue (normal and hypertrophic) does show pronounced differences. Therefore, the different TG values for normal and hypertrophic tissues cannot be due to differing degrees

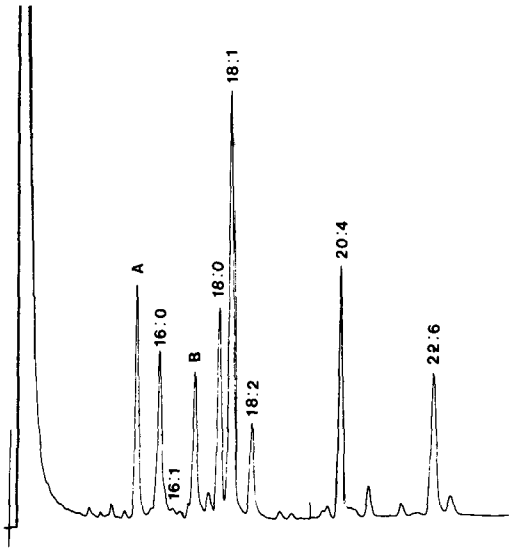

FIG. 3. Fatty acid pattern of bovine thyroid phosphatidyl ethanolamine.

of contamination with adipose tissue.

The results for CH are in good agreement with the data reported by Satyaswaroop (13), except for a much lower degree of esterification. Satyaswaroop (13) calculated the CHE content by subtracting free from total CH, while our CHE determinations were performed after TLC by a colorimetric and a GLC procedure which gave coinciding results. The fatty acid pattern of CHE is similar to that from bovine serum CHE (25). Therefore, contamination with plasma-CHE is a likely explanation for the high tissue CHE found by Satyaswaroop (13).

The presence of unidentified compounds (X_1 and X_2) in the neutral lipid fraction have not yet been reported elsewhere. A component behaving in a similar way as X_1 on TLC analysis has been found in lipid extracts of pituitary gland and identified as polyprenol (26-27). Polyprenol (dolichol) also has been found in liver extract (28). Skipski, et al., (29) mentions an unidentified compound similar to X_2 in liver extracts. Further work is now in progress to isolate these two unknown compounds on a preparative scale and to perform some further spectroscopic studies for identification.

The PL values are in good agreement with the data in the literature (13). They are slightly higher than the values for female pig thyroid and appreciably higher (± factor two) than those reported for human female thyroid (10). Total PL in hypertrophic tissue does not deviate appreciably from normal levels. The percent lipid phosphorus distribution is in good agreement with the data reported elsewhere (13). However, our values for lysoPC are much lower, while DPG and PA were not included in Satyaswaroop's data (13). Sph values for hypertrophic tissue are lower than the amount of Sph found for normal bovine thyroid tissue. PL distribution in bovine and pig thyroids are similar, while in human thyroid the PS seems to be much higher (10).

The fatty acid pattern in bovine thyroid mitochondrial and microsomal PL showed in some PL fractions the presence of high concentrations of $C_{12:0}$, $C_{14:0}$, and $C_{14:1}$, while Vilkki's and Jaakonmaki's finding (8) of nervonic acid in the PC fraction also was confirmed (13). However, as shown in Table II, we were not able to demonstrate significant amounts of these fatty acids in our PL preparations. This agrees with the results reported by Levis, et al. (10). Total PL fatty acid pattern of bovine thyroid is similar to the pattern found for human thyroid (10). Nevertheless, in the PE and PC fractions, significant differences can be noted. In human PC, ca. 30%, $C_{18:0}$; 10%, $C_{18:1}$; and 5%, $C_{18:2}$ are present. In bovine PC, the values are respectively 8, 36, and 12. In human PC, no $C_{20:4}$ was found. In bovine PC, a value of 3% was found. $C_{22:6}$ was not determined in human PL fractions. A value of 2% $C_{22:6}$ was found in bovine PC. In human PE, the $C_{18:0}/C_{18:1}$ ratio also is reversed, while the $C_{20:4}$ percentage is nearly the same for both human and bovine PE.

Our results reveal a considerable part of the PE fraction to be plasmalogens. This agrees with the values reported for human, pig, and dog thyroids (10, 12). The GLC pattern of the PE fraction represented in Figure 3 shows the presence of two peaks A and B. The ECL values for A and B were on a polar column (5% EGSS-X) 15.3 and 17.3 vs 14.9 and 16.9 on an apolar column (5% SE30). As dimethylacetals are expected to be eluted behind the corresponding fatty acid methyl esters on an apolar column, these peaks could not be identified as $C_{16:0}$ and $C_{18:0}$ dimethylacetals. A difference in ECL of 2 on both columns suggest A and B to be homologuous components. Comparing the ECL values of A and B with data reported by Krishnan, et al. (30), one can conclude that compounds A and B must be identified as the aldehydes, hexadecanal and octadecanal.

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Biosynthesis of Triglycerides in Freshly Secreted Milk from Goats

W.W. CHRISTIE, The Hannah Research Institute, Ayr, Scotland KA6 5HL

ABSTRACT

It has been confirmed that freshly secreted milk from goats contains triglyceride synthetase activity which is not stimulated by addition of the usual range of cofactors, e.g. coenzyme A, adenosine 5'-triphosphate, Magnesium ions or α-glycerophosphate. On the other hand, free glycerol did stimulate the reaction, although only low levels of glycerol-kinase could be detected. The products were principally, triglycerides, diglycerides, and phosphatidic acid, i.e. those expected of the phosphatidic acid pathway of triglyceride biosynthesis together with some phosphatidyl choline and phosphatidyl ethanolamine. In addition, some of the enzymes of the monoglyceride pathway appeared to be present since 2-O-alkylglycerols, in particular, were converted to monoacyl-monoalkyl glycerols together with trace amounts of diacyl alkyl-glycerols. The milk incorporated a wide variety of isotopically labeled fatty acids into lipids at widely differing rates (18:2 at 10 times the rate of 18:0, for example) and stereospecific analysis confirmed that fatty acids entered all three positions of the sn-triacyl glycerols according to quite specific patterns. Stearic acid was desaturated rapidly to oleic acid, and virtually all the 18:1 formed was esterified (to all three positions in the triglycerides also). Freshly secreted milk is a potentially useful alternative to tissue obtained surgically as a source of mammary triglyceride biosynthetic enzymes for the study of milk fat biosynthesis in vitro.

INTRODUCTION

More than 20 enzymes have been isolated and identified in milk, and activities of many more have been detected (1). For example, freshly secreted milks from goats (2) and sows (3) have been shown to have the capacity to synthesize triglycerides and other lipids from added isotopically labeled fatty acid and to synthesize ethyl esters of fatty acids when ethanol and labeled fatty acids were added (4). In addition in goat milk, stearic acid was desaturated to oleic acid and incorporated into lipids (5). It has not been established whether the normal mammary triglyceride synthetase was operative in freshly secreted milk, whether some unidentified nonspecific acyl transferases were responsible for the observed effects, or indeed, whether the reaction was truly enzymatic. In this study, the potential of freshly secreted milk as a source of mammary triglyceride synthetase for the study of triglyceride biosynthesis in vitro has been investigated.

EXPERIMENTAL PROCEDURES

Materials

(1-^{14}C)-*Trans*-11 octadecenoic acid was prepared biosynthetically (6). (1-^{14}C)-Pentadecanoic acid, (1-^{14}C)-heptadecanoic acid, and tri-(10,11-^{3}H)-heptadecanoin were prepared synthetically (7), as were 2-O-(1'-^{14}C)-hexadecylglycerol and 1(3)-O-(9',10'-^{3}H)-hexadecylglycerol (8). All other radioactive compounds and synthetic intermediates were purchased from the Radiochemical Centre, Amersham, England. Sigma London Chemical Co. (Kingston-upon-Thames, Surrey, England) supplied the cofactors used.

Animals and Milk Samples

Three 2 year old female goats in their first lactation were milked dry by hand at 6:30 a.m., 9:00 a.m., and 10:00 a.m. The milk from the first two milkings, together with the first and last few ml from the last milking were discarded, and the remainder of the milk from the first milking, pooled from the three goats, was used as a source of triglyceride synthesizing enzymes.

Incubations

The freshly secreted milk was cooled in ice and centrifuged at 700 x g to bring the cream to the top and deposit any heavy particles. In most experiments, the infranatant layer (10 ml portions) was incubated with isotopically labeled fatty acids (neutralized with KOH and bound to defatted bovine serum albumin) or glyceryl ethers (0.2 μCi, 0.1 μmole) bound to albumin (by ultrasonification with gentle warming) at 37 C with gentle agitation in air.

Lipid Extraction and Analysis

At the end of the incubation, lipids were extracted immediately with chloroform-methanol (2:1, v/v) by the procedure of Bligh and Dyer (9). Aliquots of the extracts were set aside for liquid scintillation counting. The main lipid classes (with unlabeled free fatty acids and

diglycerides added as carriers) were separated on thin layers of Silica Gel G (0.5 mm thick; E. Merck, Darmstadt, Germany); hexane-diethyl ether-formic acid (70:30:0.5 by volume) was the developing solvent. Lipids were detected under UV light after spraying with 2',7'-dichlorofluorescein in methanol (0.01%, w/v) and were identified by cochromatography with authentic standards. When glyceryl ethers were incubated, unlabeled glyceryl ethers were added as carriers, and, after removal of the diglyceride band from the TLC plate, the plate was replaced in the tank and redeveloped in diethyl ether-acetone (9:1, v/v) as far as the gap to ensure separation of unchanged glyceryl ether from the phospholipids. Phospholipids, recovered from the adsorbent by elution with chloroform-methanol-water (5:5:1 by volume), were fractioned further into individual phospholipid classes by chromatography on thin layers of silica gel without binder (0.5 mm thick; Camag, Muttenz, Switzerland); chloroform-methanol-acetic acid-water (25:15:4:2 by volume) was the developing solvent (10). Recoveries of radioactive lipids from the TLC plates were calculated routinely and varied between 95-102%. The methyl ester derivatives of the fatty acid constituents of lipids were prepared when required by sodium methoxide catalyzed interesterification (11).

Liquid Scintillation Counting

Radioactive samples were counted in toluene-fluor or in suspension on thin layer adsorbents in Unisolve 1 (Koch-Light Laboratories, Colnbrook, Bucks, England) in a Tricarb model 2425 liquid scintillation spectrometer (Packard Instruments, Caversham, Reading, Berks, England). Counting efficiencies in each channel were determined by means of an automatic external standard calibrated for the purpose.

Silver Nitrate Chromatography

Methyl esters of saturated and *cis*-monoenoic fatty acids were separated for liquid scintillation counting on thin layers of Silica Gel G (0.5 mm thick) containing 10% (w/w) silver nitrate. Hexane-diethyl ether (9:1, v/v) was the developing solvent. Compounds were recovered from the adsorbent by the procedure of Åkesson (12).

Stereospecific Analysis of Triglycerides

The procedure of the stereospecific analysis of triglycerides that contain isotopically labeled fatty acids has been described elsewhere (7). Tri-(10,11-^{3}H)-heptadecanoin was added as internal standard, and the amounts of the incorporated (^{14}C)-labeled fatty acid relative to that

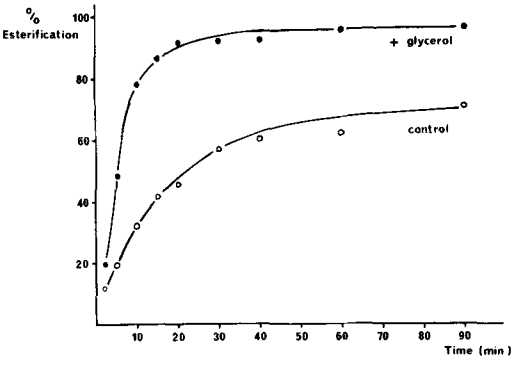

FIG. 1. The effect of the addition of glycerol (10 μmoles) on the percent esterification of (1-^{14}C)-palmitic acid (0.1 μmoles) by freshly secreted milk (10 ml) from goats with respect to time. ○ = Control, i.e. no glycerol and ● = with added glycerol. In three experiments, standard deviations were always less than 10% of the mean value.

of the standard in each of the products of the reaction were determined by dual isotope liquid scintillation counting after conversion to the methyl esters. Results for position sn-1 were obtained by analysis of the lysophosphatide produced in the final stage of the procedure, those for position sn-2 were obtained by pancreatic lipase hydrolysis and those for position sn-3 were calculated by difference from the known triglyceride composition.

RESULTS

Preliminary experiments confirmed the results of McCarthy and Patton (2) that (1-^{14}C)-palmitic acid (0.1 μmoles/10 ml) was incorporated rapidly into triglycerides, diglycerides, and phospholipids on incubation with freshly secreted milk from goats in vitro. In experiments with milk from older goats (3-6 years old), up to 30% of the label was incorporated into lipids within a few min when the reaction virtually ceased; but, with milk from young goats in their first lactation (2 years old), the initial rate was less rapid, however, esterification was still proceeding after 90 min. The latter milk was used in all the following experiments. Also, partly in confirmation of McCarthy's and Patton's results, no stimulation of lipid synthesis was obtained by addition of adenosine 5'-triphosphate (ATP) (0.5-50 μmoles), α-glycerophosphate (0.5-50 μmoles), or coenzyme A (CoA) (0.2-5 μmoles) to the milk. Similarly, addition of Magnesium ions (0.5-30 μmoles) or ethylenediaminetetraacetic acid (EDTA) (1-30 μmoles), and combinations of these cofactors had no effect. However, preincubation of the milk with ATP-ase consid-

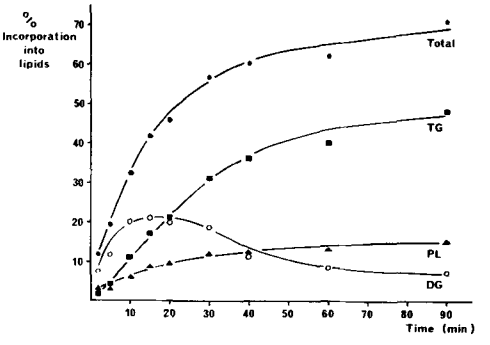

FIG. 2. The percent incorporation of (1-^{14}C)-palmitic acid (0.1 μmole) into lipid classes by freshly secreted milk (10 ml) from goats with respect to time. ● = Total esterification, ■ = triglycerides (TG), ○ = diglycerides (DG), and ▲ = phospholipids (PL). In three experiments, standard deviations were always less than 10% of the mean value.

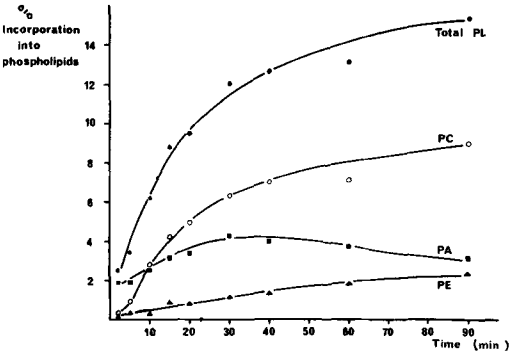

FIG. 3. The percent incorporation of (1-^{14}C)-palmitic acid (0.1 μmoles) into individual phospholipid classes by freshly secreted milk (10 ml) from goats with respect to time. ● = Total phospholipids (PL), ○ = phosphatidyl choline (PC), ■ = phosphatidic acid (PA), and ▲ = phosphatidyl ethanolamine (PE). In three experiments, standard deviations were always less than 12% of the mean value.

erably reduced the activity, and ultrasonification for 30 sec or addition of Tween 20 (10 μmoles) eliminated all activity, and the activity could not be restored by the addition of cofactors.

As Kinsella (13) had shown that freshly secreted bovine milk contained glycerol kinase, the effect of addition of glycerol to the medium upon the incorporation of (1-^{14}C)-palmitic acid was studied. In 30 min incubations, addition of 10-200 μmoles glycerol to the milk produced uniformly a 40-50% stimulation of esterification. This stimulation was confirmed by longer experiments in which samples were taken at intervals from the control and glycerol supplemented milks (containing 10 μmoles glycerol) for analysis. The results are illustrated in Figure 1. In the glycerol supplemented milk, palmitic acid was rapidly esterfied and, indeed, after 40 min, little unesterified 16:0 remained. In the control milk, the reaction was slower and was still proceeding after 90 min. The distribution of labeled 16:0 among the various lipid classes was determined and that for the control milk is illustrated in Figure 2. Triglycerides were the principal product and still were being formed at a significant rate after 90 min of incubation. Diglycerides were formed rapidly initially, reached a maximum, then diminished while the proportion of the phospholipid fraction increased steadily at first and leveled off after 60 min. Similar results were obtained by McCarthy and Patton (2). The pattern of products was, in fact, that commonly found for triglyceride biosynthesis by the α-glycerophosphate pathway, which is known to be the major mechanism of triglyceride biosynthesis in mammary tissue (reviewed recently by Patton [14] and Mooore [15]). A similar range of products was obtained when glycerol was added to the medium, except that the initial rate for all products was greater and the diglyceride maximum was higher.

In addition, the phospholipid fraction was separated from the other lipids, and individual phospholipid classes were isolated by thin layer chromatography (TLC) so that the amounts of labeled 16:0 incorporated by each could be determined. The results are illustrated in Figure 3. Phosphatidyl choline, phosphatidic acid, and phosphadtidyl ethanolamine were the main products, although some label also was found in sphingomyelin and phosphatidyl inositol. The amount of label in the phosphatidyl choline, which was the most abundant component, increased throughout the incubation. The amount of the label in the phosphatidic acid increased rapidly initially and reached a maximum (later than the diglyceride maximum) before diminishing once more. The amount of label in the phosphatidyl ethanolamine increased slowly but steadily throughout the incubation period. Phosphatidic acid is also an important intermediate in the α-glycerophosphate pathway for triglyceride biosynthesis, although normally is has a much more rapid turnover than was apparent here. Phosphatidyl choline (with some phosphatidyl ethanolamine) was the main phospholipid formed on incubation of (^{14}C)-palmitic acid with dispersed mammary cells in vitro (16).

Attempts were made to establish whether glycerol kinase was present in freshly secreted goat milk by incubating the milk with (1,3-^{14}C)-glycerol and determining the radioactivity in the glycerolipids. Some glycerol

TABLE I

Incorporation of Isotopically Labeled 1-O- and 2-O-Hexadecylglycerols into Lipids by Freshly Secreted Milk in Vitro during 30 min Incubations[a]

Compound		Percent incorporation into lipids			
		Monoalkyl diacylglycerol	Monoalkyl monoacylglycerol	Free fatty acids	Phospholipids
1-O-Hexadecylglycerol	Mean	1.46	3.14	1.90	3.06
	SD	0.32	0.17	0.41	0.42
2-O-Hexadecylglycerol	Mean	0.94	29.86	3.10	0.40
	SD	0.16	3.80	1.32	0.15

[a]Means and standard deviations (SD) of five experiments.

TABLE II

Amounts[a] of Various Isotopically Labeled Fatty Acids Incorporated into Lipids by Freshly Secreted Goat Milk in 60 min Incubations Relative to Palmitic Acid[b]

Fatty acid	Amount incorporated into each lipid		
	Triglycerides	Diglycerides	Phospholipids
16:0	100 ± 6	40 ± 6	29 ± 3
12:0	146 ± 8	27 ± 4	22 ± 2
14:0	157 ± 7	31 ± 5	33 ± 3
15:0	85 ± 6	33 ± 4	23 ± 2
17:0	62 ± 6	17 ± 3	12 ± 2
18:0	21 ± 3	7 ± 2	5 ± 1
18:1 (n-9) *cis*	232 ± 11	41 ± 7	33 ± 4
18:1 (n-7) *trans*	290 ± 16	46 ± 7	41 ± 4
18:2	233 ± 19	52 ± 6	95 ± 7
18:3	182 ± 13	34 ± 4	39 ± 4

[a]Means and standard deviations of four experiments.

[b]The amount of palmitic acid incorporated into triglycerides was given the arbitrary value of 100 (equivalent to 0.474 μmoles).

kinase activity, ca. equivalent in magnitude to the base level for bovine milk described by Kinsella (13), was observed, but it proved impossible to stimulate this activity by addition of free fatty acids or ATP, as reported by Kinsella (13).

The monoglyceride pathway for triglyceride biosynthesis also is believed to operate, to some extent, in mammary gland (reviewed by Patton [14] and Moore [15]). To test for its occurrence in freshly secreted milk, samples of milk were incubated with isotopically labeled glycerol ethers which frequently have been used to mimic monoglycerides (17), i.e. 1-O- and 2-O-hexadecylglycerols. The results are listed in Table I. Both positional isomers were acylated, presumably by endogeneous free fatty acids, but 2-O-hexadecylglycerol was esterified much more rapidly than the 1-O-equivalent. The main products in both instances were monoacylmonoalkylglycerols, and there was very little diacyl-monoalkylglycerol formation. There was some incorporation into phospholipids, and a small amount of labeled free fatty acid was found that apparently had been formed by oxidation of the glyceryl ether. No stimulation of the reaction was obtained by adding ATP, Mg^{++}, CoA, and unesterifed fatty acids to the incubation medium. Bickerstaffe and Annison (18) reported that goat mammary microsomes incorporated 2-O-glycerol ethers into lipids much more rapidly than the analogous 1-O-compounds, and it also has been shown that 2-O-glyceryl ethers infused into the arteries of lactating goats were taken up much more rapidly by the mammary gland than 1-O-compounds (19).

With such a poorly defined medium, it did not seem possible to study the kinetics of incorporation of different fatty acids into triglycerides in a systematic manner. However, a simple kinetic experiment was performed to confirm that the enzyme systems could utilize a wide variety of fatty acids, and a number of different isotopically labeled fatty acids were incubated with the milk along side palmitic acid controls. Larger amounts of each of the acids (2 μmoles) were added to the incubation with the

TABLE III

Relative Proportions of Stearic and Oleic Acids Incorporated into Individual Lipid Classes When (1-^{14}C)-Stearic Acid was Incubated with Goat Milk[a]

Lipid class	Relative proportions (%) of 18:0 and 18:1 incorporated	
	18:0	18:1
Triglycerides	72.5 ± 0.6	27.5 ± 0.6
Diglycerides	79.2 ± 0.8	20.8 ± 0.8
Free fatty acids	98.6 ± 0.3	1.4 ± 0.3
Phosphatidic acid[b]	72.1 ± 0.8	27.9 ± 0.8
Phosphatidyl choline[b]	50.2 ± 1.3	49.8 ± 1.3

[a]Means and standard deviations of four experiments.

[b]Two experiments only were performed in this instance.

intention of swamping the effect of any unesterifed fatty acids endogeneous to the milk. (An average of 0.463 μmoles/10 ml free fatty acids were found in the milk, but there was no method of determining how much was available to the synthetase). The amounts of each fatty acid incorporated into the various lipid classes relative to the palmitate control (given the arbitrary value of 100 in the triglyceride fraction) are listed in Table II. All the fatty acids examined were esterified rapidly, with the exception of 18:0 which was incorporated into triglycerides at only one-fifth the rate for palmitic acid. Other saturated fatty acids were esterified at rates comparable with that of palmitic acid, i.e. 12:0 and 14:0 slightly more rapidly and 15:0 and 17:0 slightly less rapidly. Unsaturated fatty acids were esterified to triglycerides at two to three times the rate for palmitic acid. Labeled diglycerides and phospholipids were formed in ca. similar amounts on incubating each of the fatty acids (with the exception of 18:0), although a much higher proportion of 18:2 than of any of the other acids examined was found in the phospholipid fraction.

It also has been reported elsewhere (5) that freshly secreted milk contained the enzymes necessary to desaturate stearic to oleic acid. This was confirmed by incubating (1-^{14}C)-stearic acid with the milk followed by isolation of the various products for conversion to the methyl ester derivatives of the fatty acids. The methyl esters were separated into saturated and monoenoic components by silver nitrate TLC; preparative gas liquid chromatography (GLC) was used to confirm that all the activity resided in C_{18} compounds. The results are listed in Table III. Virtually all the 18:1 formed was incorporated into lipids and a much smaller proportion appeared in the free fatty acid fraction than was expected from the study of McCarthy, et al. (5). For this to have occurred, there must have been a close association of the fatty acid desaturase system with the triglyceride synthetase. This system also was not sensitive to the addition of potential cofactors since nicotinamide adenine dinucleotide, reduced form (NADH), which is known to be necessary for bovine mammary microsomal stearyl-CoA desaturase (20), did not stimulate the reaction.

To determine whether the isotopically labeled fatty acids were entering all three positions of the sn-triacyl-glycerol molecule and then in specific proportions, triglycerides, formed by incubating the milk with a variety of different isotopically labeled fatty acids at concentrations (0.2 μmoles/10 ml) that did not swamp the endogeneous free fatty acids, were subjected to stereospecific analysis. The method used was based upon that devised by Brockerhoff (21) in which triglycerides were hydrolyzed by ethyl magnesium bromide to α,β-diglycerides for conversion synthetically to phospholipids which were in turn hydrolyzed by the stereospecific phospholipase A of snake venom. The lysophosphatide formed contained the fatty acids originally present in position sn-1; the free fatty acids released were in position sn-2 (this was confirmed by pancreatic lipase hydrolysis); and the result for position sn-3 was obtained by difference. To compensate for losses that inevitably occurred in the various steps of the analysis, tri-(10,11-^{3}H)-heptadecanoin of similar specific activity and total activity to the sample to be analyzed was added as internal standard (7). Analyses were accepted only when the results obtained for positions sn-2 and sn-3 calculated by the alternative procedures agreed within 5% (22). No difficulties were caused by the presence of triglycerides containing short chain fatty acids as reported by others (23), possibly because the

TABLE IV

Proportions (%) of Various Isotopically Labeled Fatty Acids Entering Three Positions of sn-Triacylglycerol Molecule on Incubation with Freshly Secreted Goat Milk in Vitro[a]

Fatty acid	Proportional distribution (%)					
	Found			Natural[b]		
	Positions					
	1	2	3	1	2	3
16:0	33	53	14	54	42	4
18:0	46	13	41	52	22	26
18:1 (n-9) *cis*[c]	35	26	39	26	26	48
18:1 (n-9) *cis*[d]	34	16	50		---	
18:1 (n-7) *trans*	37	19	44		---	
18:2	32	30	38	4	34	62

[a]Results are the mean of duplicate analyses. Results for positions sn-2 and sn-3 agreed within 5%. Results for position sn-2 agreed within 2%.

[b]Calculated from the results of Kuksis, et al. (23).

[c]Added to the incubation as 18:1.

[d]Formed from 18:0 in the medium.

added fatty acids were incorporated largely into triglycerides containing only longer chain fatty acids. The results are listed in Table IV.

All the fatty acids examined did, indeed, enter all three positions of the glycerol molecule and marked positional specificities were observed. For example, 13% of the 18:0 esterified entered position sn-2 as opposed to 30% of the 18:2 and 53% of the 16:0. Variations of a similar magnitude were observed in the proportions of the various fatty acids that entered position sn-3, although they were less marked in position sn-1. There were differences in the manner in which oleic acid, incubated as such, and oleic acid formed de novo from stearic acid were incorporated into triglycerides, particularly into positions sn-2 and sn-3, but this might have reflected the fact that a much greater amount of oleic acid was incorporated into triglycerides when it was added to the system in this form. In contrast, Kinsella (24) reported that bovine mammary microsomes incorporated all the 18:1 formed de novo in the system into the primary positions of the triglyceride molecules and, indeed, suggested that this 18:1 might be acylated preferentially entirely into position sn-3. The distributions obtained differed considerably from those reported to occur naturally in goat milk triglycerides (23), but a number of factors could account for this. For example, the ratio of added to endogenous free fatty acids could be important, or the particles or organelles containing the enzymic activity in milk could lack part of the biosynthetic control mechanism.

Lipases did not appear to play an important part in the reaction. The lipolytic activity of the milk was assessed by isolating the radioactive lipids from an incubation and reincubating these with fresh milk. In all cases, the lipolytic activity was less than 2% of the biosynthetic capacity. Radioactive diglycerides and phospholipids reincubated in this way were not converted further to triglycerides to any marked extent.

DISCUSSION

The triglyceride synthetase activity in freshly secreted milk from goats was certainly enzymatic, e.g. the activity was destroyed by ultrasonification or by detergents, and the possibility exists that it was a normal mammary triglyceride biosynthetic system. The findings that a variety of different fatty acids were utilized at widely different rates and were incorporated into all three positions of the sn-triacylglycerol molecule in quite specific patterns lent weight to this suggestion. In addition, the triglyceride biosynthetic capacity was associated closely with the fatty acid desaturase enzyme system. However, the possibility cannot be discounted that fatty acids are introduced by acyl transferases into preformed glycerolipid acceptors occurring naturally in milk. The lack of response of any of these enzymes to the addition of the usual range of cofactors was puzzling. McCarthy and Patton (2) suggested that this might be because there was already a sufficiency of the required cofactors in milk for the reaction to occur. On the other hand, in this study, triglyceride synthetase activity was found to be stimulated by added glycerol, presumably because the

latter was converted in situ to α-glycerophosphate. An alternative explanation of the lack of effect of other cofactors may then be that the synthetase enzymes were surrounded by a membrane barrier that permitted the passage of low mol wt compounds, such as glycerol, but not of large polar molecules like ATP or CoA. The plasma membrane that enevelopes the milk fat globule when it is secreted from the cell would have such properties for example.

One reasonable interpretation of the results described above might be that the mammary triglyceride biosynthetic system that consists of the enzymes of the α-glycerophosphate pathway was operative in freshly secreted milk, although at least some of the enzymes of the monoglyceride pathway also appeared to be present. If the enzymes or particles containing this activity can be isolated so that this interpretation can be confirmed, then freshly secreted milk has considerable potential as a source of mammary triglyceride synthesizing enzymes for studies of milk fat biosynthesis in vitro. Milk enzymes then could be used as an alternative to those from tissues obtained surgically.

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Trace Constituents in Milk Fat: Isolation and Identification of Oxofatty Acids

J.L. WEIHRAUCH[1], C.R. BREWINGTON[2], and D.P. SCHWARTZ,
Dairy Products Laboratory, Eastern Regional Research Center[3], Philadelphia,
Pennsylvania 19118

ABSTRACT

Ca. 1% of the glycerides of milk fat contain oxofatty acids. The isolation, fractionation, and characterization of oxofatty acids were accomplished using the following sequence of steps: (A) transmethylation, (B) conversion into 2,4-dinitrophenylhydrazones, (C) adsorption of the 2,4-dinitrophenylhydrazones on magnesium oxide to eliminate the colorless lipid, (D) fractionation of the 2,4-dinitrophenylhydrazones into non-oxofatty acid and oxofatty acid fractions on alumina, (E) separation of the oxofatty acid 2,4-dinitrophenylhydrazones into saturated and unsaturated classes by argentation column chromatography, (F) separation of these classes by chain length using liquid-liquid column and thin layer partition chromatography, (G) resolution of positional isomers by thin layer chromatography, (H) regeneration of the positional isomer 2,4-dinitrophenylhydrazones, and (I) analysis of the parent oxofatty acids by gas liquid chromatography-mass spectrometry. In this manner, 36 saturated and 11 unsaturated oxofatty acids were identified tentatively or positively. The saturated oxofatty acids ranged in chain length from C_{10}-C_{24}, predominantly C_{18} and C_{16}, and generally contained an even number of carbon atoms. The unsaturated oxofatty acids ranged from C_{14}-C_{18}, with C_{18} predominating.

INTRODUCTION

Application of a method for the direct isolation of carbonyl compounds from fats and oils (1) revealed that milk fat contained a class of nonvolatile carbonyl compounds subsequently identified as glycerides containing one or more esterified oxofatty acids (OFA). This class constituted ca. 1% of the milk lipids and was by far the major carbonyl-containing moiety. Studies in this laboratory and elsewhere (M. Keeney, private communication) have shown that OFA occur in both animal and vegetable lipids in varying concentrations, usually, however, lower than that in milk fat. They also occur in normal amounts in the milk fat of cows fed a synthetic diet containing little lipid, indicating that OFA cannot be derived from the feed (2).

The problem of identifying the OFA in milk fat was undertaken first by Keeney, et al., (3) who reported the occurrence of six saturated isomeric oxostearic acids using the Beckman rearrangement of the oximes to locate the oxo-group. They also reported that OFA down to C_{10} occur in lesser amounts but did not identify them. Details of the methods used were not communicated. In the only other study of OFA in milk fat, van der Ven (4) used Girard T reagent to extract the OFA, reduced them to hydroxy fatty acids, and determined those hydroxy acids which lactonized. In this manner, three 4-OFA and three 5-OFA were identified indirectly.

In a problem related to the biosynthesis of OFA in milk fat, Katz and Keeney (5) isolated an oxostearic acid fraction from the lipids of rumen digesta which consisted mainly of 16-oxostearate. They also reported that the 8-oxo- through the 15-oxopositions were present.

Aside from the work on milk fat and rumen lipids, relatively few OFA have been identified in biological material: lactarinic (6-oxostearic) acid from the mushroom fungus *Lactarius refus* (6,7); 13-oxodotriacontanoic acid from the cochineal insect, coccerin (8); licanic (4-oxo-9,11,13-octadectrienoic) acid in the seed fat of *Licania rigida*(9) and other species of this genus (10); 9-oxo-*trans*-2-decenoic acid, socalled Queen Substance from bees (11), which also has been considered to be the sex attractant for drones (12); 8-oxohexadecanoic acid which occurs in small amounts in the oil of the spores of Lycopodium species of the clubmoss *Lycopodium* (13); 6-oxotetradecanoic acid from hydrolized lac resin (14); 10-oxooctadecanoic acid from the lipids of the Tubercle bacillus (15); and 17-oxo-*cis*-20-hexacosenoic, 15-oxo-*cis*-18-tetracosenoic, and 19-oxo-*cis*-octacosenoic acid from the seed oil of *Cuspidaria pterocarpa* (16).

This report concerns a reinvestigation of the

[1]Present address: Consumer and Food Economics Institute, USDA, Washington, D.C. 20250.

[2]Present address: Animal and Plant Health Inspection Service, USDA, Washington, D.C. 20250.

[3]ARS, USDA. Research conducted while Dairy Products Laboratory was located in Washington, D.C.

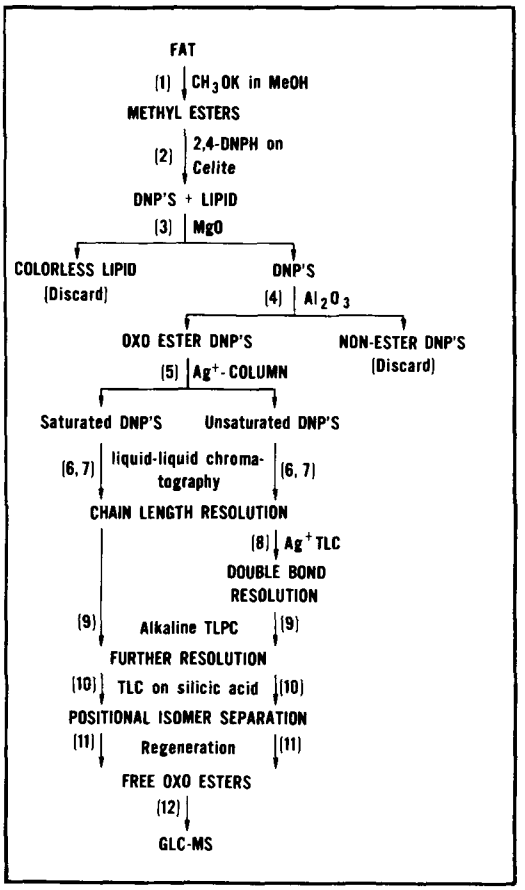

FIG. 1. Schematic of steps used in the isolation, fractionation, and characterization of oxofatty acids from milk fat. DNPH = 2,4-dinitrophenylhydrazine, GLC-MS = gas chromatography-mass spectrometry, DNP = 2,4-dinitrophenylhydrazones, TLC = thin layer chromatography, and TLPC = thin layer partition chromatography.

OFA of milk fat using new, improved wet microchemical techniques and mass spectrometry. Our analysis has revealed that the OFA fraction is highly complex, containing over 60 components, 47 of which have been either positively or tentatively identified.

EXPERIMENTAL PROCEDURES

The methods for isolating, fractionating, and identifying the OFA in milk fat are outlined in Figure 1 and are described in the following steps:

Step 1—Transmethylation

Milk fat (46 g), prepared from mixed herd winter milk obtained from the USDA, Beltsville, Md., was transmethylated by the procedure of Luddy, et al., (17) using 460 ml 0.5 N methanolic potassium methoxide. Following transmethylation, 460 ml 0.5 N HCl was added and the esters extracted with 5 x 500 ml portions of carbonyl-free n-hexane (18, 19).

Step 2—Preparation of 2,4-Dinitrophenylhydrazones (DNPs)

The hexane extract was divided into 3 equal portions and passed over three 30 g columns of Celite impregnated with 2,4-dinitrophenylhydrazine, prepared according to Schwartz and Parks (19), to derivatize the carbonyl compounds. Analysis of the effluents indicated that reaction had been incomplete. The effluents were, therefore, recycled over the columns which resulted in quantitative derivatization relative to results obtained on a small aliquot.

Step 3—Adsorption of DNPs on MgO

The combined effluents from step 2 were evaporated to ca. 300 ml and passed over a 50 g column containing Seasorb 43: Celite 545 (1:1) (1). Nitrogen pressure was applied to force the solution through the column so that an unbroken stream of effluent issued from the column continuously. The sides of the column were washed down with hexane and the column washed under pressure with 1 liter hexane to remove the last traces of colorless lipid. The total time involved to this point was ca. 30 min. The DNPs then were desorbed with a 25% solution of nitromethane in chloroform and the solvent evaporated under nitrogen until the odor of nitromethane was absent.

Step 4—Fractionation of DNPs on Al_2O_3

The residue from step 3 was dissolved in 50 ml hexane and passed over a 50 g column of 8% hydrated Al_2O_3 (19). Aldehyde and ketone DNPs containing no ester function (band 1) were eluted with hexane:benzene (1:1) until all color below the next (major OFA) band was removed. The major OFA band (yellow-orange, band II) was eluted with benzene. The next band (light red, band III) which contained geometric isomers of the major OFA band (see "Results and Discussion") was eluted with dichloromethane. Spectrophotometric analysis of band II in chloroform at its absorption maximum (365 nm) indicated 185 μmoles and that of band III (adsorption maximum, 355 nm) of 32 μmoles. Band I was discarded.

Step 5—Argentation Column Chromatography

Separation of band II into saturated and unsaturated OFA DNPs was effected by Ag^+ column chromatography. Absolute separation was not achieved, but the majority of the satu-

rated and unsaturated DNPs moved with their respective classes. Supplemental testing with iodine monochloride, hydrogenation, and mass spectrometry was sufficient to establish whether the OFA was unsaturated.

Silicic acid (10 g) (Mallinckrodt's 100-200 mesh) was slurried with 10 ml 10% solution of $AgNO_3$ in acetonitrile, poured into an evaporating dish, heated 1 hr at 100 C, cooled, and ground briefly to eliminate clumps. The silicic acid was slurried in hexane, poured in a column (20 x 2 cm), and packed under air pressure. The DNPs dissolved in a minimum of hexane were applied to the column. The majority of the saturated DNPs was removed as a single band with dichloromethane:hexane (1:1), and the remaining bands on the column were removed with 5% methanol in chloroform. The "saturated" DNP fraction was rechromatographed as above and separated into a major and a minor band (ca. 9%). The major band was collected and designated as the saturated fraction. The minor band was pooled with the "unsaturated" DNP fraction from the initial chromatogram. This was designated as the unsaturated fraction. Spectrophotometric analysis (at 365 nm, E = 22,500) gave an estimate of ca. 69% saturated and 31% unsaturated DNPs.

Band III from step 4 was subjected to the above procedure giving a fraction comprised of 55% saturated and 44% of unsaturated DNPs.

Step 6—Column Partition Chromatography

The saturated fraction from band II was chromatographed on a 25 g acetonitrile-Celite column as described by Corbin, et al. (20). Fractions (15 ml) were collected. Separation was followed visually and spectrophotometrically at 340 nm. The fractions comprising a given peak were pooled.

The unsaturated fraction from band II and the saturated and unsaturated fractions from band III were chromatographed similarly.

Step 7—Thin Layer Partition Chromatography (TLPC) of Saturated Fractions on Neutral Plates

Each peak from the saturated fractions obtained in step 6 was streaked across a 20 x 8 in. TLPC plate and developed in the hexane system described by Schwartz, et al. (21). The bands were scraped from the plate, the color eluted with benzene, and any contaminating bands were pooled with the appropriate subsequent fraction. All fractions then were freed of stationary phase (polyethylene glycol 400) by passing the benzene solution over a column of 8% hydrated alumina (19) (ca. 1 g in a disposable pasteur pipette), and the column was eluted with benzene until all color was removed.

Step 8—Thin Layer Argentation Chromatography of Unsaturated Fractions

Mylar sheets (8 x 8 in.) precoated with silica gel (Baker-Flex 1B, J.T. Baker Co., Phillipsburg, N.J.) were drawn through a 10% silver nitrate solution in acetonitrile and dried 10 min at 100 C. The peaks obtained for the unsaturated fractions in step 6 were streaked across the sheet and the chromatogram developed repeatedly (usually 3 times) in chloroform:benzene (3:2) to resolve the derivatives more or less according to the degree of unsaturation. The bands were scraped from the sheet and eluted with chloroform or ethyl acetate.

Step 9—TLPC on Alkaline Plates

Each band obtained in steps 7 and 8 was streaked across the origin of a 20 x 8 in. alkaline TLPC plate (21) and developed in hexane and hexane:benzene (65:35). This system afforded further separation according to chain length which was not attained on either the partition column or on the neutral TLPC plates. This was especially evident with the longer chain ($>C_{16}$) OFA DNPs. At the same time, some DNPs gave different colors (see "Results and Discussion"). The bands were scraped from the plate, eluted with benzene, and purified on small alumina colums as in step 7.

Step 10—Resolution of Positional Isomers by Thin Layer Chromatography (TLC)

The bands from the saturated fraction, cut and purified from the plates in step 9 and presumably containing OFA DNPs of a given chain length, were streaked across the origin of a silica gel sheet and the chromatogram repeatedly developed with 5% ethyl acetate in hexane to resolve positional (oxogroup) isomers. A clean cut separation of the 2-oxo- through 8-oxopositions was achieved. The 9-oxo- through 12-oxopositions were not as cleanly resolved, but relatively pure fractions were obtained by scraping successive narrow segments from the partially resolved band. The 13-oxo- through 16-oxopositions (in the stearates) could not be resolved. Each band was scraped from the plate, eluted with chloroform, and checked for saturation or unsaturation using iodine monochloride (22). Saturated DNPs are not retarded on TLPC plates, whereas most unsaturated DNPs are retarded from 30-80%, depending usually upon the degree of unsaturation. All bands also were purified by dissolution in 10% dichloromethane in hexane and passage over a small Al_2O_3 column as described in step 7. The column was washed with 5 ml 10% dichloromethane in hexane and the effluent discarded. The colored band then was eluted with dichloromethane.

Step 11—Regeneration of OFA

A volume of a dichloromethane solution of each purified fraction (obtained in step 10) containing ca. 5 μg OFA DNP was transferred to a mp capillary and the solvent removed under vacuum. A 1% solution (10 μliter) of concentrated hydrochloric acid in acetone (prepared each day) was added and the DNP dissolved by repeatedly drawing up and expelling the solution with the hypodermic syringe. The solution was allowed to stand for 15 min to complete the regeneration (M. Keeney, private communication).

Step 12—Gas Liquid Chromatography-Mass Spectrometry (GLC-MS) of the Regenerated OFA

The entire solution from step 11 was injected into a gas chromatograph and the effluent vapor analyzed by MS. The LKB-9000 spectrometer was used. OFA with 16 carbons or less were chromatographed on a 5 ft x 1/8 in. stainless steel column packed with 7.5% ethylene glycol adipate and 2% phosphoric acid on 90-100 mesh Anakrom ABS (Analabs, North Haven, Conn.). OFA containing 17 or more carbons were chromatographed on a 10 ft x 1/8 in. stainless steel column packed with 3% OV-1 on 100-200 mesh Chromosorb Z. Both columns were operated isothermally, the former at a temperature of 160-180 C, depending upon chain length, and the latter at 190-210 C, also depending upon the chain length. Helium was the carrier gas and was maintained in all instances at 40 psi. Other standard operating conditions were: flash heater and separator, 230 C; ion source, 290 C. Mass spectra were obtained at a constant accelerating voltage of 3500 V with an electron energy of 70 ev and a scanning time of 4.5 sec over a m/e range of 12-450. Chromatographic peaks were scanned repeatedly, and the strongest area at the apex was compared to a similar area of an authentic compound when availble.

Additional Methodology

Hydrogenation: In a few instances, hydrogenation of unsaturated OFA was conducted to compare their spectra with those established for saturated OFA. The OFA was regenerated, as in step 11, and the excess acetone removed under vacuum. The residue was taken up in the minimum volume of dimethoxypropane, transferred to a microcolumn of Celite impregnated with palladium chloride and hydrogenated as described by Schwartz, et al. (23).

Location of double bond position: The position of the double bond in monounsaturated OFA was determined directly on the DNP using the periodic acid column procedure of Weihrauch and Schwartz (24) when the mass spectrum indicated that the double bond occupied a position between the oxogroup and end of the chain. When the mass spectrum indicated that the double bond was between the ester and oxogroups, a potassium permanganate-periodic acid column procedure was employed on the DNP (J.L. Weihrauch and D.P. Schwartz, unpublished data) because the former procedure does not give the semialdehyde. Briefly, this procedure consisted of application of the unsaturated OFA DNP in dichloromethane to a column of Celite (0.5 g) which had been ground (on the day of use) with 12 drops of a saturated aqueous solution of potassium permanganate and 3 drops of a saturated aqueous solution of potassium carbonate. The OFA DNP was permitted to remain on the column for 30 min. It then was eluted and passed over the periodic acid column system (24).

Reference oxoacids: Synthesis of 6-oxomyristic, 7-oxomyristic, 6-oxopalmitic, 7-oxopalmitic, 8-oxopalmitic, 8-oxoheptadecanoic, and 8-oxostearic acids were carried out according to Hünig, et al. (25,26). The acids were converted to methyl esters overnight in methanolic hydrochloric acid. The 4- and 5-oxodecanoates and 4- and 5-oxododecanoates were prepared from the corresponding γ- and Δ-lactones by transmethylation in methanolic hydrochloric acid, extraction with hexane, passage of the hexane extract over a chromic acid column (27) to oxidize the hydroxy ester to the oxoester, conversion of the oxoester to the DNP, and purification by preparative TLC.

RESULTS AND DISCUSSION

The saturated OFA positively or tentatively identified are listed in the first section of Table I. A compound is listed as being identified positively only when an authentic sample was identical both chromatographically and mass spectrometrically.

The oxostearates comprised the major portion of the total oxoesters which confirms the findings of Keeney, et al. (3). Ca. 85% of the oxostearates are comprised of the 13-oxo-, 9-oxo-, and 10-oxoisomers in decreasing prevalence. The 11-oxoisomer was found in only trace amounts. Keeney, et al., (3) reported the order of prevalence to be 9-oxo-, 10-oxo, and 13-oxostearates. They also found a significant amount (ca. 10%) of the 11-oxoisomer. The occurrence of all oxostearates (8-oxo through 13-oxo) reported by Keeney, et al., (3) with the exception of the 12-oxoisomer, was confirmed in the present study.

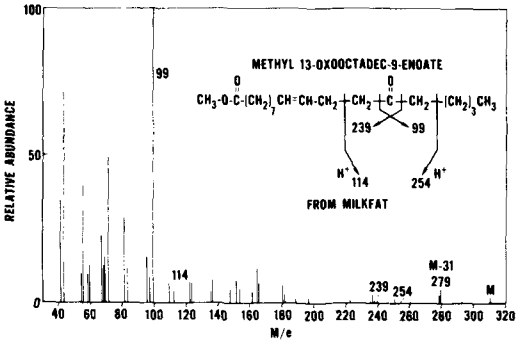

FIG. 2. Mass spectra of authentic methyl-9-oxooctadec-12-enoate and an oxofatty acid methyl ester isolated from milk fat.

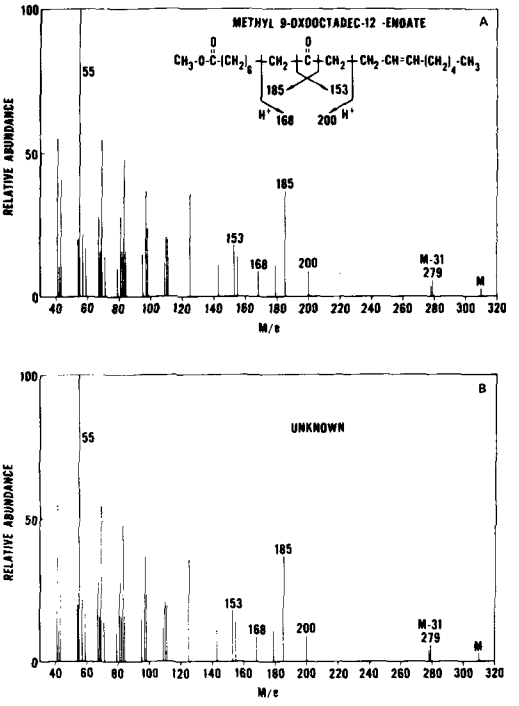

FIG. 3. Mass spectrum of an oxofatty acid methyl ester isolated from milk fat and tentatively identified as methyl 13-oxooctadec-9-enoate.

The oxopalmitates occured in the next highest concentration (ca. 20% of the total saturates) and was comprised predominantly of the 11-oxoisomer.

The oxomyristates, oxolaurates, and oxodecanoates were found in decreasing amounts. The most abundant of these oxoesters were 9-oxomyristate, 5-oxolaurate, and 5-oxodecanoate. The occurrence in milk fat of 4-oxolaurate, 5-oxodecanoate, and 5-oxolaurate indirectly identified as lactones by van der Ven (4) is substantiated by our data. However, the other reported oxoacids (4-oxodecanoate, 4-oxoundecanoate and 5-oxooctanoate) were not detected.

Very small amounts of odd carbon oxoacids were identified and were estimated to occur in the amount of ca. 1 μg/g milk fat.

OFA with more than 18 carbons were found, their amounts decreasing with increasing chain length. Only traces of the oxotetracosanoates were detected.

Unsaturated OFA: Analysis of the unsaturated OFA fraction yielded only 11 identifiable compounds. These are given in the second section of Table I.

Methyl 9-oxooctadec-12-enoate and methyl 13-oxooctadec-9-enoate were the predominant unsaturated OFA. Besides those unsaturated OFA given in Table I, the presence of the following OFA was indicated: a C_{15} monounsaturate, a C_{16} diunsaturate, a C_{17} mono-, di-, and triunsaturate, a C_{18} di- and triunsaturate, a C_{19} mono- and diunsaturate, and a C_{20} monounsaturate.

Both saturated and unsaturated OFA DNP fractions contained bands which regenerated, but the fragmentation pattern was not interpretable outside of being recognized as methyl esters of OFA. Another set of bands was not methyl esters, and a third set of bands did not regenerate under the conditions used in step 11. In addition, a series of bands displaying a lavender color on the alkaline TLPC plates was seen, and also a series of blue bands was observed. The former were reminiscent of the color shown by alk-2,4-dienal or alk-2,4-dienone DNPs but were not investigated further. Both classes were present in small amounts, probably less than 1% of the total OFA.

Mass spectrometry: The mass spectra of the oxofatty acids isolated from milk fat are given in Table I. The location of the carbonyl group is made readily as both α- and β-cleavage occur on either side of the carbonyl group. The molecular ion which is usually less than 1% of the base peak is readily discernible. Mass spectra of the methyl esters of the oxostearates were published by Ryhage and Stenhagen (28). Our spectra were generally in good agreement with theirs.

For monounsaturated OFA, the mass spectrum reveals also the side of the carbonyl group in which the double bond occurs. Figure 2 shows mass spectra of authentic methyl 9-oxooctadec-12-enoate and an OFA from milk fat with an identical spectrum as examples of unsaturated OFA with the double bond between the carbonyl group and the end of the chain. A

TABLE I

Mass Spectra of Oxofatty Acids Positively or Tentatively Identified in Milk Fat

	Mass spectrum, m/e (relative intensity)
Saturated n-oxofatty acids	
Methyl-5-oxodecanoate	43(100), 112(61), 71(60), 101(59), 99(57), 129(42), 144(36), 169(19), 200(M^+) (0.14)
Methyl-4-oxolaurate	98(100), 130(65), 115(56), 71(43), 111(23), 141(21), 197(9), 228(M^+) (0.4)
Methyl-5-oxolaurate	57(100), 112(74), 55(55), 43(48), 144(38), 129(37), 127(29), 197(12), 228(M^+) (0.3)
Methyl-7-oxolaurate	43(100), 55(59), 71(57), 69(43), 99(43), 172(13), 157(9), 197(4), 228(M^+) (0.4)
Methyl-5-oxomyristate	112(100), 43(67), 55(58), 144(56), 101(47), 129(44), 155(22), 225(12), 256(M^+) (0.5)
Methyl-6-oxomyristate	158(100), 141(77), 143(53), 156(24), 225(7), 197(5), 256(M^+) (1.3)
Methyl-7-oxomyristate	57(100), 115(41), 125(37), 127(29), 142(14), 172(14), 157(10), 225(1.3), 256(M^+) (0.4)
Methyl-9-oxomyristate[a]	43(100), 55(71), 71(62), 99(52), 114(15), 185(15), 200(8), 225(6), 256(M^+) (0.2)
Methyl-4-oxopentadecanoate[a]	130(100), 115(59), 111(41), 239(9), 169(4), 270(M^+) (1)
Methyl-5-oxopentadecanoate[a]	112(100), 43(63), 55(60), 144(58), 41(47), 129(43), 169(17), 239(10), 270(M^+) (0.3)
Methyl-4-oxopalmitate[a]	98(100), 130(91), 55(62), 115(50), 43(54), 57(49), 197(10), 253(4), 284(M^+) (0.5)
Methyl-5-oxopalmitate[a]	112(100), 144(67), 43(51), 55(51), 57(47), 129(40), 183(15), 253(10), 284(M^+) (0.5)
Methyl-6-oxopalmitate	158(100), 143(42), 169(42), 184(15), 235(7), 253(4), 284(M^+) (1.0)
Methyl-7-oxopalmitate	43(100), 55(90), 71(73), 172(38), 155(35), 170(23), 157(21), 253(3), 284(M^+) (0.13)
Methyl-8-oxopalmitate	141(100), 171(86), 156(68), 154(64), 144(50), 186(46), 253(13), 211(9), 284(M^+) (0.13)
Methyl-9-oxopalmitate	127(100), 143(87), 142(71), 185(36), 200(26), 168(26), 253(21), 284(M^+) (1.9)
Methyl-11-oxopalmitate	43(100), 71(62), 55(58), 99(50), 114(16), 213(10), 228(7), 253(5), 284(M^+) (0.3)
Methyl-8-oxoheptadecanoate	171(100), 170(64), 186(53), 267(7), 169(7), 298(M^+) (1.4)
Methyl-5-oxostearate	112(100), 144(66), 43(51), 55(49), 57(46), 129(37), 211(7), 281(6), 312(M^+) (0.24)
Methyl-8-oxostearate[a]	171(100), 154(75), 169(73), 184(59), 186(59), 281(15), 312(M^+) (1.6)
Methyl-9-oxostearate	43(100), 55(96), 71(71), 155(24), 170(20), 185(19), 200(16), 281(4), 312(M^+) (0.2)
Methyl-10-oxostearate	55(100), 43(99), 57(90), 156(51), 141(46), 214(24), 199(24), 281(14), 312(M^+) (0.75)

Methyl-11-oxostearate	43(100), 127(19), 142(14), 213(7), 281(9), 228(6), 312(M^+) (0.8)
Methyl-13-oxostearate	43(100), 55(66), 71(61), 99(49), 114(17), 241(10), 256(7), 281(5), 312(M^+) (0.6)
Methyl-16-oxostearate	57(100), 55(57), 43(54), 72(49), 41(42), 209(13), 283(10), 281(3), 312(M^+) (1.5)
Methyl-11-oxononadecanoate[a]	43(100), 141(37), 156(36), 228(19), 213(18), 295(8), 326(M^+) (0.5)
Methyl-9-oxoeicosanoate[a]	43(100), 185(13), 183(9), 200(9), 198(8), 309(4), 340(M^+) (0.5)
Methyl-11-oxoeicosanoate[a]	43(100), 170(30), 155(23), 228(15), 213(13), 309(7), 340(M^+) (0.8)
Methyl-15-oxoeicosanoate[a]	43(100), 99(40), 114(16), 269(8), 284(7), 309(6), 340(M^+) (0.7)
Methyl-11-oxodocosanoate[a]	43(100), 198(11), 228(11), 183(10), 213(7), 337(4), 368(M^+) (0.41)
Methyl-12-oxodocosanoate[a]	184(100), 189(94), 242(71), 227(55), 337(80), 368(M^+) (9.4)
Methyl-13-oxodocosanoate[a]	170(100), 155(87), 256(59), 241(45), 337(37), 368(M^+) (4.4)
Methyl-14-oxodocosanoate[a]	141(100), 156(97), 255(41), 270(39), 337(59), 368(M^+) (7.0)
Methyl-15-oxodocosanoate[a]	127(100), 142(44), 269(19), 284(11), 337(56), 368(M^+) (6.6)
Methyl-14-oxotetracosanoate[a]	184(100), 169(93), 270(53), 255(44), 365(16), 396(M^+) (5)
Methyl-15-oxotetracosanoate[a]	155(100), 170(96), 269(21), 284(11), 365(16), 396(M^+) (5)
Unsaturated n-oxofatty acids	
Methyl-5-oxotetradec-9-enoate[a]	55(100), 112(75), 41(71), 144(39), 129(28), 153(6), 223(5), 231(2), 254(M^+) (1.5)
Methyl-5-oxotetradec-10-enoate[a]	55(100), 112(75), 41(71), 144(39), 129(28), 153(6), 223(5), 231(2), 254(M^+) (1.5)
Methyl-9-oxotetradec-5-enoate[a]	43(100), 99(87), 71(57), 223(4), 222(4), 254(M^+) (3), 198(1.5)
Methyl-7-oxohexadec-10-enoate[a]	157(100), 172(50), 153(42), 251(11), 250(10), 282(M^+) (6)
Methyl-11-oxohexadec-7-enoate[a]	99(100), 43(89), 71(64), 94(47), 55(44), 251(4), 282(M^+) (2), 226(1.8), 211(1.3)
Methyl-11-oxohexadec-9-enoate[a]	99(100), 43(92), 71(65), 94(47), 55(47), 251(4), 282(M^+) (2.1), 226(1.6), 211(1.4)
Methyl-9-oxooctadec-12-enoate	55(100), 41(56), 69(53), 185(29), 153(16), 200(8), 168(7), 279(6), 310(M^+) (2)
Methyl-9-oxooctadec-13-enoate[a]	43(100), 55(92), 41(81), 185(36), 153(22), 279(15), 200(9), 310(M^+) (6)
Methyl-9-oxooctadec-15-enoate[a]	55(100), 43(75), 41(67), 185(34), 153(19), 168(8), 200(8), 279(8), 310(M^+) (4)
Methyl 13-oxooctadec-7-enoate[a]	99(100), 43(72), 71(49), 55(45), 41(37), 279(5), 310(M^+) (2), 254(0.7), 239(0.6)
Methyl-13-oxooctadec-9-enoate[a]	99(100), 43(72), 71(50), 55(40), 41(35), 279(5), 310(M^+) (2), 254(0.7), 239(0.6)

[a]Tentative identification.

similar spectrum has been reported by Kleiman and Spencer (29) for methyl 17-oxo-*cis*-20-hexacosenoate.

Figure 3 is the mass spectrum of an unsaturated OFA isolated from milk fat in which the double bond occurs between the ester and carbonyl functions. It tentatively has been identified as methyl-13-oxooctadec-9-enoate based upon the spectra and identification of methyl azelaaldehydate following oxidation on the potassium permanganate-periodic acid column.

Geometrical isomerism in the DNPs: The well known geometrical isomerism in DNPs was evident also with the DNPs of some of the OFA. This phenomenon created some confusion and also a great deal more work, as many additional bands had to be scraped from the plates, purified, regenerated, and examined in the mass spectrometer. Although two isomers for each OFA DNP undoubtedly were present, they were not always separable. The DNPs of the OFA with the oxogroup in the 2-8 positions were separable into 2 isomers. The 9 position and higher were not. The closer the oxofunction was to the ester group, the more marked the separation of isomers became. In this study, the 5-oxoposition occurred most frequently among the positions up to 9-oxo (Table I); consequently, the geometric isomers of the DNP derivative of the 5 OFA were encountered most frequently. The main isomer was always the slower moving spot or band on the plates. The minor, faster moving spot or band usually moved as if it had two more carbon atoms. Thus, it was not unusual to find, for example, some 5-oxodecanoate as a contaminant in the oxolaurate band. The problem was complicated further by reformation of two isomers from each isomer cut from the plate, thereby doubling the number of isomer bands each time a separation was made.

Despite the shortcoming of geometrical isomerism in the DNPs, it is felt that the information obtained using the methods described more than justified the additional work involved.

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Lipid Changes during Life Cycle of Marine Copepod, *Euchaeta japonica* Marukawa

RICHARD F. LEE[1] and **JUDD C. NEVENZEL**, Scripps Institution of Oceanography, La Jolla, California 92037, and **A.G. LEWIS**, Institute of Oceanography, University of British Columbia, Vancouver, British Columbia

ABSTRACT

All stages from egg to adult of the North Pacific copepod, *Euchaeta japonica* contained wax esters in their lipid stores, while triglycerides were important only in the eggs, early naupliar stages, and adults. The large lipid reserves of the eggs were wax esters and triglycerides (58% and 19% of the lipid, respectively), both of which were used rapidly during the early stages of development. Wax esters continued to decrease after triglycerides had been utilized completely for energy. The slow metabolism of lipid during starvation indicated that lipid stores in adult females may be conserved for egg production. The dominant alcohols of the wax esters of all stages were tetradecanol (24-42% of the total) and hexadecanol (25-65%). Only minor amounts of polyunsaturated alcohols were observed. There was, however, a high proportion of polyunsaturation in the wax ester fatty acids, even though octadecenoic was generally predominant (16-46% of the total wax ester fatty acids). The polyunsaturation of the wax esters fatty acids and the presence of 21:6 hydrocarbon suggest phytoplankton in the diet of adults and in the younger stages. Cholesterol was the main sterol, but there were minor amounts of desmosterol (1-12% of the total sterols) present. The latter sterol has not been found previously in copepods, although reported from Cirripedia and Decapoda.

INTRODUCTION

The calanoid copepods are an important part of the marine food web because of their predominance in the zooplankton. In previous work, dealing mainly with adult forms, we have noted that wax esters are an important reserve lipid in many species of copepods (1-3). The type of lipids in the early developmental stages of copepods and the possible presence of wax esters in these stages are largely unknown. *Euchaeta japonica* is a common copepod in the North Pacific (4), and adults of the genus *Euchaeta* are known to contain large stores of wax esters (3). The life history and techniques for maintaining the early life history stages are well known from the work by Campbell (5) and Lewis and Ramnarine (6,7). The life history consists of an egg stage, followed by six naupliar and six copepodid stages (the copepodid VI is the adult).

We present, in this article, the results of lipid analyses of the various stages of *Euchaeta japonica*. Both field collected and laboratory reared stages were used. In addition, the rate of utilization of lipids by the adults is given.

METHODS

E. japonica adults (both male and female), copepodid V, copepodid II, and copepodid I stages were collected for analysis by vertical net hauls (0-200 m) at Indian Arm, an inlet of the Strait of Georgia near Vancouver, British Columbia, Canada. The bright blue egg clusters were detached from females and some of the eggs were kept for analysis while the remainder were placed in large glass bowls and raised in the laboratory at 10 C. The colorless first naupliar stage is spent in the egg. So called late eggs were eggs which showed less of the blue color, thus indicating the presence of a high proportion of first naupliar stage individuals; these eggs were analyzed separately. Naupliar stages 2-4 were kept in filtered sea water without feeding. Nauplius stage 5-copepodid II were fed a mixture of algae, *Dunaliella tertiolecta* and *Phaeodactylum tricornutum*. Immature adults (200) were picked from plankton samples and transferred to filtered sea water for starvation experiments. At selected intervals, females were removed for analysis as described below.

The lipids of all stages were extracted by covering live animals with chloroform:methanol (2:1 v/v) and grinding gently with a glass rod. Contact at room temperature (30 min) was sufficient to extract all the lipid. The carcass left after lipid extraction was weighed to allow determination of lipid as a percent of dry wt. All subsequent work was carried out under nitrogen. The lipid was weighed and, for those stages where more than 6 mg was available, fraction-

[1]Present address: Skidaway Institute of Oceanography, Savannah, Georgia 31406.

TABLE I

Lipid Changes during Development of *Euchaeta japonica*[a]

Stage	Lipid/individual (mg)	Lipid (% dry wt)	Wax ester (% of lipid)	Triglyceride (% of lipid)
Eggs	0.59[b,c]	64.4	58	19
Eggs (late stage)	0.39[b]	58.1	50	17
Nauplius 2	0.02	43.8	61	17
Nauplius 3	0.02	30.8	56	5
Nauplius 4	0.02	25.0	20	3
Nauplius 5	0.04	21.2	15	1
Nauplius 6	0.04	17.0	12	absent
Copepodid I	0.03	14.2	9	absent
Copepodid I (field collected)	0.05	23.6	29	absent
Copepodid II	0.03	11.6	12	absent
Copepodid IV	0.20	31.2	40	3
Copepodid V	0.52	50.1	81	2
Adult ♀ (immature)	0.44	41.3	54	18
Adult ♀ (mature)	0.60	52.2	60	17
Adult ♂ (mature)	0.58	49.2	78	9

[a]Egg sacs were removed from females and placed in filtered sea water. Naupliar stages 2-4 were reared in filtered sea water at 10 C without feeding. Naupliar stages 5-copepodid II were raised in the laboratory by feeding them a mixture of phytoplankton. The remaining stages were field collected.

[b]Per egg cluster.

[c]Average 0.04 mg lipid/egg.

ated on a silicic acid column. Different lipid classes were eluted with solvents of increasing polarity, as described by Nevenzel, et al., (8) and weighed. The procedures for analyzing the five different lipid fractions (hydrocarbons, wax esters, triglycerides, sterols, and phospholipids) or their component acids and alcohols by gas liquid chromatography (GLC) are given in a previous paper (9). A Varian Aerograph (series 1800) gas chromatograph equipped with a digital print-out for peak areas was used for the work. The two columns used were a 2.4 m x 3.2 mm (outside diameter) column of 10% diethylene glycol succinate polyester (DEGS) and a 1.8 m x 3.2 mm column of 3% OV-1 on 60-80 mesh Gas-Chrom P (both supplied by Applied Science Laboratories, State College, Pa). Several different temperatures were used, depending upon the compounds being analyzed. Hydrocarbons were run at 150 and 170 C on OV-1 and DEGS, methyl esters of fatty acids at 160 and 190 C on DEGS, and at 170 and 200 C on OV-1, trifluoroacetate derivatives of long chain alcohols at 150 and 180 C on DEGS and 160 and 180 C on OV-1, and the sterol trifluoroacetates were run at 250 C on OV-1. Hydrogenation of fatty acid and alcohol mixtures was carried out to aid in identifications. The structures of some fatty acids were verified using an LKB gas chromatography-mass spectrometer (GC-MS) (model 9000).

When less than 6 mg total lipid was available, the lipids were separated by silicic acid thin layer chromatography (TLC), and the amounts of wax esters and triglycerides were determined spectrophotometrically by the procedure of Armenta (10) using dichromate digestion and measurement at 350 nm.

The procedures used to separate, identify, and quantitate the phospholipids were essentially those described by Parsons and Patton (11) using TLC and phosphorus analysis. The identities of the predominant phospholipids were confirmed by IR spectrum (Perkin-Elmer Infracord spectrometer, model 137) of the lipids after deposition as thin films on KBr pellets.

RESULTS

Changes in Lipid during Development

Wax esters were present in all stages of the life history of *E. japonica*, but triglycerides were important constituents only in the eggs, the early naupliar stages (to N2), and the adults (Table I). The eggs when first removed from the female had 0.59 mg lipid/egg cluster (0.04 mg/egg), but, for eggs in the late stage of development, this value had dropped to 0.39 mg of lipid/egg cluster (0.03 mg/egg). Lipid made up a decreasing proportion of the dry wt in the stages from nauplius 2 to copepodid II, although the absolute amount of lipid/animal was at a minimum until nauplius 4 (in nature, feed-

ing commences with the third naupliar stage but is not appreciable until nauplius 4) and then increased to a somewhat higher plateau from nauplius 5 until copepodid II. The lipids then increased rapidly to high contents (31-52%) in copepodid IV to adults. Triglycerides were used preferentially as energy source until, by nauplius stage 3, they had been largely depleted, and the major utilization of wax esters began. By nauplius stage 6, triglycerides had been used up completely, and even the wax ester content was approaching the minimum seen in copepodid stages I and II (ca. 0.4 μg wax esters/individual).

So called immature and mature adults (females) were analyzed separately. Mature adults showed internal evidence of developing eggs, whereas immature adults had no evidence of eggs. Mature adults were higher in lipid and wax ester content than immature adults, yet still did not have sufficient lipid to account for the total in the eggs. Thus, our mature adults may have been in the process of accumulating lipid stores for later transfer to the eggs.

Lipid Utilization by Starved Adults

Since the eggs are so high in lipid, the female must use most of her lipid stores for the production of eggs. One female apparently can produce several clutches of eggs, since animals with attached egg sacs were observed to be forming additional eggs. Although *E. japonica* reproduces throughout the year (5, 6), we assume that, during the winter, the availability of food is quite low so that utilization of lipid by adults must be carefully controlled during this period to produce eggs with sufficient fat stores. In starved immature females, the amount of total lipid went down slowly, confirming the hypothesis of careful control over wax ester mobilization. For the first seven days of starvation, they utilized triglycerides, with little or no depletion of wax ester stores (Table II). After the seventh day, when most of the triglycerides had been used, the wax esters began to be metabolized, and, by the end of the experiment, this lipid type was reduced to ca. a third of its original content.

Starvation experiments with mature females also showed rapid utilization of triglycerides but much slower utilization of wax esters, so that only after starvation for 37 days had the wax esters dropped to a third of their original content.

TABLE II

Starvation Experiments with *Euchaeta japonica*[a]

Starvation time (days)	Lipid (% dry wt)	Wax ester (% of lipid)	Triglyceride (% of lipid)
0	41	54	18
7	39	68	2
10	32	46	absent
12	33	38	absent
15	27	26	absent

[a]Immature adult females were collected in the field and transferred to filtered sea water (at 10 C).

Fatty Acids and Long Chain Alcohols

Table III presents the fatty acids and alcohols of the wax esters, triglycerides, and phospholipids in several stages of *E. japonica*, including eggs, copepodid V, and adults. Immature, mature, and seven day starved immature adults were analyzed. Only the data for adult females is reported, but the fatty acid and alcohol analyses for mature males were similar.

The 14:0 and 16:0 alcohols accounted for 21-24% and 52-65%, respectively, of the total alcohols in eggs and mature adults (copepodid Vs also showed a predominance of these alcohols). Beside major amounts of 14:0 and 16:0 alcohols, immature adults had 15% of 20:1 and 9% of 22:1 homologues; however, in the eggs, the latter two constituents accounted for less than 1% of the alcohols. There was no correlation between the fatty acids and alcohols of the wax esters in any stage of *E. japonica*.

There were a broad spectra of fatty acids in the different stages, and several interesting observations can be made. In most stages, the 18:1 fatty acid predominated in the wax esters, constituting 41-46% of the total in eggs and copepodid V and 16-27% in adults. The wax esters of immature adults were characterized by 40% of 16:2 fatty acid, while in other stages this fatty acid never accounted for more than 4% of the total. In a second collection of immature adults the amount of 16:2 acid in the wax esters was 12% and subsequent collections of immature adults has revealed that the amount of this acid is highly variable. The identification of the 16:2 acid from *Euchaeta* was based upon the fact that it had an identical retention time on the polar column with authentic 16:2 acid prepared from spinach chloroplasts. The one peak seen for 16:1, 16:2, and 16:3 acids on the nonpolar column was equal in amount to the sum of these individual peaks seen on the polar column. Also, hydrogenation gave the expected amount of 16:0 acid. The 14:1 fatty acid of the wax esters of immature adults was identified tentatively on similar grounds. The predominant fatty acids of triglycerides were 16:0, 18:1, and 20:5. The fatty acids of wax esters and triglycerides of different stages were similar, with the exception that in

TABLE III

Compositions (wt %) of Fatty Acids and Long Chain Alcohols of Different Stages of *Euchaeta japonica*[a]

Homologue	Eggs				Copepodid V				Immature adults				Mature adults				Immature adults[c]			
	WE	WE	TG	PL	WE	WE	TG	PL	WE	WE	TG	PL	WE	WE	TG	PL	WE	WE	TG	PL
	AL	FA	FA	FA	AL	FA	FA	FA	AL	FA[b]	FA	FA	AL	FA	FA	FA	AL	FA	FA	FA
14:0	21	2	9	9	42	1	7	12	36	3	7	3	29	8	9	9	26	2	7	5
16:0	65	3	20	21	53	1	31	29	29	5	17	14	52	4	18	14	50	1	14	14
16:1	ND	14	10	3	ND	16	7	2	1	6	10	1	ND	10	11	7	ND	10	9	5
16:2	ND	2	ND	ND	ND	2	1	Tr	ND	39	1	ND	ND	4	1	Tr	ND	1	2	Tr
16:3	ND	ND	ND	ND	ND	ND	1	ND	ND	4	Tr	ND	ND	4	11	ND	ND	1	14	ND
18:0	3	Tr	Tr	10	2	Tr	2	11	2	1	Tr	5	4	Tr	1	5	8	Tr	2	8
18:1	5	47	30	19	1	41	16	14	3	16	29	10	4	27	25	16	9	53	23	12
18:2	ND	3	3	1	Tr	3	4	1	Tr	Tr	4	1	Tr	5	5	2	Tr	4	2	3
18:3	ND	1	3	3	ND	2	3	1	ND	3	4	Tr	ND	6	3	Tr	ND	2	5	Tr
18:4	ND	Tr	3	ND	ND	2	3	ND	ND	3	3	ND	ND	2	2	ND	ND	3	2	ND
20:1	1	1	Tr	1	Tr	Tr	2	5	15	3	3	1	3	3	1	11	3	2	1	2
20:4	ND	Tr	Tr	2	ND	Tr	3	1	ND	Tr	2	2	ND	Tr	1	8	ND	1	1	Tr
20:5	ND	10	17	8	ND	17	10	5	ND	3	14	11	ND	14	7	9	ND	10	10	11
22:1	1	ND	ND	ND	Tr	Tr	ND	ND	9	ND	ND	Tr	2	ND	ND	ND	1	ND	ND	ND
22:5	ND	ND	Tr	ND	ND	Tr	Tr	Tr	ND	ND	ND	4	ND	Tr	ND	Tr	ND	1	ND	2
22:6	ND	15	3	15	ND	10	6	9	ND	1	4	40	ND	8	2	17	ND	6	4	30
24:1	1	ND	ND	ND	Tr	ND	ND	ND	ND	ND	ND	1	ND	ND	ND	ND	1	ND	ND	ND

[a]Only the principal components are given and they are rounded off to the nearest whole percent. AL = long chain alcohols, FA = fatty acids, WE = wax esters, TG = triglycerides, PL = phospholipids, ND = not detected, and Tr = trace.

[b]A principal fatty acid of this wax ester was 14:1 (9%).

[c]Starved seven days.

all cases the 16:0 fatty acid was a major component of triglycerides (14-30%), but only minor amounts of this fatty acid were in the wax esters (1-5%).

The phospholipid fatty acids were highly unsaturated in the adults, but a predominance of saturated fatty acids was evident in the phospholipids of eggs and copepodid V. As expected, the phospholipid fatty acid pattern in eggs was closer to that of mature adults than to that of immature adults, since mature adults contain developing eggs. During starvation, there were no changes in the phospholipid fatty acids, but the fatty acid composition of wax esters was altered dramatically, especially with respect to the relative amounts of 14:1, 16:2, 18:1, 18:2, and 20:5 fatty acids.

Our first analyses of the copepodid V phospholipid fatty acids were questioned because of low values 20:5 and 22:6. Because of this criticism and to verify the other data, we made a second collection of copepods from the same area and at the same time of the year. Analysis of all phospholipids was completed within two days of the end of the cruise, and the relative amount of 20:5 and 22:6 in copepodid V, as reported here, was higher than we previously found, although still much lower than the levels of these fatty acids in the adults. The fatty acids and alcohols of the other lipid classes were not found to be significantly different, with the exception of the 16:2 acid in immature adults noted above, for copepod stages collected during the two cruises.

Lipid Composition Data

Wax esters were the dominant lipid type in egg, copepodid V, and adult stages, and neutral lipids (wax esters plus triglycerides) accounted for ca. 80% of the total lipid; the structural lipids (phospholipids and sterols) amounted to less than 20%. The chain length (alcohol plus fatty acid) composition of wax esters showed no major differences in the different stages with a range of carbon numbers from C_{26}-C_{40} (Table IV). In all stages, C_{32} and C_{34} were the major components, totaling 47-93% of the wax esters and C_{36}, C_{38}, and C_{30} (generally in that sequence) accounting for most of the remainder. The predominance of C_{32} and C_{34} wax esters would be expected, since 18:1 was the main fatty acid and 16:0 and 14:0 were the dominant alcohols.

Hydrocarbons were less than 2% of the lipid, with pristane as the major hydrocarbon in all stages (30-50% of the total hydrocarbon). Of special interest was the presence in all stages of 21:6 hydrocarbon which was previously reported in phytoplankton (12-14). Between 30-40% of the hydrocarbons of adults and copepodid V and 7% of the egg hydrocarbons were composed of this polyunsaturated hydrocarbon. In addition, we noted minor amounts of a series of straight chain saturated and monounsaturated hydrocarbons ranging in length from C_{17}-C_{26}.

The so called polar lipid fraction, including free fatty acids, pigment, and sterols, accounted for 8-12% of the lipid. The main pigment was astaxanthin in all stages. As collected, the eggs were a bright blue, but, when extracted with chloroform, a red-orange solution was obtained. In benzene solution, the egg pigment had an absorption spectrum identical to that of authentic astaxanthin. The eggs of the barnacle *Lepas fasicularis* also contain a blue chromoprotein in which the main pigment is astaxanthin (15). Recently, Zagalsky and Herring (16) purified a blue carotenoprotein containing astaxanthin from the pontellid copepod, *Labidocera acutifrons*. The astaxanthin of the eggs is metabolized in the early naupliar stages of *E. japonica* so that late naupliar stages are colorless. The copepodid stages apparently have the ability to synthesize astaxanthin.

The major sterol of all stages was cholesterol (0.1-0.8 wt percent of the total lipids). Desmosterol, generally a minor constituent (less than 1%), amounted to 12 and 26% of the total sterols in, respectively, the seven day starved adults and the eggs. The source of this desmosterol is most probably dietary, since it generally is accepted that crustaceans, like other arthropoda, cannot biosynthesize cholesterol de novo but can convert dietary phytosterols to cholesterol via desmosterol (17). Why desmosterol should seemingly accumulate during starvation is not clear.

The phospholipids of eggs, adult male, and adult female were analyed by TLC. The major phospholipids of the adults were phosphatidyl choline (51-62%), phosphatidyl ethanolamine (32-47%), sphingomyelin (3-5%), phosphatidyl inositol (5-7%), and lysophosphatidyl choline (1-2%), while the egg phospholipids were phosphatidyl choline (67%), phosphatidyl ethanolamine, and phosphatidyl inositol (1%). No sphingomyelin was detected in the egg phospholipids.

DISCUSSION

The data presented here demonstrate the important role of wax esters in providing sustenance for the young naupliar stages of *E. japonica*. The wax esters and triglycerides of the eggs must provide all the energy for the nauplius 1 and nauplius 2 stages since these are nonfeeding stages, and, in fact, *Euchaeta* can be

TABLE IV

Wax Ester Compositions of *Euchaeta japonica* Stages (wt %)

Stage	Eggs	Late eggs	Naupliar 2	Naupliar 5	Naupliar 6	Copepodid I	Copepodid II	Copepodid IV	Adult females			Adult males
									Mature + eggs	Mature − eggs	Starved[a]	
Chain lengths of wax esters												
C_{28}		0.06				0.05			0.29	0.06	0.37	1.2
29		0.3								0.3		
30	3.8	4.9	4.4	4.4	9.1	4.9	3.9	7.3	10.6	10.2	9.1	8.1
31	0.6			0.8	1.4		0.2	0.9				
32	19.0	28.1	27.7	28.7	40.1	31.0	35.9	34.3	35.7	37.2	35.1	36.6
33	0.8	1.4		2.2				1.4				
34	28.4	42.6	42.1	46.3	38.8	50.2	57.8	26.6	34.0	37.2	34.7	37.8
36	14.1	18.4	14.3	12.8	10.6	12.0	2.2	18.1	12.1	11.9	12.0	10.3
38	16.3	4.1	11.4	4.7	ND[b]	1.8	ND	11.4	7.2	2.9	7.8	4.5
40	6.7	0.07	ND	Tr[c]		ND		Tr	ND	0.2	0.7	0.5
42	6.6	ND		ND				ND		ND	ND	0.3
44	2.3											

[a]Immature, starved 7 days.

[b]ND = not detected.

[c]Tr = trace.

raised to copepodid I without feeding (18 and A.G. Lewis, unpublished data). Since *E. japonica* reproduces throughout the year (6), the ability of young stages to use lipid stores would be important in the winter when food supply is low.

The egg clusters from females averaged 0.59 mg lipid, while mature adults with developing eggs had a total of only 0.60 mg lipid. Our mature copepods still may have been feeding so that their lipid stores had not yet reached maximum values at the time of analysis. Another explanation may be that the mature adults which we collected in September would not be producing their egg clusters until October or later; at this later time, there are fewer eggs/cluster (6) and possibly less lipid/egg. Maximum egg production takes place during the spring when lipid stores in the adult are probably at their maximum. Littlepage (19) has noted a large increase in lipid in *Euchaeta antarctica* during the summer when egg production occurs in this copepod. Corkett and McLaren (20) suggested that *Pseudocalanus* lengthens its period between egg production during times of low food. The slow utilization of lipid by starving females (Table II) indicates that lipid stores may be saved for egg production.

The eggs of *Euchaeta media*, collected off California, and *Euchaeta marina*, collected near Tahiti, were also rich in lipid with wax esters accounting for 72 and 58% of their lipid, respectively (21). Thus, most species of *Euchaeta*, regardless of environment, are assumed to have wax esters in their egg lipid stores. Lipid stores are present in the eggs of many marine invertebrates (22-24), and the thorough study of lipids in the young stages of the barnacles, *Balanus balanoides* and *Balanus balanus*, by Dawson and Barnes (25) demonstrated that the eggs of the barnacles were rich in triglycerides, which were consumed rapidly during development.

The wax esters of the filter feeding copepods, such as *Calanus* and *Rhincalanus* resemble their phytoplankton diet in having high proportions of polyunsaturated fatty acids (3, 9, 26). However, the wax ester fatty acids of deep water predatory copepods are mainly saturated and monounsaturated, with 18:1 accounting for over 50% of the total (3). Our data indicate that *E. japonica* is omnivorous, since the wax ester fatty acids show both high polyunsaturation and high 18:1 content (16-50%). This agrees with Pandyan's (27) observation that adult *E. japonica* would feed on both large phytoplankton and zooplankton. The immature adults seem to be exceptions with their high content of 16:2, a characteristic fatty acid (together with the 16:3, 18:3, and 18:4 homologues) of phytoplankton (28, 29). Immature adults collected at different times had quite variable contents of 16:2 acid. This could result if they graze extensively on phytoplankton for brief periods, since marine phytoplankton fatty acids may contain up to 14% of the 16:2 homologue (29). Similarly, in marine fish, the presence of small amounts of such acids, variable with season, has been interpreted as temporary survival from dietary phytoplankton (30). In both *Euchaeta* and fish, the characteristic phytoplankton acids are significant constituents only in the neutral storage lipids. The dominance of hexadecanol and absence of appreciable polyunsaturation in the wax ester alcohols suggest that the carbon chains of phytoplankton fatty acids are not converted directly into alcohols in *E. japonica*. Hexadecanol previously has been noted as the dominant alcohol of the wax esters of deep water copepods and fish (3, 31).

The presence in *E. japonica* of 21:6 hydrocarbon, which is synthesized by phytoplankton (12), also indicates the presence of phytoplankton in the diet of the various stages. The presence of 21:6 hydrocarbon in the eggs shows that the adult is able to transfer this hydrocarbon to the eggs. Because of the sizeable amounts of 21:6 hydrocarbon in the adults and copepodid V, it would appear that phytoplankton is important in the diet of these stages. Among all the other copepod species examined, including herbivorous species of *Calanus* (12), only *Rhincalanus nasutus* contained this hydrocarbon. The reason for its retention by *Euchaeta japonica* and *Rhincalanus nasutus* is not clear.

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Lipid Peroxidation in Chronic Ethanol Treated Rats: In Vitro Uncoupling of Peroxidation from Reduced Nicotine Adenosine Dinucleotide Phosphate Oxidation

MARY KOES, Biochemistry Section, Cancer Research Center, Columbia, Missouri 65201, and **TOM WARD** and **SAM PENNINGTON,** Department of Biochemistry, School of Medicine, East Carolina University, Greenville, North Carolina 27834

ABSTRACT

Chronic ethanol treated rats were found to have enhanced ethanol metabolism and to metabolize ethanol in vivo in the presence of an inhibitor of alcohol dehydrogenase. In vitro studies of the hepatic microsomal system thought to be responsible for this activity showed it to be markedly induced. Lipid peroxidation also was enhanced in the ethanol treated animals. The lipid peroxidation was shown to be uncoupled from the microsomal nicotinamide adenine dinucleotide phosphate, reduced form, oxidase activity by a low concentration of azide.

INTRODUCTION

The liver enzyme that catalyzes the oxidation of ethanol, alcohol dehydrogenase (ADH), is among the most studied of proteins. The result of these studies has been to demonstrate that chronic ethanol consumption has little effect upon the biochemical behavior of ADH (1,2). Because of this result, alternate mechanisms for the formation of the biochemical lesion associated with chronic ethanol consumption in certain individuals have been investigated.

In 1965, Orme-Johnson and Zeigler (3) reported that liver microsomes could oxidize ethanol to acetaldehyde in the presence of nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) and oxygen. This work was confirmed by several workers (4,5). Attempts to determine the mechanism of this reaction (6,7) have indicated that the reaction appears similar to the microsomal drug metabolizing system, in that cytochrome P-450, NADPH, and O_2 are required for maximum activity, but variations exist (6).

Further research has indicated the microsomal ethanol oxidizing system (MEOS) is inducible by chronic exposure to ethanol. The MEOS contains cytochrome P-450 and NADPH oxidase activities, both of which are induced by ethanol exposure (4,5).

Recent in vitro experiments (8) have indicated that NADPH oxidase is not necessary for ethanol oxidation, i.e. P-450 alone may catalyze the reaction; however, the reduced cofactor (NADPH) and O_2 are required. We have confirmed these findings (9), but, because of the extremely low activity associated with purified P-450, as compared to total microsomal activity, we still are concerned with NADPH oxidase activity in chronically exposed animals. This concern is based upon several reports of a lipid peroxidation reaction that occurs in conjunction with the oxidation of NADPH by this enzyme.

Experiments have been carried out that indicate that the MEOS may contribute significantly to ethano metabolism in vivo in animals chronically exposed to ethanol. We have verified these findings and wished to determine the role played by NADPH oxidase in the peroxidation reaction.

EXPERIMENTAL PROCEDURES

Administration of Ethanol

Ethanol was administered daily to 20 female Holtzman rats (150-200 g) at a dose rate of 3 g/1000 g body wt. The dose was given by stomach tube with the ethanol mixed 50/50 (v/v) with normal saline. Control animals were administered equal volumes of saline only. After 3 weeks, experimental and control animals were fasted for 24 hr. The animals then were administered ethanol, after which blood samples were drawn by heart puncture at 8, 16, and 24 hr.

A second group of experimental animals was used to determine the effect of ethanol consumption upon certain of the intermediates of the MEOS system. The ethanol administration in this group of animals followed the method of Porta and Gomez-Dumm (10), with the exception that the sucrose was 20% (w/v) and the ethanol 25% (w/v). We previously had found that rats readily accept ethanol in this form, plus allowing them to have access to rat chow which we feel to be a more normal situation that a totally liquid diet.

Ethanol Determination

The blood samples, collected as outlined above, were collected in tubes containing a few crystals of ethylenediaminetetraacetic acid (EDTA). Blood (0.1 ml) was added to a clean centrifuge tube along with 0.1 ml 5% $ZnSO_4$ and 0.1 ml 0.1 N $Ba(OH)_2$. The resulting mixture was centrigued at 8000 x g for 10 min and 1.0 μliter of the clear supernatant injected into a gas chromatograph.

The Bendix model 2100 chromatograph used for the analyses was equipped with a 6 ft x 1/4 in. outside diameter Teflon (Dupont, Wilmington, Del.) column packed with Poly Pak 2 (Hewlett-Packard Co., Avondale, Pa.) and operated at 135 C; the detector (flame ionization) was operated at 220 C using hydrogen (50ml/min) and oxygen (400 ml/min). Nitrogen carrier gas was used at a flow rate of 50 ml/min. The instrument was calibrated using known amounts of ethanol in water.

Ethanol metabolism by ADH was measured by a modification of the method of Greenburger, et al. (1), in that the amount of ethanol formed was quantitated by gas liquid chromatography (GLC).

Alcohol Dehydrogenase Inhibition

Pyrazole (1,2-diazole) was used to inhibit ADH (11) in the clearance rate studies. The compound was administered (intraperitoneal) in water at a concentration of 18 mg/100 g body wt, 2 hr before the alcohol dose. To determine that the pyrazole concentration remained at sufficiently high levels to inhibit ADH during the experiment, pyrazole was assayed at the end of the 24 hr period by a procedure developed in this laboratory (12).

Microsome Isolation

Livers were excised and homogenized in 3 volumes of 1.15% KCl. The homogenate was centrifuged at 600 x g for 20 min and the pellet discarded. The supernatant was spun further at 9000 x g for 60 min and the pellet removed. The remaining supernatant was centrifuged at 105,000 x g for 90 min and the pellet saved as the source of microsomal enzymes. The 105,000 x g supernatant was used as a source of ADH. All operations were carried out at 0-4 C.

NADPH Oxidase

NADPH oxidase activity was measured by recording the decrease in absorbance at 340 nm of a solution containing NADPH (0.25 μmoles), nicotinamide (100.0 μmoles) and enzyme (0.1 ml 105,000 x g pellet suspended in 15.0 ml 0.1 M phosphate buffer pH 7.4) in a total volume of 3.0 ml 0.1 M phosphate buffer, pH 7.4. The reference solution was identical, except no NADPH was added. The rates were recorded using a Beckman DB-G spectrophotometer equipped with a log chart recorded.

Malonaldehyde (MA) Reagent (Thiobarbitaric Acid [TBA])

TBA reagent was prepared in Tris buffe (0.01 M, pH 7.0) and contained 0.1 M TBA Enzyme reaction mixtures were treated wit 5% trichloroacetic acid (TCA) to deproteinize The analytic solution contained 2.0 ml enzym mixture, 2.0 ml TBA reagent, and 0.5 ml 0.5 N HCl. The color was developed by heating at 100 C for 10 min, and a solution of 1,1,3,3-tetraethoxypropane was used as a standard. The solutions were read at 535 nm after centrifugation.

P-450 Assay

The P-450 content was assayed by measuring the different spectra of a preparation following the procedure of Omura and Sato (13). The Δ optical density between 450-490 nm was used to quantitate the samples. The factor of 91 cm^{-1} $mmole^{-1}$ was used to convert absorbance to mmoles.

All data obtained for enzyme activities and ethanol metabolism were placed on a per mg of protein basis by running a standard Lowry, et al., (14) protein determination on all tissue samples.

RESULTS AND DISCUSSION

It was the working hypothesis that ethanol, by virtue of its dual nature with respect to lipid and water solubilities, confuses the normal metabolic system during chronic consumption. The normal route of metabolism (via ADH) functions in all cases, but the normal individual has only low (basal) levels of the MEOS. When confronted with excessive amounts of ethanol, the microsomal system is induced markedly, leading to peroxidative destruction of large amounts of fatty acids associated with phospholipids.

The first aim of this research was to verify that chronic ethanol exposure induces the various components of the MEOS and that a lipid peroxidation reaction was coupled to this induction, such that the peroxidation was enhanced by ethanol exposure. The peroxidation of lipid has been suggested as the initial step in formation of the biochemical lesion associated with ethanol consumption (11), and several experiments have demonstrated that antioxidants inhibit the adverse effects of ethanol in animals.

The data presented in Figure 1 confirm the

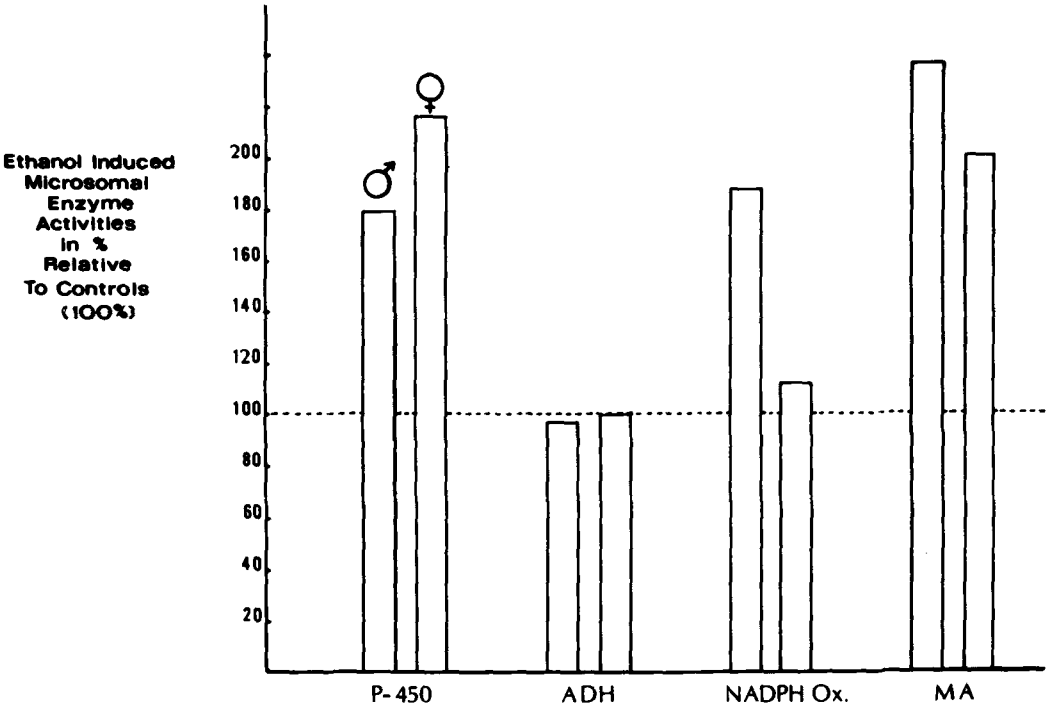

FIG. 1. Various microsomal activities from ethanol treated animals. Values relative to control animals (100%). The malonaldehyde (MA) was measured in each tube following microsomal ethanol oxidation as described by Lieber and DeCarli (4). Alcohol dehydrogenase (ADH) was measured in a 105,000 x g supernatant. The assay procedures were as described in the text. Normal values (females) were as follows: P-450 = 0.32 ± 0.05 (standard deviation) nmoles/mg; ADH = 54 ± 5 (standard deviation) μmoles/min/mg; MA control = 0.98 μg/mg, ethanol treated = 1.88 μg/mg; nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) oxidase = 2.65 ± 0.51 x 10^{-2} Δ optical density/min/mg for controls and 3.29 ± 0.81 x 10^{-2} Δ optical density/min/mg for ethanol treated animals ($p<0.05$). Peroxidation experiments were corrected for blanks containing no NADPH. Generally, little, or no, peroxidation was observed in any samples when NADPH was absent.

inducibility of various intermediates of the MEOS, as well as demonstrating a lipid peroxidation (measured as MA formation) associated with ethanol metabolism by the microsomal preparation. As predicted, the ADH levels were not influenced by prior exposure to ethanol, while cytochrome P-450 and NADPH oxidase were elevated. The activity that most closely follows the peroxidation levels is that of NADPH oxidase (both evelated, males more than females although not proportionally). This result is as expected in light of previous reports that the action of this enzyme in other systems is coupled to a simultaneous lipid peroxidation reaction (15,16).

The second aim of this study was to determine if the induced microsomal system makes a contribution to ethanol metabolism in vivo such that lipid peroxidation might be expected to occur. The data given in Figure 2 represents blood ethanol levels determined at various times on 4 groups of animals. These groups are control animals, control animals treated with pyrazole, chronic ethanol treated animals, and chronic ethanol treated animals further treated with pyrazole. The data are presented using the 8 hr blood level as a reference point which allows comparison of clearance rates for animals with different absorption levels. The data for control and ethanol treated animals indicates that all the ethanol was cleared by 25 hr, with some indication being given that the ethanol treated animals cleared the alcohol faster than did the control animals. Better evidence is seen in the comparison between control and ethanol induced animals treated with pyrazole. It is evident that the ethanol treated animals cleared ca. 20% of the 8 hr value, whereas the control animals actually had more ethanol at 16 and 24 hr. The data in Figure 2 represent 20 animals carried through the procedure and have a $p<0.05$ for differences between the means of control and experimental groups. Similar findings regarding ethanol metabolism

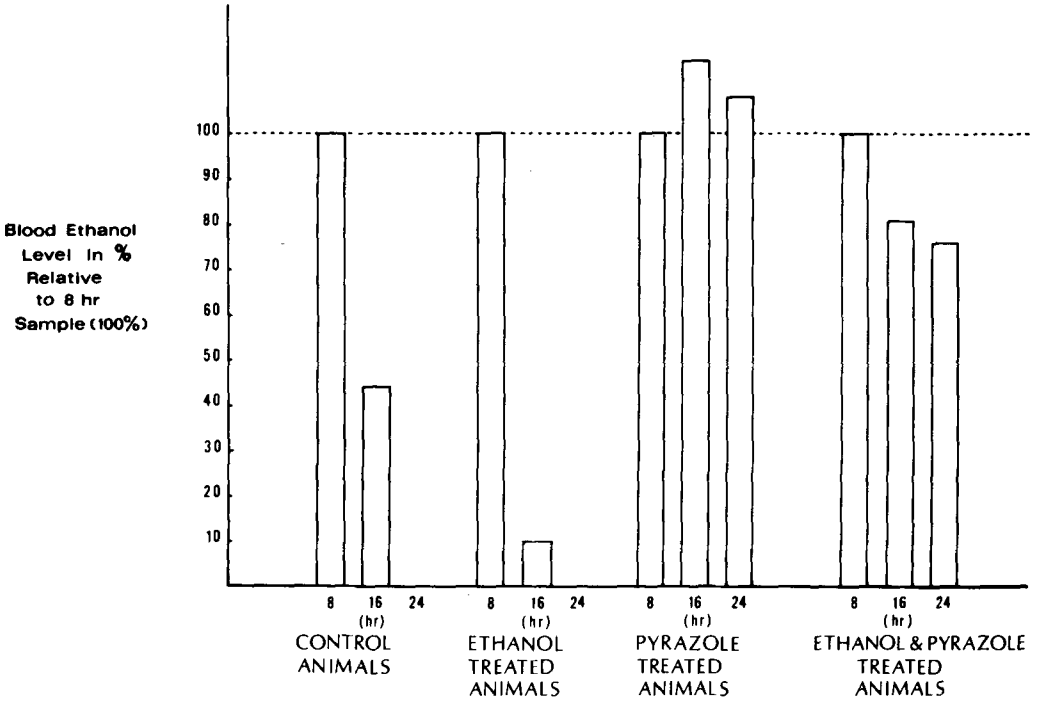

FIG. 2. Blood ethanol clearance by control and chronic ethanol treated animals. In certain experiments, the control animals and chronic ethanol treated animals were treated further with pyrazole. All values are relative to blood levels (100%) 8 hr following an intoxicating dose (6 g/kilo) of ethanol. Assay conditions are given in the text. The data represent 20 animals carried through the experiment, 5 in each group. Differences between the control group and the ethanol and ethanol plus pyrazole group were significant at the 0.05 level. Wide variability in the ethanol absorption was observed, especially in the pyrazole treated animals.

by the MEOS have been reported by Lieber and DeCarli (7).

To ensure that the ethanol treated animals did not metabolize pyrazole faster than controls, and thus have functional ADH, the circulating levels of pyrazole in control and ethanol treated animals were determined at the end of 24 hr. Both groups had comparable levels of pyrazole.

To test the coupling between NADPH oxidase and the generation of MA, an experiment was carried out using isolated microsomes in a system similar to the assay media for NADPH oxidase, except that NADPH was added at 40 min intervals for 2 hr. The oxidase activity was monitored spectrophotometrically, and samples were withdrawn from the cuvette every 20 min for analysis for peroxidized lipid content. The microsomes from ethanol treated animals generated elevated levels of MA, and the appearance of the peroxidized materials in the solution followed the addition of substrate for the NADPH oxidase enzyme. This suggests that NADPH oxidation lies near the rate determining step for the formation of peroxidized lipid in the microsomal preparations.

A possible mechanism for this reaction has been suggested (17) to involve hydrogen peroxide generated according to the equation:

$$NADPH + H^+ + O_2 \xrightarrow[\text{Oxireductase}]{\text{NADPH}} NADP^+ + H_2O_2$$

$$\text{Unsaturated fatty acids} \xrightarrow{\text{Catalase}} \text{Oxidation products (MA)}$$

The actual peroxidation reaction is thought to be carried out via a catalase or peroxidase enzyme. This mechanism is consistant with the idea that NADPH oxidase is the rate limiting step for peroxidation, since the generation of a supply of H_2O_2 should be the limiting factor for the peroxidation reaction. Such a reaction, i.e. H_2O_2 plus catalase, also would oxidize ethanol, and various studies have investigated the relationship between the MEOS and catalase (9).

An attempt was made to provide support for the proposed lipid peroxidation mechanism by the use of the classical catalase and peroxidase inhibitor, azide ion. Control and chronic etha-

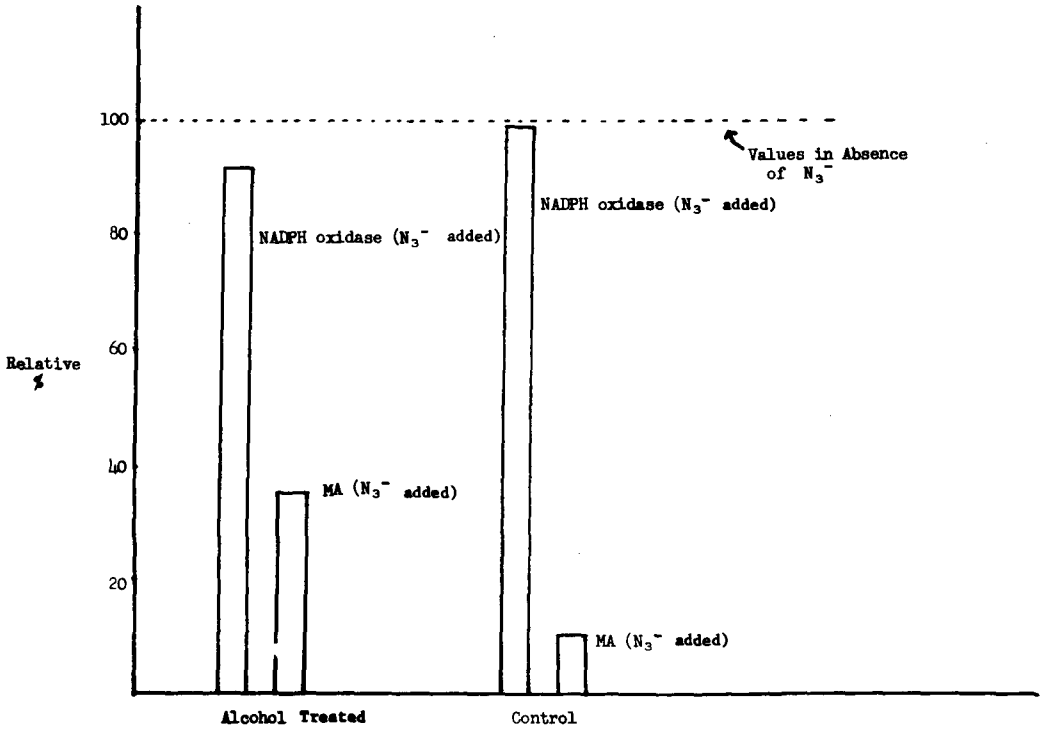

FIG. 3. Comparison of nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) oxidation to malonaldehyde (MA) formation in control and chronic ethanol-treated rats in the presence of added azide ion. All values relative to experiments in the absence of azide (100%). Total oxidase activity in the ethanol treated rats was ca. 1.5 times the control values in the absence of azide. Other normal values are given in Figure 1 for comparison; NADPH oxidase activity in the absence of azide ion was 4.1 μmole/min/mg for controls and 5.2 μmole/min/mg for alcohol treated. Each value was determined for four animals.

nol treated animals were used as sources of microsomes, and the NADPH oxidase and peroxidation (as MA) were measured both in the presence and absence of added azide. Comparison of NADPH oxidase from control and ethanol treated animals showed enhancement of the oxidase due to ethanol feeding, but this is not evident from Figure 3 due to presentation in terms of percentages. What is apparent is that, relative to experiments without azide (100% line), the NADPH oxidase activity is not influenced greatly by the addition of azide, while the formation of peroxidized lipid (MA) in the same reaction tube was depressed significantly. This result would indicate that the peroxidation of lipids is not linked irreversibly to NADPH oxidation, as has been suggested (15,16).

Further experiments are underway to determine the amount of lipid peroxidation that occurs in such systems in the presence or absence of ethanol with or without added azide. These experiments hopefully will determine whether ethanol oxidation per se causes the peroxidation or, as indicated by the above experiments, whether ethanol merely induces the oxidase enzymes which can contribute to lipid peroxidation whenever a reduced cofactor is available.

An important difference in our incubation system for NADPH oxidation and MA formation, as compared to those reported in the literature, should be noted. The system used in our experiments does not require the addition of ferric iron or ADP for maximum activity or either NADPH oxidase of MA formation. This may be the result of running the experiments in a phosphate buffer system, as phosphate is known to stimulate the peroxidation reaction (R. Reitz, University of North Carolina, personal communication). Contamination of phosphate salts by Fe^{+++} may explain the maximal stimulation observed in phosphate buffers.

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Properties of Palmityl-CoA: L-α-Glycerolphosphate Acyl Transferase from Bovine Mammary Microsomes

M.J. GROSS and **J.E. KINSELLA**, College of Agriculture and Life Sciences, Department Food Science, Cornell University, Ithaca, New York 14850

ABSTRACT

Palmityl-coenzyme A: L-α-glycerolphosphate acyltransferase is the most active acyltransferase of bovine mammary microsomes, with a specific activity ranging from 8-20 nmoles min^{-1} mg^{-1} protein. Corresponding acylation rates of 2.2, 1.4, 2.1, and 0.6 nmoles min^{-1} mg^{-1} were obtained for myristyl-, stearyl-, oleyl- and linoleyl-coenzyme A, respectively. Optimum pH of palmityltransferase was 7.7, and activity was not affected by buffer molarity in range 25-150 mM. Inhibitory effects of palmityl-coenzyme A (10 μM/0.1 mg microsomal protein) was relieved by bovine serum albumin. Sonication magnesium and ethylenediaminetetraacetic acid enhanced activity. Delipidation of microsomes reduced activity by 84%; restoration of extracted lipids achieved 70% of original activity. Apparent Km and Vmax values of 4.1 and 260 μM and 9.5 and 8.2 nmole min^{-1} mg^{-1} were determined for palmityl-coenzyme A and D,L-α-glycerolphosphate, respectively, using untreated microsomes. The enzyme was stable as lyophilized microsomes when stored at -30 C. Phosphatidic acid was the major product and marked quantities of diglycerides were formed, especially when microsomal protein was increased.

INTRODUCTION

The mammary gland of the lactating cow acylates ca. 1 kg fatty acids/day, indicating the possession of active acyl transferase(s). The activity of these enzymes markedly increases with the initiation of lactation which is apparently related to the synthesis of secretory triglycerides (1-3). Acyl transferases of bovine mammary microsomes may be unique, because they mediate, in a nonrandom manner, the acylation of predominantly saturated substrates having a wide range of mol wts (4). They specifically locate saturated acids (mostly palmitic and myristic) on position sn 2 (4-5), and they preferrentially locate endogenous short chain and oleic acid on position sn 3 of milk triglycerides (1,6). Bovine mammary acyl transferases also may perform the unique function of assembling heterogeneous (structural and compositional) triglycerides to ensure fluid milk fat droplets at physiological temperatures and facilitate secretion.

Whether different acyl transferases are involved in acylation of positions sn 1, sn 2, and sn 3 of milk glycerides is being investigated. Because of their importance, we studied mammary acyl transferases, particularly palmityl-CoA: L-α-glycerolphosphate (LαGP) acyl transferase, which is the most active in vitro (7).

MATERIALS AND METHODS

Preparation of microsomes: Mammary tissue excised from cows immediately after slaughter was minced in a meat grinder. This was homogenized in a regular Waring blender at top speed for 30 sec, after 1:2 dilution with phosphate bicarbonate buffer pH 7.4 (70 mM $KHCO_3$, 85 mM K_2HPO_4, and 9 mM KH_2PO_4), and rehomogenized in a Waring blender with a polytron assembly for 20 sec. All manipulations were carried out at 4 C.

The homogenate was centrifuged in a Sorvall refrigerated centrifuge using a G.S.A. rotor (r = 5.75 in.) at 1020 x g for 10 min to remove cellular debris. The supernatant was centrifuged at 14,600 x g for 15 min to remove mitochondria. This supernatant was centrifuged in a Beckman model L2-65 ultracentrifuge with type 21 rotor at 44,000 x g for 75 min. The microsomal pellets were pooled and freeze-dried, and small lots were stored in teflon sealed vials at -30 C.

Preparation of enzyme solution: Appropriate amounts of freeze-dried microsomes were dissolved in potassium phosphate buffer (66 mM, pH 7.4) using a tissue homogenizer with teflon pestle. The solution was sonicated for 1 min at 4 C in a model 8845-3 sonicator (Cole-Parmer Instrument Co., Chicago, Ill.), and the microsomes then were assayed for enzyme activity. Protein was determined by the method of Lowry (8) using crystalline bovine serum albumin as standard.

Enzymatic assay: Enzyme activity was measured spectrophotometrically (9) and by radioactive assay. Incubation media contained: acyl-CoA 5-20 μM; D,L-α-glycerolphosphate (DL-αGP) 400 μM; 5,5′ dithiobis-2-nitrobenzoic

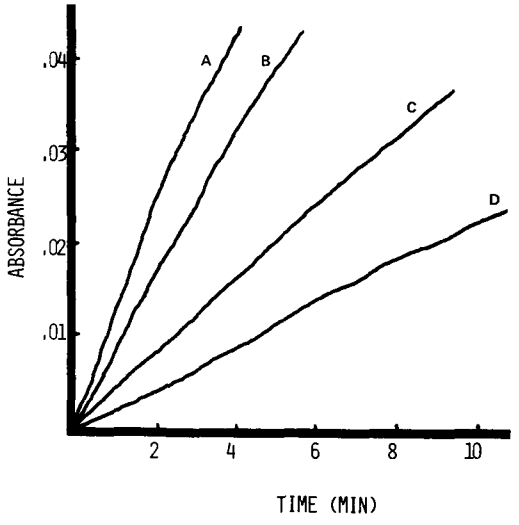

FIG. 1. Spectrophotometric tracing (at 412 nm) showing initial velocity of bovine mammary palmityl-CoA: L-α-glycerolphosphate acyl transferase as a function of various protein levels. Assay medium as in Table I. A = 0.3 mg protein, B = 0.2 mg protein, C = 0.1 mg protein, and D = 0.05 mg protein.

acid (DTNB) 1.0 mM; bovine serum albumin 3 mg, 0.1 mg microsomal protein in 1 ml Tris-HCl buffer (66 mM, pH 7.4). The incubation medium was equilibrated at 31 C for 3 min before acyl-CoA was added. The reference (control) sample lacked L-α-glycerolphosphate. Acyl-CoA: L-α-glycerolphosphate acyl transferase activity was quantified by the reaction of liberated CoASH with DTNB as measured continuously at 412 nm with a Perkin-Elmer model 356 spectrophotometer. A molar absorbance of 13,600 was used.

Delipidation: To determine the role of microsomal lipids on the activity of L-α-GP-acyl transferase, samples of microsomes were delipidated and assayed. Batches of freeze-dried microsomes (15 mg) were twice extracted with 20 ml acetone, acetone:water (9:1 v/v), or benzene. The extracted microsomes were dried carefully by nitrogen (4 C) and stored at -30 C. The extracted lipids, in ether solution, were stored for addition to the delipidated microsomes in restoration experiments.

Sonication of enzyme: Enzyme solutions were sonicated for a total of 60 sec at 4 C. One solution was sonicated using a bath sonicator model 8845-3 (Cole-Parmer Instrument Co.) Others were sonicated at different settings using a probe sonicator with a sonifer power supply (Bronson Sonic Power, 20,000 Hertz, Bronson Instruments, Danbury, Conn.). After sonication, the microsomes were assayed quickly for enzyme activity.

Analysis of products: To determine the products of the acyl transferase reactions, U-^{14}C-L-αGP or alternatively 1-^{14}C-palmityl-CoA or 1-^{14}C-stearyl-CoA was used in assays using similar conditions as described in the regular assay. The assay, containing appropriate amounts (0.1 mg) of microsomal protein, acyl-CoA, and DL-αGP, were incubated with continuous shaking at 30 C for 10 min, and the reaction was terminated by extracting the lipids according to the procedure of Folch, et al. (10). The solvent was evaporated, and the lipids dissolved in chloroform. The total radioactivity in each sample was determined. Lipids were fractionated by thin layer chromatography (TLC) using the system described by Lamb and Fallon (11), i.e. a solvent system of chloroform-methanol-3.5M NH_4OH (65:35:8). Standard lipid mixtures (Applied Science, State College, Pa.), including phosphatidic acid, were cochromatographed with these lipids. The lipid spots were located using iodine vapor. After evaporation of the iodine, the spots containing the identified lipids were transferred to scintillation vials, and 10 ml toluene base scintillation solution, containing 5.0 g 2,5-diphenyloxazole and 0.3 g 1,4-bis 2(4 methyl-5 phenyloxazolyl)benzene (Amersham/Searl Corp., Chicago, Ill.)/liter toluene, was used in each vial. Then radioactivity was quantified in a Packard TriCarb scintillation spectrometer. From these data, the extent of esterification into the glycerolipids was calculated.

Materials: All chemicals were reagent grade Bovine serum albumin (BSA) (fraction V powder), 5,5[1] DTNB, disodium DL-αGP, and CoA (trilithium salt) were purchased from Sigma Corp., St. Louis, Mo.). Stearyl-CoA, palmityl-CoA, oleyl-CoA, myristyl-CoA, linoleyl-CoA, and lecithin (bovine) were purchased from P.L. Biochemical (Milwaukee, Wisc.). L-α-glycerol 3-phosphate (U-^{14}C), 1-^{14}C-palmityl-CoA and 1-^{14}C-stearyl-CoA were purchased from New England Nuclear Corp. (Boston, Mass.).

RESULTS

Palmityl-CoA; L-αGP acyl transferase was dependent upon the addition of active enzyme preparation, palmityl-CoA, and DL-αGP. Acyl transferase activity was linear over a range of protein concentrations (Fig. 1). The rate of acylation of palmityl-CoA was proportional to the microsomal protein up to 0.3 mg. All subsequent incubations containing 0.1 mg microsomal protein were assayed at 31 C.

The optimum pH of palmityl-CoA: L-αGP acyl transferase was 7.4-8.0. However, the enzyme was active over a pH range, i.e. 6.5-8.5.

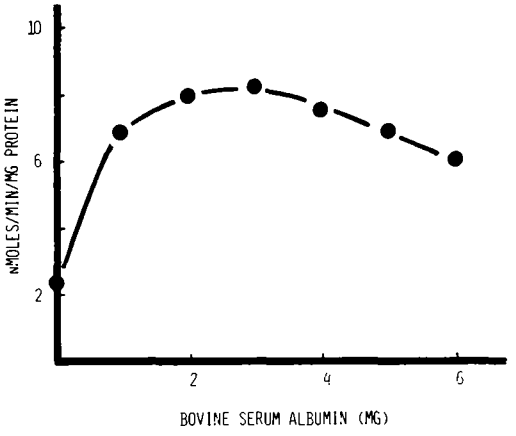

FIG. 2. Effect of bovine serum albumin upon specific activity of palmityl-CoA: L-α-glycerolphosphate acyl transferase from bovine mammary microsomes. Assay contained palmityl-CoA, 10 μM and D,L-α-glycerolphosphate, 450 μM. Other conditions as described in the text.

Increasing molarity of buffer in range 25-150 mM did not affect specific activity of the enzyme.

DTNB up to 1.0 mM stimulated release of CoA but inhibited the reaction above this level.

Magnesium ions enhanced activity, i.e. 0, 0.25, 0.5, and 1.0 mM (magnesium chloride) gave rates of 8.0, 9.2, 10.7 and 12.5 nmoles palmityl-CoA acylated min^{-1} mg^{-1} protein. The addition of ethylenediaminetetraacetic acid (EDTA) (2 mM) to the normal assay media doubled the rate of acylation, and the inclusion of magnesium ions (1.0 mM) in excess of the EDTA further accelerated the reaction 4-fold over untreated microsomes. This observation is being studied in detail.

Inhibition of palmityl-CoA: LαGP acyl transferase by acyl-CoA was relieved by the addition of BSA, with 3 mg/ml providing optimum activity (Fig. 2).

TABLE I

Effect of Sonication on Palmityl-CoA: L-α-Glycerolphosphate Acyl Transferase Activity from Bovine Mammary Microsomes[a]

Treatment	Acylation rate nmoles/min/mg protein
Normal	8.0
Sonicated (bath)	10.4
Sonicated (probe) 1	12.0
Sonicated (probe) 2	9.0
Sonicated (probe) 3	7.2

[a]In all cases, the enzyme solutions were sonicated for a total of 60 sec at 4 C. Complete assay contained: palmityl-CoA, 10 μM; dithiobis-2-nitrobenzoic acid, 1 mM; D, L-α-glycerolphosphate, 450 μM; 0.1 mg microsomal protein; Tris-HCl, 66 mM; and pH 7.4.

Sonication increased acyl transferase activity (33%) over that of a control, nonsonicated, enzyme preparation (Table I). The inclusion of small amounts of ethanol in the assay did not affect acyl transferase activity, whereas from 2-10% ethanol caused a gradual depression in activity, i.e. from 5-80% of normal.

Removal of microsomal-bound lipids reduced the specific activity of the acyl transferase. The effect was modified with the solvent used, i.e. acetone:water which removed more of the polar lipid components had the most pronounced effect (Table II). Addition of the extracted lipids even with sonication failed to restitute original activity; however, 70% of activity was restored by egg phospholipids, and phosphatidyl choline restored activity by 54%.

The enzyme became relatively saturated at low concentrations of palmityl-CoA, but increasing the acceptor, DL-αGP, to ca. 500 μM increased activity (Fig. 3). Using low levels of palmityl-CoA and saturating concentrations of

TABLE II

Effects of Solvent Extraction and Lipid Restoration upon Activity of Palmityl-CoA: L-α-Glycerolphosphate Acyl Transferase from Bovine Mammary Microsomes[a]

Treatment	Specific activity nmoles/min/mg protein
Normal microsomes	8.2
Acetone (100%) extracted microsomes	5.7
Acetone:water (9:1 v/v) extracted microsomes	1.3
Plus extracted lipids	2.1
Plus egg phospholipids	5.7
Plus bovine phosphatidylcholine	2.0
Benzene (100%) extracted microsomes	3.6
Plus extracted lipids	4.4

[a]Assay medium as in Table I.

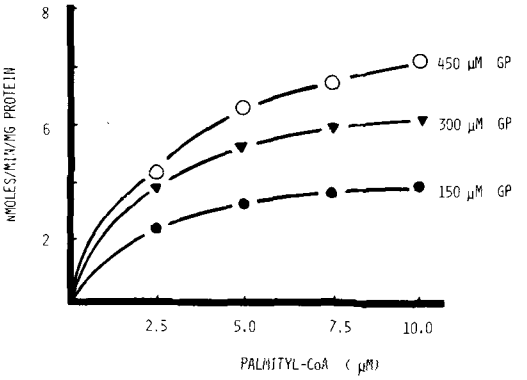

FIG. 3. Effects of increasing substrate concentrations upon acylation by palmityl-CoA: L-α-glycerolphosphate acyl transferase from bovine mammary microsomes. α-Glycerolphosphate denotes D,L-α-glycerolphosphate. Assay conditions as described in the text. GP = glycerolphosphate.

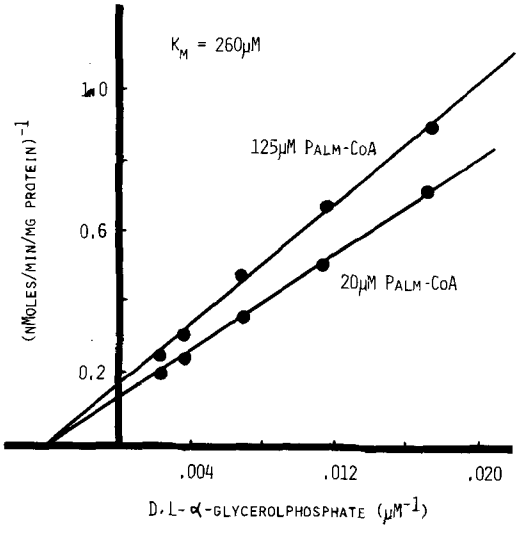

FIG. 4. Lineweaver-Burk plots showing effect of D,L-α-glycerolphosphate concentrations upon the rate of acylation at two levels of palmityl-CoA by palmityl-CoA: L-α-glycerolphosphate acyl transferase from bovine mammary microsomes. Assay medium contained bovine serum albumin, 6 mg; two fixed levels of palmityl-CoA, 20 and 125 μM; and varying levels of D,L-α-glycerolphosphate. All other conditions described in the text.

DL-αGP (500 μM), an apparent Km of 4.1 μM for palmityl-CoA was determined. The apparent Km of the palmityl-transferase for DL-αGP was 260 μM. Noteworthy was the inhibition of the enzyme by higher levels of palmityl-CoA (Fig. 4). At low levels of microsomal protein, i.e. 0.1 mg, the acyl transferase activity was inhibited by palmityl-CoA concentrations above 25 μM. This inhibition could be relieved by increasing

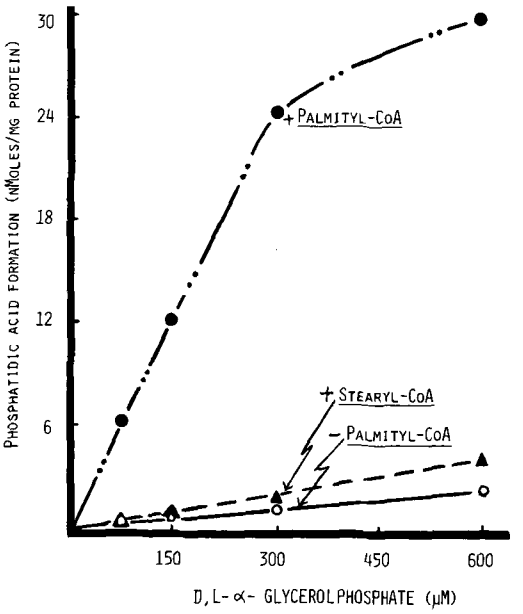

FIG. 5. The incorporation of L-α-glycerolphosphate (U-^{14}C) into phosphatidic acid in the absence, (○) and presence of (●) palmityl-CoA (20 μM) or (▲) Stearyl-CoA (20 μM) using 0.1 mg microsomal protein from bovine mammary microsomes incubated for 5 min at 35 C.

the microsomal protein or BSA content of the medium.

The apparent Vmax for the enzyme was 9.5 and 6.2 nmole/min/mg untreated microsomal protein for palmityl CoA and DL-αGP, respectively. The specific activity of palmityl-CoA: αGP acyl transferase from mammary tissue of different cows varied widely, i.e. from 1-22 nmoles palmityl-CoA acylated/min/mg protein. Most of the mammary tissue assayed was obtained from animals in late lactation, hence the values are probably lower than those obtaining in vivo at peak lactation. The enzyme used in the present study demonstrated remarkable stability, i.e. its specific activity decreased slightly, from 9.0-8.2 nmoles/min/mg protein, during storage for 9 months at -30 C as a freeze-dried powder.

Using labeled (U-^{14}C) DL-αGP and palmityl-CoA in normal assays and subsequent analysis by TLC, the principal product was phosphatidic acid (Table III). The presence of diglycerides probably reflects the activity of microsomal-bound phosphatidate phosphohydrolase, because acylation of monoglycerides was negligible in these preparations. Negligible radioactivity occurred in the triglycerides. When saturating levels of acyl-CoA were employed in the presence of 0.1 mg microsomal

TABLE III

Distribution of Radioactivity in Ester Lipids Following Incubation of U-^{14}C-L-α-Glycerolphosphate with Bovine Mammary Microsomes and Palmityl-CoA[a]

Lipid class	Incubation time 3 min	6 min
L-α-GP incorporated (nmoles/min)	3 ± 1.0	5.0 ± 1.0
	Distribution of radioactivity	
	Percent	
Lysophosphatidic acid	4 ± 1.0	3.5 ± 0.5
Phosphatidic acid	59 ± 2.1	63 ± 2.0
Diglycerides	25 ± 1.6	26 ± 3.0
Phospholipids	9.5 ± 1.5	5.0 ± 1.0

[a]Incubation tubes contained dithiobis-2-nitrobenzoic acid, 1 mM; bovine serum albumin, 3 mg; microsomal protein, 0.4 mg; palmityl-CoA 30 μM; and D, L-α-glycerolphosphate (U-^{14}C), 100 μM in 1 ml sodium phosphate buffer (66 mM, pH 7.2).

protein, phosphatidic acid formation increased with the levels of L-αGP provided (Fig. 5). The rate of phosphatidic acid synthesis observed using radioactive substrate was consistent with that observed in spectrophotometric assays. Incorporation was low when stearyl-CoA was used as substrate, and, in absence of acyl-CoA, phosphatidic formation acid was negligible, indicating a low concentration of endogenous acyl-CoAs in these bovine mammary microsomes.

Palmityl-CoA was the preferred acyl substrate under our assay conditions (Table IV). Acylation rates obtained with myristyl-CoA, stearyl-CoA, and oleyl-CoA were comparable but much less than with palmityl-CoA. The preference for palmityl-CoA was observed consistently with microsomal preparations of varying specific activities obtained from three different lactating animals.

TABLE IV

Comparative Rates of Acylation of D, L-α-Glycerolphosphate (450 μM) with Different Acyl-CoA Species by Acyl-CoA: L-α-Glycerolphosphate Acyl Transferase from Bovine Mammary Microsomes

Substrate	Rate nmoles/min/mg protein	
	Substrate concentration	
	10 μM	25 μM
Myristyl-CoA	1.7	2.2
Palmityl-CoA	8.2	9.0
Stearyl-CoA	1.0	1.4
Oleyl-CoA	1.4	2.1
Linoleyl-CoA	0.4	0.6

DISCUSSION

The involvement of acyl transferases in milk lipid synthesis has been reported for several species (7, 12-19). Our data indicate that microsomal enzymes are involved and that the properties of the enzymes from bovine mammary are quite similar to those from other animal tissues (11, 20-25).

The broad pH optimum with maximum activity around pH 7.5 for L-αGP acyl transferase agrees with the findings of others (21, 22, 26, 27). Lamb and Fallon (11) and Brandes, et al., (28) reported an optimum of 6.5 for the acyl transferase from rat and guinea pig liver, respectively. The negligible effects of altering the molarity of the buffer observed in this study corroborates the results of Jezyk and Lands (29) who showed that changes in concentrations of the buffer (8-80 mM) did not significantly affect the rate of αGP acylation by rat liver microsomes. However, Kuwahara (30) reported that acyl transferase of rat brain microsomes was affected by nature of buffer and ionic strength of the medium and that these effects could be ascribed to the state of hydration of the microsomes. However, it also could be related to the availability of free cations.

DTNB, a thiol reagent at low concentrations slightly stimulated acylation of L-αGP by bovine mammary microsomes in both spectrophotometric and radioactive assay systems. There are no previous reports of such stimulation. In fact, N-ethylmaleimide inhibited acyl transferase of pigeon liver microsomes by binding thiol groups (31), and Lands and Hart (32) reported that DTNB actually inhibited the acyl transferase mediating the initial acylation of αGP.

The effect of BSA in facilitating palmityl-CoA transferase has been well documented for this enzyme from other tissues and for other

enzymes which use acyl-CoA as substrates (20, 21, 28, 32,33). BSA reversibly binds palmityl-CoA and, at relatively low concentrations, prevents the formation of micelles, relieves inhibition of the acyl transferase, and conceivably ensures a monomolecular solution of substrate palmityl-CoA, as indicated by linearity of acylation rate. Furthermore, the BSA binds the free fatty acids released by the acyl-CoA hydrolase present in bovine mammary microsomes (1, 34). In the absence of BSA, nonlinear rate curves were obtained, as observed by Tanioka, et al. (15).

Mild sonication conceivably enhanced the acyl transferase by facilitating access of substrate to the enzyme via exposure of more microsomal surface area. It also may alter kinetic properties of these enzymes (36). Excessive sonication impaired activity, presumably as a result of protein denaturation. Similar effects were observed with bovine mammary microsomal stearyl-CoA desaturase (34).

The observation of Jezyk and Lands (29) that ethanol inhibited acyl transferase was confirmed in this study. It is probable that the ethanol solubilized some of the lipids required by this enzyme and thereby impaired function. This mechanism was corroborated by demonstration of the absolute lipid requirement for lipid, particularly polar lipids, by this enzyme. The observation that phosphatidyl choline was effective in restoring activity is consistent with the knowledge that this lipid is the major component of mammary microsomal membranes (35), and it confirms the report of Abou-Issa and Cleland (24) and Yamashita, et al., (23, 36) concerning the lipid requirement of this enzyme.

The apparent Km of DL-αGP for palmityl-CoA transferase (260 μM), while being lower, is within the general range, i.e. 0.2, 0.4, 0.5, and 0.67 mM, reported by Yamashita and Numa (36), de Jimenez and Cleland (37), Abou-Issa and Cleland, (24) and Lamb and Fallon (11) respectively, using L-αGP with rat liver microsomes but higher than the .05 mM reported by Husbands and Lands (31) for pigeon liver microsomes. It compares favorably with the Km of this enzyme of mammary microsomes from lactating rat and guinea pig. Mammary tissue of guinea pig and cow may contain limiting concentrations of L-αGP. Concentrations of ca. 90 and 80-250 μM L-αGP have been measured in guinea pig and bovine mammary (13, 38), respectively. These values vary depending upon nutritional and physiological status of these lactating animals. However, they indicate that availability of L-αGP may affect rate of glycerolipid synthesis in mammary tissue because of the apparent high Km of this substrate for the enzyme.

The Km for palmityl-CoA (4 μM) occurs at ca. the critical micellar concentration (cmc) for palmityl CoA (33) and is identical to Km for rat mammary microsomes which was measured at low substrate concentrations in the absence of BSA (15). Values of 10 and 50 μM were reported by Yamashita and Numa (36) and Lamb and Fallon (11). These are above the cmc, but these disparities can be explained by the difficulties involved in determining accurate Kms of amphipathic molecules, like long chain acyl-CoA species, as documented by several researchers (20, 27, 33, 34, 39) and discussed by Gatt, et al. (40).

In this respect, the problem of reconciling observations made on enzymes in vitro with the situation obtaining in vivo is challenging. In vitro low protein to substrate ratios usually are required for linearity, whereas in vivo higher protein to substrate ratios prevail. Okuyama and Lands (27) have studied this problem using the acyl transferases of rat liver microsomes. Relatively high concentrations of acyl-CoA exist in mammary tissue, e.g. 44 μM in guinea pig (13), and free fatty acids may attain concentrations of >4 mM (18) in cow mammary tissue. These facts should be considered when discussing specificity of acylation based upon in vitro observations.

The marked preference of the bovine mammary L-αGP-acyl transferase for palmityl-CoA has been discussed in relation to synthesis and the unique structure of bovine milk glycerides (7). The observed specificity provides an explanation for the nonrandom distribution of acyl groups in milk glycerides (4). Our preliminary data (7) and previous reports (5) would support the suggestion that a combination of partial segregation of fatty acids and specificity of acyl-CoA transferases accounts for the unique, nonrandom pattern of fatty acids in milk triglycerides.

The rapid, preferential acylation of position sn 1 and sn 2 of αGP with palmityl-CoA by these mammary microsomes is consistent with the events in vivo, wherein most of palmitic acid is located on sn 1 and sn 2 of milk glycerides (4). Furthermore, the observed preference of this enzyme for palmityl-CoA probably explains the preferential absorption and esterification of palmitic acid by bovine mammary cells (41) and its facile acylation by mammary homogenates (18). The acyl transferase from rat mammary showed a similar substrate preference, though not as exclusive as the bovine (15). Several other researchers have reported that palmityl-CoA is the most effec-

tive acyl donor in acylation of L-αGP by microsomes (11, 25, 26, 28, 42-44).

The rate of acylation of palmityl-CoA by mammary microsomes was ca. 10-fold greater than its rate of desaturation (34), whereas, by contrast, the respective activities for stearyl-CoA were similar. This observation provides an explanation for the greater generation of oleic compared to palmitoleic acid in bovine mammary microsomes and conceivably in lactating tissue also (34).

Phosphatidic acid was the principal product in these assays. Some workers reported that lysophosphatidic acid was the principal product (11, 28, 32, 45). However, several researchers have determined phosphatidic acid as the major product of acylation of L-αGP by microsomal preparations (13, 21-28, 31, 37, 39, 43-46). Significant amounts of radioactivity occurred in 1-sn-acylglycerolphosphate only when small amounts (50-100 mM) of (1-^{14}C) palmityl CoA were provided in our assays. The acylation of 1-acyl-sn-glycerolphosphate by bovine mammary microsomes (7) was more rapid (3-4 times) than the initial acylation, and the preference for palmityl-CoA was not as pronounced as in the acylation of DL-αGP. Our observations support the suggestion (41) that the L-αGP pathway is involved in bovine mammary glycerolipid synthesis and indicates that, in this respect, bovine mammary tissue is similar to mammary tissue of goat (16,17), rat (15), and guinea pig (12,13).

The phosphatidic acid formed by bovine mammary microsomes is dephosphorylated rapidly to sn-1,2 diglyceride and, in fact, only can be detected easily when low levels of microsomal protein (0.1-2 mg) are used and products are analyzed during the initial (linear) phase of the reaction. This indicates the presence of an active phosphatidate phosphohydrolase in bovine mammary tissue, as has been reported for mammary tissue of other species (15-17). It also validates the interpretation of precursor product curves obtained following the incubation of bovine mammary cells with radioactive acetate and glycerol in earlier separate experiments (47,48).

ACKNOWLEDGMENTS

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Preparation of Fully Deuterated Fatty Acids by Simple Method

C.Y.Y. HSIAO, C.A. OTTAWAY, and D.B. WETLAUFER,
Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota 55455

ABSTRACT

An economical, simple, efficient system of deuterating saturated fatty acids at atmospheric pressure has been developed. Exchange is carried out between fatty acids and deuterium gas over palladium on charcoal catalyst at 195 C. The method was applied to even membered fatty acids containing from 6-14 carbon atoms. The method yielded perdeuterated fatty acids of high isotopic purity with no evidence of other reaction products.

INTRODUCTION

Perdeutero fatty acids are in increasing demand for a variety of investigations. Preparation of perdeutero fatty acids from algae (1) or by some synthetic methods (2,3) is either expensive or inconvenient. Atkinson, et al., (4) reported palladium (Pd) catalyzed hydrogen exchange of several hydrocarbons at atmospheric pressure. Their method appeared simple enough and chemically plausible to apply to straight chain fatty acids. This article reports the results of a feasibility study. By an adaptation of the method of Atkinson, et al., (4) saturated straight chain fatty acids can be deuterated completely with relative simplicity and convenience.

EXPERIMENTAL PROCEDURES

An Aerograph hydrogen generator, model A-650, was used to produce deuterium gas by the electrolysis of a 20% solution of KOD in D_2O. KOD solution was prepared by weighing and thinly slicing metallic potassium (Mallinckrodt, St. Louis, Mo.) on a piece of smooth ceramic tile in a nitrogen tent (Instruments for Research and Industry, Cheltenham, Pa.). The potassium slices in nitrogen atmosphere were washed free from kerosene with light petroleum ether and blotted with soft tissue paper. The potassium then was transferred to a shallow nickel dish on the shelf of a desiccator in the nitrogen tent. D_2O (99+% D isotope) had been placed at the bottom of the same desiccator in a large dish (14 cm diameter). The desiccator was closed and provided with a pressure release device (Bunsen valve) to permit escape of the D_2 reaction product. It took ca. 5 days for 50 g sliced potassium metal to be converted completely to a concentrated solution of KOD with 100 ml D_2O. The desiccator was stored in the nitrogen tent at room temperature during that period. The KOD solution was diluted with D_2O to give the desired (20%) concentration. The concentration was calculated from the wt of potassium metal and the volume of solution.

The deuterium gas generated was passed through a drying tower packed with granular anhydrous $CaSO_4$ before introduction into the reaction vessel. The gas flow rate was 35-40 ml/min. Pd on charcoal (5% or 10% Pd) (Matheson, Coleman, & Bell, Norwood, Ohio) was employed as catalyst. Catalyst (4 or 5 g) was added to 25 g caproic, caprylic, capric lauric, or myristic acid (Sigma grade, Sigma, St. Louis, Mo.) in a 50 ml Erlenmeyer flask. The reaction mixture: catalyst slurried in neat fatty acid, was stirred magnetically and heated in an oil bath (Fisher wax, mp 60 C) at 195 C ± 5 C. A cylindrical Pyrex container with a diameter of 8-9 cm, wrapped with heating tape (Electroflex, Heat, Bloomfield, Conn.) and insulted with a winding of asbestos strings, was adequate for an oil bath. An asbestos board was inserted between the oil bath and the magnetic stirrer to reduce heat loss and protect the magnetic stirrer. Temperature was controlled by a variable transformer between the mains and the heating tape. The reaction vessel was conntected to a water condenser and then (in series) to a chilled ethanol condenser (-30 C) to lower the rate of fatty acid loss by volatilization. Solid fatty acid was noticed in the alcohol cooled condenser when the cooling temperature was <-15 C. The alcohol chilled condenser was thawed once every 24 hr to drain out the condensed mixture of volatilized fatty acid and water. A series of fatty acids could be deuterated simultaneously, in series, to economize on the use of deuterium gas and to save time. Two dry ice-cooled cold fingers were used as traps between each fatty acid reaction mixture to safeguard against carry-over from one reaction mixture to the next. Glass joint apparatus with Teflon sleeves was used. Tight seals were required to prevent condensation of atmospheric water vapor in the system. The alcohol cooled condenser was designed with an outlet at the top for the deuterium gas to pass to the cold finger and also a trap with a needle valve at the bottom of the same condenser to drain out water condensed from moisture originally in the system. A Calrod (500 watt) heated tube filled with CuO, ar-

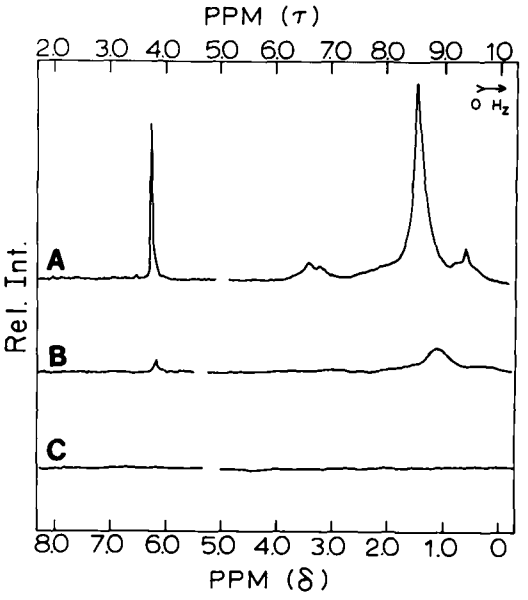

FIG. 1. Proton magnetic resonance (PMR) spectra of dodecanoic acid after varying times of deuteration. A-zero time; B-100 hr; C-194 hr. The spectra were obtained at 50 C under identical conditions of filter band width (4 Hz), radio frequency field (0.005 right, 0.02 left), sweep time (250 sec.), sweep width (250 Hz right, 1000 Hz left), and spectrum amplification (1.6 x 1 right, 1.0 x 10 left). Rel. Int. = relative intensity.

ranged according to Wiberg (5), was assembled to oxidize and recover the ^1H-diluted deuterium gas passing out of the last reaction vessel. However, the above mentioned arrangement often will not be necessary, because the consumption of heavy water was only ca. 100 ml in a 100 hr run.

The deuteration process was monitored by proton magnetic resonance (PMR) (Varian A-60D analytical NMR instrument) analysis. Samples were taken for analysis periodically with pasteur pipettes and Büchner filtered using extremely retentive filter paper (Carl Schliecher and Schuell, no. 576). Fatty acids that were solid at room temperature were Büchner filtered in an oven at temperatures 5-10 C above their mp, and the PMR measurements on these samples were taken at 70 C.

IR spectra were obtained from thin films of the neat acids with a Perkin-Elmer 2373 spectrometer.

Mass spectroscopy was carried out with a Hitachi RMU6D mass spectrometer.

Gas liquid chromatography (GLC) was carried out on methyl esters of the acids, employing a Hewlett-Packard research chromatograph 5750, and 10% diethyleneglycol succinate on

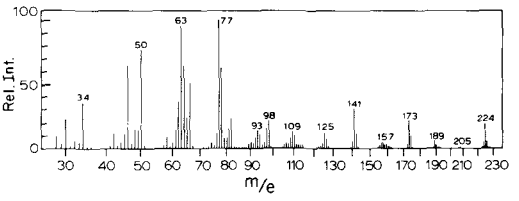

FIG. 2. Mass spectrum of exhaustively deuterated (194 hr) dodecanoic acid, obtained at 70 eV. Rel. Int. = relative intensity.

80-100 mesh Chromosorb W-BP. The methyl esters were prepared by the methanol-BF_3 method of Morrison and Smith (6).

RESULTS AND DISCUSSION

The exchange of deuterium for hydrogen on the fatty acids takes several days for virtual completion under these conditions, but the reaction requires little attention once initiated. Different parts of the fatty acid molecule exchange at different rates. Figure 1 shows, as an example, how the PMR spectrum of dodecanoic acid changed progressively with deuteration. The peak at 6.2 ppm represents the -COOH proton. Of all the features of the original spectrum, this peak disappeared most rapidly on deuteration. The relative contributions of the different methylene protons also are seen to have changed after 100 hr (compare the top and middle curves).

The neutralization equivalent of caproic acid before deuteration was found to be 119 (Theory: 116). After deuteration, a neutralization equivalent of 135 was found (theory: 128). There is no indication here of appreciable reduction of the acid.

The major peaks in the GLC of the perdeutero esters were retarded in comparison with the perhydro esters. We saw no significant change in the chemical composition of the samples following complete deuteration.

IR spectra of the acids were obtained before and after deuteration. For example, the IR spectrum of deuterated caproic acid (neat) showed absorption bands of medium intensity at 1054, 1085, 1298, and 2100 cm^{-1}; strong bands at 1357, 1710, and 2215 cm^{-1}; a broad band of medium intensity at 2535 cm^{-1}; and broad bands of low intensity at 2910 and 3425 cm^{-1}. The following bands, apparent in perhydro caproic acid, were absent in the perdeutero compound: 935, 1105, 1235, 1280, 1412 (present but very weak), 1466, 2855, 2865, 2930, 2960 cm^{-1}. Also absent in the perdeutero caproic acid spectrum was the broad absorption envelope from 3000-330 cm^{-1}. The IR spectra

observed were consistent with those reported by Jones (7) for perhydro and perdeutero fatty acids.

The mass spectra obtained for the perdeuterated fatty acids are exemplified by the fragmentation pattern of exhaustively deuterated dodecanoic acid shown in Figure 2. The pattern is qualitatively similar to that obtained from the perhydro-dodecanoic, with mass replacements of ^1H by ^2H in the fragments.

Caprylic and myristic acids were deuterated to the extent of $\geq$ 95% in 113 hr under the above conditions, starting with 24 g perhydro acids and 4 g 10% Pd on charcoal. The uncorrected yields of deuterated acids were 65% and 75%, respectively. We attribute losses to volatilization over the exchange period, withdrawal of samples for analysis, and mechanical losses in filtration. There were no obvious nonmechanical losses. In limited comparisons, the catalyst containing 10% Pd appeared to be more effective than that containing 5% Pd.

A higher temperature and a higher catalyst concentration might be expected to speed the exchange process. However, the practical temperature limit for this bath fluid was ~ 195 C based upon volatility. The amount of Pd-charcoal catalyst is limited by the requirement of maintaining a reaction slurry sufficiently fluid that effective stirring can be achieved. Effective stirring is essential, since there exists the ultimate in heterogeneity: a three phase system composed of liquid fatty acid, solid catalyst, and gaseous D_2.

In summary, we have shown that Pd catalyzed deuteration of fatty acids occurs under preparatively feasible conditions. Since a similar method already has been shown effective for deuteration of hydrocarbons, the present procedure may be applicable for deuteration of additional classes of organic compounds.

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Complex Formation and Reversible Oxygenation of Free Fatty Acids

I.M. CAMPBELL, Department of Zoology and Scarborough College, University of Toronto, West Hill, Ontario, Canada, **R.B. CATON,** Department of Chemistry and Scarborough College, University of Toronto, West Hill, Ontario, Canada, and **D.N. CROZIER,** Cystic Fibrosis Clinic, Hospital for Sick Children, Toronto 2, Ontario, Canada

ABSTRACT

Combined manometric and spectrophotometric studies reveal that prior to true oxidation unsaturated fatty acids undergo an oxygenation which is reversible in response to changes in oxygen pressure. The oxygenation seems to result from the oxygen molecule having a specific affinity for pairs of olefinic bonds.

INTRODUCTION

Berry and Turner (1) demonstrated that the rate of oxygen uptake by intestinal mucosa and liver homogenate of rat is stimulated by fatty acid esters of sucrose, linoleates having a much greater effect than stearates. The rate of oxygen uptake by fatty acids and their esters, in bulk at 37 C, increases directly with the number of unsaturated bonds, and linoleates take up oxygen 25 times faster than do oleates (2). Decreased metabolic activity, in both plants and animals, frequently is associated with an increase in the oleic and a decrease in the linoleic content of the constituent lipids, particularly the phospholipids (3-7). Such observations suggested to us that lipids may be involved in the facilitation and regulation of oxygen at the cellular level. Our hypothesis assumes that fatty acids form complexes with oxygen and that these interactions are readily reversible in response to changes in oxygen pressure. The results reported here, on the effect of uptake of oxygen upon the UV absorption spectra of free fatty acids, indicate that, prior to true oxidation, the unsaturates, but not the saturates, undergo a reversible oxygenation. For the purposes of this article, we define oxygenation as a readily reversible physical interaction, in contrast to oxidation, which we define as an irreversible formation of new chemical species. The oxygenation seems to result from the oxygen molecule having a specific affinity for pairs of unsaturated carbon-carbon bonds.

MATERIALS

For spectrophotometric studies, the following fatty acids were used: stearic (Fisher reagent grade), oleic, linoleic (Serdary, 99%, gas liquid chromatography [GLC]), and linolenic (Aldrich > 99%). Oxygen uptake of bulk samples was determined using reagent grade fatty acids whose purity, on the basis of UV and IR spectra, was greater than 97%. The spectral absorbance due to impurities coincided mainly with that of the conjugated derivatives of the parent species.

METHODS

Manometry

To measure the quantity of oxygen taken up by a fatty acid, a 100 ml round bottom flask containing a 10 ml sample was attached to a high vacuum (1×10^{-6} Torr, 1×10^{-4} Nm^{-2}) manifold, which was equipped with a multiple range recording capacitance monometer (MKS Baratron). To ensure that there was little or no residual free oxygen in the acid at the beginning of each experiment, the sample was degased at 1×10^{-6} Torr and 23 C, with slow stirring, for 24 hr. An oxygen pressure of 700 Torr was established in the manifold and flask, and changes in pressure were recorded continuously, while the flask was maintained at 40 ± 0.5 C. The experiment was carried on with continual replenishment of the oxygen until there was little or no further uptake of oxygen by the sample.

Spectrophotometry

The reversibility of oxygenation of fatty acids was assessed by measuring their UV absorption under oxygen pressures of 160 Torr (2.1×10^{4} Nm^{-2}) and 1×10^{-3} Torr (1×10^{-3} Nm^{-2}). A cyclohexane (Spectrograde) solution of each fatty acid was placed in a Suprasil quartz vacuum cuvette (1.0 mm path length) fitted with a greaseless teflon stopcock for attachment to a high vacuum (1×10^{-6} Torr, 1×10^{-4} Nm^{-2}) manifold. For determinations on the conjugated derivatives of linoleic acid, the spectra of 6.4×10^{-3} M solutions were scanned from 350-205 nm in a Bausch and Lomb 505 spectrophotometer. For the other samples, the spectra of solutions (ca. 6.6×10^{-4} M) were scanned from 350-185 nm in a Cary-14 spectrophotometer. (To determine the spectra between 185-180 nm, one oxygen-

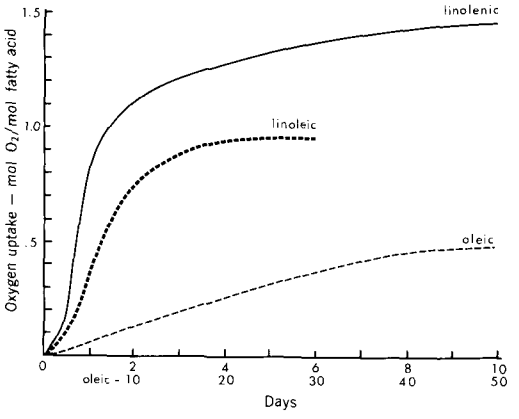

FIG. 1. The uptake of oxygen by oleic, linoleic, and linolenic acids, in bulk, at 40 ± 0.5 C. The ordinate is in units of number of moles of oxygen/mole of fatty acid. Note that the upper time scale is for linoleic and linolenic, whereas the lower time scale is for oleic.

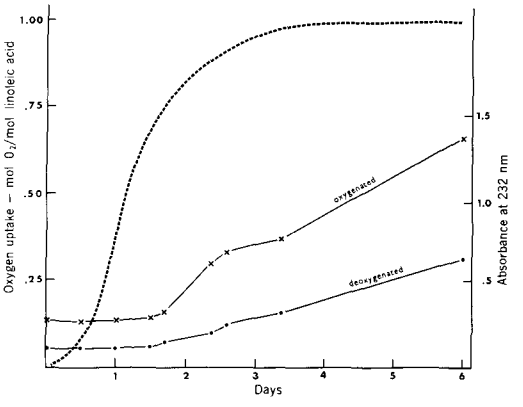

FIG. 2. The relation between the uptake of oxygen by linoleic acid and its autoxidation to conjugated derivatives at 40 ± 0.5 C, as measured by changes in absorbance at 232 nm for 6.4×10^{-3} M solutions in cyclohexane at oxygen pressures of 160 Torr (2.1×10^{4} Nm^{-2}) and 1×10^{-5} Torr (1×10^{-3} Nm^{-2}), labeled oxygenated and deoxygenated, respectively. The dashed curbe is taken from Figure 1.

ated and one deoxygenated sample of each fatty acid was scanned in a Beckman DK 2A spectrophotometer.) Following the initial scan, the sample was degased by the standard technique of free-thaw cycling at 1×10^{-5} Torr until there was no measurable decrease in absorbance. To ensure that the observed changes in absorbance resulted from decreased oxygen pressure, the solution was reoxygenated at 160 Torr. In every instance, the intensity of the absorbance returned to that of the initial nondegased solution. As controls, other degased samples similarly were regased with pure nitrogen or helium. In each instance, the absorbance remained close to that of the degased sample.

Because it was found that the absorption of the cyclohexane solvent was also sensitive to oxygen at wavelengths shorter than 232 nm (8), absorption spectra were determined for both oxygenated and deoxygenated solvent. These spectra were subtracted from the appropriate spectra for oxygenated and deoxygenated fatty acid.

To demonstrate that the difference between the gased and degased spectra was not due to free molecular oxygen, the spectrum of 1 atm pure oxygen vs vacuum was recorded. There was no measurable absorption in this wavelength range.

Oxygen Uptake by and Autoxidation of *cis*-9, *cis*-12 Octadecadienoic (Linoleic) Acid

Following the determination of oxygen uptake by the various fatty acids, 10 ml samples of linoleic acid were treated similarly, and the formation of conjugated derivatives was monitored by spectrophotometry. At intervals, a small aliquot was withdrawn from the flask and dissolved in cyclohexane. The UV absorption spectrum was scanned for each solution in the oxygenated, deoxygenated, and reoxygenated states. The absorbance at 232 nm was taken as a measure of the relative concentration of conjugated derivatives (hydroperoxides) of linoleic acid.

RESULTS AND DISCUSSION

The great difference in the rate of oxygen uptake by bulk samples of oleic and linoleic acids (Fig. 1) indicates a synergistic effect between olefinic bonds within the same carbon chain which are separated by one methylene group. That the existence of a third olefinic bond, as in the linolenic acid molecule, results in only a relatively small further increase in the rate of oxygen uptake suggests that the synergism probably occurs between pairs of methylene interrupted olefinic bonds. Since the oxygen to fatty acid molar ratios at maximal oxygen uptake by the monene, diene, and triene bulk fatty acids were ca. 0.5, 1.0, and 1.5, respectively, it seems that a pair of olefinic bonds is required for the reaction with each oxygen molecule.

If enhancement of UV absorptivity due to conjugated derivatives is taken as a measure of autoxidation, then the fact that such enhancement lags considerably behind the uptake of oxygen (Fig. 2) suggests that the uptake of oxygen by linoleic acid is neither caused by nor necessarily coincident with autoxidation. Indeed, more than 75% of the total oxygen ab-

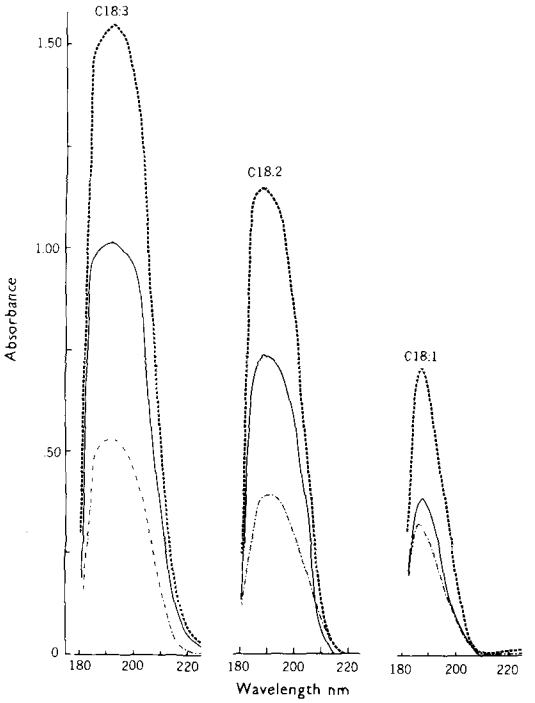

FIG. 3. UV absorption by solutions of linolenic (6.58×10^{-4} M), linoleic (6.44×10^{-4} M), and oleic (6.34×10^{-4} M) acids in the oxygenated (- - - -) and deoxygenated (- - - - - -) state. Absorption resulting from the formation of FA:O_2 complexes (——) was derived by subtracting the absorbance in the deoxygenated state from that in the oxygenated state.

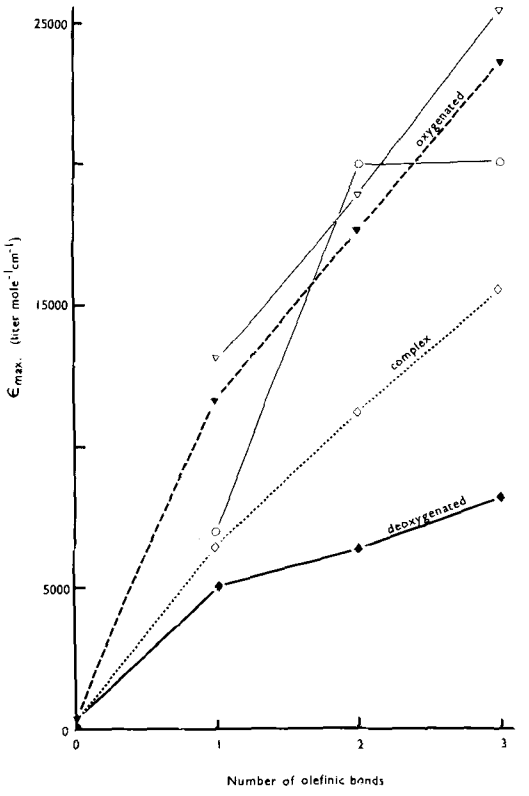

FIG. 4. Molar absorptivities ($\epsilon_{max.}$) of various fatty acid:oxygen species at absorption maxima vs number of methylene interrupted olefinic bonds for stearic (N=0), oleic (N=1), linoleic (N=2), and linolenic (N=3) acids. Data for (▽) from reference 10 and for (0) from reference 12. The line marked complex is the difference between the lines marked oxygenated and deoxygenated.

sorbed was taken in before there was any measurable increase in the concentration of the conjugated derivative. Since the rate of oxygen uptake did not increase when autoxidation occurred, it is evident that the oxidative reaction was with oxygen which had been absorbed previously. Privett and Blank (9) found a similar increase in UV absorption by methyl linoleate in the presence of oxygen. They found, however, that there was no measurable uptake of oxygen for 16-20 hr after exposure of their samples to air, although a considerable increase in UV absorption due to conjugated derivatives occurred during this period of time. The latter observation suggests that there must have been oxygen dissolved in their samples before uptake was monitored. This conclusion is consistent with their description of experimental procedures. Our samples were degased under high vacuum for a long time before the experiment; whereas, Privett and Blank agitated their small samples vigorously under an air atmosphere for 15 min prior to beginning their measurements.

The effect of oxygen upon the UV absorption spectra of linolenic, linoleic, and oleic acids is shown in Fig. 3. Oxygen had no effect upon UV absorption by the saturated acid, stearic, in this region of the spectrum. The effect of oxygen upon the spectra of the unsaturated fatty acids (Fig. 3) was to increase greatly the intensity of UV absorbance without any marked influence upon the wavelength of maximal absorbance (λ_{max}) or the width at the halfht of the absorption band. Oleic acid had a relatively weak band to ca. 205 nm with maximal absorption at 186.5 nm (Fig. 3). Each additional nonconjugated olefinic bond in the chain increased the intensity of absorption, the width of the band, and the wavelength of maximal absorbance. The absorption maxima for linoleic and linolenic acids were 187.8 and 190.5 nm, respectively. Binder, et al., (10) reported absorption maxima of 184, 190, and 192 nm for methyl oleate, linoleate, and linolenate, respectively. Presumably, the difference between their

results and ours reflects a difference between the methyl esters and the acids. The absorption maxima, the intensity of absorption, and the shape of the absorption bands determined on the Cary-14 were the same as those which we determined on a Beckman DK 2A. Areas under the absorption bands of the deoxygenated fatty acids (Fig. 3) were calculated from absorbance vs frequency spectra as the product of the band ht with the band width at half ht. These areas are in the approximate ratio of 3:2:1 for triene: diene:monene. This ratio is that expected on the basis of no interaction between the nonconjugated chromophores (11).

Figure 4 gives the molar absorptivities (ϵ_{max}) at the wavelength of maximal absorbance (λ_{max}) for the oxygenated, deoxygenated, and fatty acid: oxygen ($FA:O_2$) complexes for the three nonconjugated olefinic acids from our study and those for fatty acids or their methyl esters from two earlier studies (10,12).

The spectra which we associate with $FA:O_2$ complexes are merely the differences between oxygenated and deoxygenated spectra, which we take to be measures of the contact charge-transfer spectra. Whether these species should be referred to as complexes, other than in the electronically excited, charge-transfer state, will be discussed below. There may be further enhancement of the contact charge-transfer spectra, since they are computed on the basis of the total initial free fatty acid concentration and deoxygenation was not necessarily complete. The oxygenated solution spectra in Figure 3 appear to correspond to maximal absorption, since the intensity does not increase when the oxygen pressure above the solution is increased. Our data (Fig. 4) suggest that the discrepancies among the reported extinction coefficients for these olefinic compounds may be attributable to variation in the amount of dissolved oxygen. Our values for the oxygenated fatty acids agree closely with those reported for the methyl esters by Binder, et al. (10). The small consistent differences probably reflect a difference between the acids and their methyl esters. The extinction coefficients of Binder, et al. (∇ in Fig. 4) clearly refer to the oxygenated species. UV spectra of the fatty acids frequently are used for qualitative and quantitative analysis, as well as characterization of fats, oils, and other fatty acid containing biological materials. Since the spectra are even more sensitive to oxygen than previously expected, care should be taken in interpreting such analyses unless the utmost precaution has been exercised in controlling the oxygen concentration.

The complex is viewed as a loose π-complex or contact donor-acceptor complex, similar to those oxygen complexes already described (13-15). In the context of this model, the UV absorption spectrum corresponds to a transition from the weakly bound ground state of the complex ($FA:O_2$) to an excited electronic state which is largely a charge-transfer state ($FA^+:O_2^-$). The terminology contact charge-transfer implies that a stable, isolable complex does not form but that close encounters between FA and O_2 molecules lead to a physical (nonbonded) interaction. Details of this interpretation, in terms of the overlap of donor (FA) and acceptor (O_2) π-molecular orbitals, already have been described (11, 13, and 15-18).

It is interesting that the qualitative idea of loosely bound oxygen compounds playing a role in autoxidation of fats appeared in the literature in 1927, when Holm, et al., (20) termed these species moloxides. It is clear that their interpretation is consistent with the formation of a sort of $FA:O_2$ complex.

The results indicate that prior to and during, at least, the initial stages of true oxidation, like hemoglobin and myoglobin, olefinic fatty acids can undergo reversible oxygenation in response to changes in oxygen pressure. The mechanism seems to result from the oxygen molecule being attracted to and physically bound by pairs of olefinic bonds. These pairs of olefinic bonds may be either in two different molecules (intermolecular), as in the case of oleic acid, or in the same molecule (intramolecular) when they are either conjugated or separated by a single methylene group. Preliminary studies on the dissociation of the olefinic oxygen complexes indicate that, whereas 50% of the dissociation of the linoleic complex occurs between 2-15 Torr, which is within the effective pressure range of tissues, the oleic acid complex is totally associated at 1 Torr, which is well below the effective pressure range. These apparent affinities are in the reverse order to the rates of oxygen uptake noted above. The kinetic data alone cannot be used directly to predict the thermodynamic stability of the ground state of the $FA:O_2$ complexes. Likewise, the intensity of the charge-transfer spectra bears no direct relationship to thermodynamic stability (17). Further experiments are necessary to determine the stabilities of the ground states of these complexes and to investigate whether kinetic or thermodynamic properties are more important in the oxygen regulatory role of fatty acids. However, since a pair of olefinic bonds seems to be required for the reversible binding of a molecule of oxygen, it seems possible that substitution of a molecule of oleic for one of linoleic in membrane phospholipids, which may be otherwise totally saturated, might eliminate oxygen

transport sites within the membrane and thereby the availability of oxygen for oxidative respiration. Studies now are underway to explore this possibility.

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Analysis of Sterol Fractions from Twenty Vegetable Oils

TAE MYOUNG JEONG[1], TOSHIHIRO ITOH, TOSHITAKE TAMURA, and TARO MATSUMOTO,
College of Science and Technology, Nihon University, Chiyodaku, Tokyo, 101 Japan.

ABSTRACT

The unsaponifiables separated from 20 vegetable oils were divided into sterol and three other (less polar compound, triterpene alcohol, and 4-methylsterol) fractions by preparative thin layer chromatography. The amounts of the sterol fractions were more than ca. 30% in the unsaponifiables from all of the oils, except tohaku, pumpkin seed, and fagara seed oils. Composition of the sterol fractions were determined by gas liquid chromatography. Individual components of the sterol fractions were identified by gas liquid chromatography and combined gas liquid chromatography-mass spectrometry. β-Sitosterol was found as the most predominant component in the sterol fractions from all oils, except two, i.e. the sterol fraction from pumpkin seed oil contained no detectable amount of β-sitosterol and the sterol fraction from akamegashiwa oil contained Δ^5-avenasterol as the most abundant component. Campesterol, stigmasterol, Δ^5-avenasterol, Δ^7-stigmastenol, and Δ^7-avenasterol and also trace amounts (at the very least) of cholesterol and brassicasterol were found in most of the oils analyzed. It may be noted that a large amount (ca. 9%) of cholesterol was detected in the sterol fraction from capsicum seed oil. The presence of 24-methylenecholesterol and Δ^5-avenasterol in the sterol fraction of akamegashiwa oil was demonstrated by isolation of these sterols.

INTRODUCTION

It was reported in the previous article (1) that cholesterol, brassicasterol, Δ^5-avenasterol, Δ^7-avenasterol, and Δ^7-stigmastenol, in addition to campesterol, stigmasterol, and β-sitosterol, are widespread in 19 vegetable oils. In a subsequent study (2) on the sterol fractions from *Theaceae (Camellia japonica* L., *Camellia Sasanqua* Thumb. and *Thea sinensis* L.), alfalfa, garden balsam and spinach seed oils, and shea butter, it was found, however, that the sterol fractions of these oils consisted almost exclusively of Δ^7-sterols, i.e. α-spinasterol and Δ^7-stigmastenol, as predominant components together with Δ^7-avenasterol and 24-methylcholest-7-enol.

Our interest in ascertaining whether there exists any consistent relationship between the sterol composition and the taxonomical arrangement of plants has led us to a more extensive study of the sterol fractions from various kinds of vegetable oils. In this study, the unsaponifiables of 20 vegetable oils, hitherto little investigated, were separated into four fractions by preparative thin layer chromatography (TLC), and the sterol fractions were analyzed by gas liquid chromatography (GLC) and gas liquid chromatography-mass spectrometry (GLC-MS). Δ^5-Avenasterol and 24-methylenecholesterol from akamegashiwa oil were separated and identified by GLC-MS and IR and NMR spectroscopy.

EXPERIMENTAL PROCEDURES

Materials

Nine oils, i.e. pecan nut, cashew nut, pistachio nut, pine nut, almond nut, akamegashiwa (*Mallotus japonicus* Muell. Arg.) seed, capsicum seed, pumpkin seed, and fagara seed oils, were prepared from the corresponding dried nuts and seeds by Soxhlet extraction using methylene chloride, with the exception of ether extracted akamegashiwa oil. The oil contents of these dried nuts and seeds, as well as saponification and iodine values, and the unsaponifiable contents of these oils are indicated in Table I. Eleven oils were prepared commercially: walnut, tohaku (*Benzoin obtusilobum* O. Kuntze), chaulmoogra, perilla, tung, hemp seed, mustard and poppy seed oils, sal fat, illipé butter and Japan wax.

A specimen of pure cholesterol and a sterol fraction consisting of campesterol, stigmasterol, and β-sitosterol were supplied by Riken Vitamin Oil Co. (Tokyo, Japan). Specimens of α-spinasterol and Δ^7-stigmastenol were isolated from tea seed oil (2). Four fractions, containing brassicasterol, Δ^5- and Δ^7-avenasterol, and 24-methylcholest-7-enol as their respective main components, were prepared from rapeseed, castor, safflower, and spinach seed oils, respectively. These specimens were used as reference substances for the determination of the relative retention time (RRT) of the respective

[1] Present address: Department of Agricultural Chemistry, Gyeong-Sang College, Chilam-Dong, Jinju City, 620 Korea.

TABLE I

Content of Oils in Dried Seeds and Nuts, Content of Unsaponifiables in Oils, and Yields of Four Fractions from Unsaponifiables by Thin Layer Chromatography

Oil	Content of oil in seed and nut, percent	Saponification value	Iodine value	Unsaponifiables in oil, percent	Fraction[a] from unsaponifiables, percent 1	2	3	4
Walnut (*Juglandaceae*) Japan	----	190.6	150.9	0.3	12	24	31	33
Pecan nut (*Juglandaceae*)[b] U.S.	50	190.3	102.2	0.4	53	9	6	32
Cashew nut (*Anacardiaceae*)[b] India	49	181.0	80.6	0.6	30	12	6	52
Pistachio nut (*Anacardiaceae*)[b] Iran	48	166.3	92.2	0.6	25	8	8	59
Japan wax (*Anacardiaceae*) Japan	----	204.6	20.0	1.1	37	23	7	33
Pine nut (*Pinaceae*)[b] China	43	185.8	148.6	0.4	25	6	9	60
Almond nut (*Rosaceae*)[b] U.S.	38	190.1	100.3	0.9	52	4	4	40
Sal fat (*Dipterocarpaceae*) India	----	188.4	38.4	1.2	24	20	5	51
Tohaku (*Lauraceae*) Korea	----	240.0	71.2	2.5	66	9	11	14
Chaulmoogra (*Flacourtiaceae*) India	----	200.8	85.4	0.4	35	10	8	47
Perilla (*Labiatae*)	----	192.9	182.8	0.9	22	27	12	39
Tung (*Euphorbiaceae*) China	----	189.6	164.1	0.5	42	6	11	41
Akamegashiwa (*Euphorbiaceae*)[b] Japan	42	194.5	113.1	0.6	37	9	9	45
Hemp seed (*Moraceae*)	----	191.9	135.0	0.6	27	11	11	51
Mustard (*Cruciferae*)	----	178.4	117.5	0.8	17	5	3	75
Illipé butter (*Sapotaceae*)	----	195.7	30.9	1.1	40	10	8	42
Poppy seed (*Papaveraceae*)	----	192.0	136.8	0.5	32	7	6	55
Pumpkin seed (*Cucurbitaceae*)[b] China	28	187.0	110.8	1.4	54	7	9	30
Capsicum seed (*Solanaceae*)[b] Korea	26	189.5	138.2	1.8	17	46	6	31
Fagara seed (*Rutaceae*)[b] Korea	37	207.2	118.7	1.4	71	8	7	14

[a]Fraction 1 = less polar compounds (hydrocarbons, etc.), fraction 2 = triterpene alcohols (4,4-dimethylsterols), fraction 3 = 4-methylsterols (4-monomethylsterols), and fraction 4 = sterols (4-desmethylsterols).

[b]Laboratory extracted oil.

TABLE II

Relative Retention Time of Sterols Occurring in Twenty Vegetable Oils

Sterol	Position of double bond	Other structural characteristics	RRT[a]
Cholesterol	5	--	0.61
Brassicasterol	5, 22	24R-CH_3	0.70
Campesterol	5	24R-CH_3	0.81
24-Methylenecholesterol	5, 24(28)	24-CH_2	0.82
Stigmasterol	5, 22	24S-C_2H_5	0.88
24-Methylcholest-7-enol	7	24-CH_3	0.95
β-Sitosterol	5	24R-C_2H_5	1.00
α-Spinasterol	7, 22	24S-C_2H_5	1.03
Δ^5-Avenasterol	5, 24(28)	24Z-C_2H_4	1.12
Δ^7-Stigmastenol	7	24R-C_2H_5	1.18
Δ^7-Avenasterol	7, 24(28)	24Z-C_2H_4	1.32

[a]Relative retention time (RRT) in Table II and III is expressed by the ratio of the retention time for the substance under examination to the retention time (30 min) for β-sitosterol.

sterols and for the identification of the sterols occurring in the oils examined in this work. Table II shows RRT of the sterols determined under the GLC conditions used in this work. An authentic specimen of fucosterol used as a reference substance in NMR measurements of sterol components of akamegashiwa oil was supplied by N. Ikekawa, Laboratory of Chemistry for Natural Products, Tokyo Institute of Technology.

Saponification

Oil (30 g) in 300 ml alcoholic 1.0 N potassium hydroxide was refluxed for 1 hr under nitrogen. The reaction mixture was diluted with 600 ml water, and the unsaponifiable material was extracted with one 300 ml portion and three 200 ml portions of isopropyl ether (IPE). The combined IPE extract was washed first with 300 ml 0.5 N aqueous solution of potassium hydroxide followed by 5 washings with 200 ml portions of water and dried over anhydrous sodium sulfate, and the IPE was removed by evaporation. The content of unsaponifiables in oil was expressed by wt percent.

Preparative TLC

Preparative TLC was performed as described previously (1). Unsaponifiable material (30 mg/plate) was applied uniformly along a line 1.5 cm from 1 edge of a 20 x 20 cm plate coated with a 0.5 mm layer of Wakogel B-10 (Wako Pure Chemical Industries, Osaka, Japan) and developed with hexane-ether (7.5:2.5) for 1 hr using a Toyo continuous flow development preparative TLC. After developing, the plate was sprayed with a rhodamine-6G solution in ethanol and observed under UV light (3600 A) for marking the separated zones. Four separated zones, containing less polar compounds, triterpene alcohols, 4-methylsterols, and sterols, respectively, were cut off and thoroughly extracted with ether. After evaporation of the ether, the dried extracts were weighed for the quantification of individual fractions in unsaponfiables. The sterol fraction was purified further by repeated preparative TLC for subsequent GLC analysis.

Preparative Argentation TLC

TLC plates (20 x 20 cm) coated with a 0.5 mm layer of 10% silver nitrate impregnated Wakogel B-O (Wako Pure Chemical Industries) were used for a further fractionation of sterol mixtures in the form of their acetates. The acetates were prepared by acetylation of free sterols (10 mg) in acetic anhydride (0.5 ml) and pyridine (0.5 ml) overnight at room temperature. Developing of the acetates on TLC plates was conducted with hexane-benzene (6:4) for 40 min.

GLC

GLC of sterol fractions was performed, as mentioned previous (1). An OV-17 column (1.5%) was used for these analyses. RRT is expressed by the ratio of the retention time for the substance under examination to the retention time (30 min) for β-sitosterol.

GLC-MS

Analyses were performed, as described previously (1), on a Shimadzu LKB-9000 gas chromatograph-mass spectrometer (Shimadzu Seisakusho, Kyoto, Japan). An OV-17 column (1.5%) was used for GLC. Operating conditions were: column, 246 C; helium carrier gas, 30 ml/min; molecular separator, 290 C; ion source, 310 C; ionizing voltage, 70 eV; trap

TABLE III

Composition (%) of Sterol Fractions of Twenty Vegetable Oils Determined by Gas Liquid Chromatography

Relative retention time of individual sterol[a]	Percent composition								
	I 0.61	II 0.70	III 0.81	IV 0.88	V 1.00	VI 1.12	VII 1.18	VIII 1.32	Others
Walnut oil	1	tr[b]	4	tr	91	4	tr	tr	
Pecan nut oil	tr		4	2	80	12	1	1	
Cashew nut oil	1		8	tr	82	9	tr	tr	
Pistachio nut oil	tr		6	2	83	8	1	tr	
Japan wax	tr	tr	12	1	85	2	tr	tr	
Pine nut oil	tr		10	tr	61	28	1	tr	
Almond nut oil	tr		4	3	77	12	2	2	
Sal fat	tr	tr	24	15	56	5	tr	tr	
Tohaku oil	tr		7	1	90	2	tr	tr	
Chaulmoogra oil	tr	tr	18	17	58	7	tr	tr	
Perilla oil	1	tr	28	9	49	11	2	tr	
Tung oil	tr	tr	5	13	77	2	2	1	
Akamegashiwa oil	tr	tr	35[c]	6	20	36		3	
Hemp seed oil	tr	tr	21	18	54	3	3	1	
Mustard oil	tr	11	30[c]	tr	57	1	1	tr	
Illipé butter	tr	tr	16	7	70	6	1	tr	
Poppy seed oil	tr	tr	22	3	68	2	2		3
Pumpkin seed oil	tr	tr	6	2			28[d]	12	52[e]
Capsicum seed oil	9	1	17[c]	13	45	14	1	tr	
Fagara seed oil	1		24	1	67	6	1	tr	

[a] I = cholesterol, II = brassicasterol, III = campesterol, IV = stigmasterol, V = β-sitosterol, VI = Δ^5-avenasterol, VII = Δ^7-stigmastenol, and VIII = Δ^7-avenasterol.

[b] tr = trace, less than 0.5%.

[c] Mixture of campesterol and 24-methylenecholesterol.

[d] Mixture of Δ^7-stigmastenol and unknown sterol.

[e] 24-methylcholest-7-enol (RRT 0.95), 3%; α-spinasterol (RRT 1.03), 39%; and unknown (RRT 1.09), 10%.

current, 60 μA; and accelerated high voltage, 3500 V.

NMR spectra were measured with a JNM-C-60 HL (60 MHz, Japan Electron Optics Laboratory Co., Tokyo, Japan), in deuterochloroform. The IR spectra were taken in KBr tablets on a type IRA-2 diffraction grating IR spectrophotometer (Japan Spectroscopic Co., Tokyo, Japan). UV spectra were taken in ethanol (spectro grade) on a Hitachi 124 type spectrophotometer (Hitachi Co., Tokyo, Japan). All mp were determined on a micro melting point apparatus (Yanagimoto Seisakusho, Kyoto, Japan).

RESULTS

Unsaponifiables

The unsaponifiables were separated by preparative TLC in the same manner as described in the previous article (1) into four fractions: less polar compounds (hydrocarbons, etc., fraction 1), triterpene alcohols (fraction 2), 4-methylsterols (fraction 3), and sterols (fraction 4). Fraction 1 was closest to the solvent front and fraction 4 to the start line, as shown in Table I.

Relative proportions of four fractions were estimated under an assumption that the material losses caused by preparative TLC procedures were random for all components without specific losses for different components. Fraction 4 followed by fraction 1 was the major fraction, while fractions 2 and 3 were minor fractions accounting for ca. 10% of the unsaponifiables for most of the oils with a few exceptions. Almont nut, pecan nut, tohaku, pumpkin seed, and fagara seed oils are distinguished from other oils by a large proportion of fraction 1. Capsicum seed oil also is easily distinguishable from other oils by the preponderance of fraction 2 (46%).

Sterols

Table III shows approximate compositions of the sterol fractions of 20 vegetable oils determined by GLC. Estimations of these approximate compositions are based upon area percent values obtained from GLC peaks using the triangulation method. Differences in GLC response, if any, for different sterols are disregarded. Hence, the data recorded in Table III may not be precise but provide a measure sufficient enough to know relative proportions of component sterols. The sterol fractions in most

of the oils consisted mainly of campesterol (III), stigmasterol (IV), β-sitosterol (V), and Δ^5-avenasterol (VI), among which β-sitosterol was most predominant, accompanied with trace or minute amounts of cholesterol (I), brassicasterol (II), Δ^7-stigmastenol (VII), and Δ^7-avenasterol (VIII). The fractions from akamegashiwa and pine nut oils contain relatively larger amounts of Δ^5-avenasterol than do the fractions from other oils. The sterol fraction from pumpkin seed oil is characterized by containing Δ^7-sterols, such as α-spinasterol and Δ^7-stigmastenol, as predominant components. Identification of individual sterols was carried out in the following manner.

Brassicasterol, campesterol, stigmasterol, β-sitosterol, and Δ^7-avenasterol were identified by comparing their RRT with those of the reference specimens by GLC.

Cholesterol in capsicum oil: The sterol-I (RRT 0.61) which accounted for as much as 9% of the sterol fraction of capsicum oil was analyzed by GLC-MS. Its fragmentation pattern obtained agreed with that of a reference specimen of cholesterol and also that reported by Knights (3), molecular ion (M^+) at m/e 386 (calculated for $C_{27}H_{46}O$, MW 386) with other principal ions at m/e 371, 368, 353, 301, 275, 273, and 247. Hence, the sterol-I in capsicum oil is identified as cholesterol.

24-Methylcholest-7-enol in pumpkin seed oil: 24-Methylcholest-7-enol, α-spinasterol, and Δ^7-stigmastenol in pumpkin seed oil were identified by GLC-MS. The sterol with RRT 0.95 (footnote e, Table III) gave M^+ at m/e 400 (calculated for $C_{28}H_{48}O$, MW 400) with other ions at m/e 385, 382, 367, 273, 255, 246, 229, and 213.

These fragmentations agree with those given for Δ^7-sterols by Knights (3) and are basically similar to those of 24-methylcholest-7-enol in spinach seed oil recently reported by Itoh, et al. (2). Consequently, the sterol with RRT 0.95 reasonably is identified as 24-methylcholest-7-enol.

α-Spinasterol in pumpkin seed oil: The mass spectrum of the sterol with RRT 1.03 (footnote e, Table III) in pumpkin seed oil showed M^+ at m/e 412 (calculated for $C_{29}H_{48}O$, MW 412) with other abundant ions at m/e 397, 394, 379, 369, 351, 273, 271, 255, 246, 231, 229, and 213. The fragmentation giving the ions at m/e 369 ($M - C_3H_7$) and 351 ($M - C_3H_7 - H_2O$) was suggested by Knights (3) to involve the isopropyl group at the end of the side chain and appears to be characteristic for Δ^7-sterols. The fragmentation pattern of the sterol with RRT 1.03 was found to be basically similar to that of authentic α-spinasterol. Hence, this sterol is recognized as α-spinasterol.

Δ^7-Stigmastenol in pumpkin seed oil: The mass spectrum of the sterol-VII (RRT 1.18) in pumpkin seed oil showed two molecular ions at m/e 414 and 412 accompanied with the ions corresponding to $M - CH_3$, $M - H_2O$, and $M - CH_3 - H_2O$, respectively. Hence, the GLC peak with RRT 1.18 is considered to consist of two sterols, one of which (M^+, m/e 414) is considered to be Δ^7-stigmastenol (RRT 1.18), while the other one (M^+, m/e 412) is presumably a C_{29}-sterol with two double bonds in the molecule. An unknown sterol with RRT 1.09 also was found in the sterol fraction of pumpkin seed oil by GLC. Identification of these unknown sterols is still in progress.

24-Methylenecholesterol isolated from akamegashiwa oil: The sterol-III (RRT 0.82) in the sterol fraction from akamegashiwa oil gave two molecular ions at m/e 400 and 398 by GLC-MS, indicating that it is a mixture of two sterols, one (M^+, m/e 400, $C_{28}H_{48}O$) of which is presumed to be campesterol (MW 400). The other one (M^+, m/e 398, $C_{28}H_{46}O$) was isolated and identified as 24-methylenecholesterol, as described below.

The sterol fraction (540 mg), separated by preparative TLC from the unsaponfiable matter (1200 mg) of akamegashiwa oil (200 g), was acetylated. The acetate was separated into three zones by preparative argentation TLC. Recrystallization from acetone-methanol (1:1) followed by repeated preparative argentation TLC of the zone closest to the start line gave a sterol acetate in the form of fine plates: 62 mg; RRT 1.10; GLC purity, 91%; and mp, 136-137 C. Free sterol (38 mg) derived from the acetate showed RRT 0.82 and mp 144-145 C. IR spectrum of the acetate gave the bands at 1730, 1248 and 1038 cm^{-1} (–OAc), 1668, 842, 830, and 802 cm^{-1} (trisubstituted olefin) (4-6), 1370 cm^{-1} (geminal dimethyl) and 3080 and 1640 cm^{-1} (terminal methylene). NMR spectrum of the free sterol showed methyl signals at 0.70 (C-18 methyl) and 1.02 (C-19 methyl), giving the same values of chemical shift as those of the corresponding signals observed for cholesterol (6). The doublet at 1.03 ppm (J = 7.2 Hz) is probably due to C-26 and C-27 dimethyl protons affected by C-24 terminal methylene group, since this doublet has been observed also in the spectra of 4-methylsterols and triperpene alcohols containing C-24 terminal methylene group, such as gramisterol (24-methylenelophenol) (4), cycloeucalenol (7), and 24-methylenecycloartanol (8). Multiplets at 3.37-3.69 (C-3α H), 5.25-5.41 (C-6 H) and 4.71 ppm (terminal methylene protons) also appeared in the

NMR spectrum. Mass spectrum of the free sterol showed M^+ at m/e 398 (calculated for $C_{28}H_{46}O$, MW 398) with other principal ions at m/e 383, 380, 365, 314, 313, 296, 281, 273, and 271. The presence of the ions at m/e 314 ($M - C_6H_{12}$), 296 ($M - C_6H_{12} - H_2O$), 281 ($M - C_6H_{12} - H_2O - CH_3$) suggests that the sterol has a Δ^5-bond with C-24 terminal methylene group (1,2,9). The fragmentation pattern of the mass spectrum is basically similar to that of 24-methylenecholesterol reported by Knights (3). Hence, this sterol is identified reasonably as 24-methylenecholesterol.

Under the GLC conditions in this work, the peaks of campesterol (RRT 0.81) and 24-methylenecholesterol (RRT 0.82) could not be resolved. Hence, the fractions corresponding to the peak III of 9 oils, i.e. pine nut oil, sal fat, chaulmoogra oil, perilla oil, hemp seed oil, mustard oil, illipé butter, capsicum seed oil, and fagara seed oil, in addition to akamegashiwa oil described above, were analyzed by GLC-MS with the results that the presence of 24-methylenecholesterol in mustard and capsicum seed oils was indicated with certainty.

Δ^5-Avenasterol isolated from akamegashiwa oil: The middle zone obtained by the foregoing argentation TLC of the sterol acetate fraction was subjected to repeated argentation TLC to give a sterol acetate (RRT 1.48). The acetate (75 mg) recrystallized from acetone-methanol (1:1) showed mp 136-137 C and GLC purity 92%. The free sterol (41 mg RRT 1.12) obtained by hydrolysis of the purified acetate showed mp 139-140 C. IR spectrum of the acetate gave the bands at 1730,1250, and 1038 cm^{-1} (–OAc) and at 1370 cm^{-1} (geminal dimethyl) (4,7,10). Absorptions at 812 and 802 cm^{-1} are attributable to trisubstituted olefin (11). NMR spectrum of the free sterol showed the singlets at 0.70 (C-18 methyl) and 1.02 (C-19 methyl) ppm, giving the same values of chemical shift as those of the signals of cholesterol and other Δ^5-sterols (6). Doublets at 0.98 (J = 6.0 Hz, C-26, and C-27 dimethyl) and 1.58 ppm (J = 6.0 Hz, C-29 methyl) (12) and quartet centered at 5.11 ppm (J = 7.2 Hz, C-28 proton) (12) also were observed. A heptet centered at 2.85 ppm (J = 7.2 Hz), due to the C-25 proton affected by C-29 methyl group, was observed. Bates, et al., (13) reported that the heptet could be observed at 2.8 ± 0.1 ppm for 24Z-ethylidene sterols, i.e. Δ^5-avenasterol, and at 2.2 ± 0.1 ppm for 24E-ethylidene sterols, i.e. fucosterol. The NMR spectrum measured in this laboratory for an authentic specimen of fucosterol gave the heptet centered at 2.23 ppm. Accordingly, the sterol isolated from akamegashiwa oil must have 24Z-ethylidene group. Mass spectrum of the free sterol gave M^+ at m/e 412 (calculated for $C_{29}H_{48}O$, MW 412) with other ions at m/e 397, 394, 379, 314, 296, 281, 273, and 271. The strong ions at m/e 314 ($M - C_7H_{14}$), 296 ($M - C_7H_{14} - H_2O$), 281 ($M - C_7H_{14} - H_2O - CH_3$) can be derived by McLafferty rearrangement and indicate the presence of $\Delta^{24(28)}$-bond in the molecule (11,14). Based upon the spectral data described above, the sterol from akamegashiwa oil is identified as Δ^5-avenasterol.

DISCUSSION

It is seen by reference to Table III that the sterol fractions of all oils except pumpkin seed oil consist mainly of Δ^5-sterols while Δ^7-sterols are present only in small proportions at the most. The sterol fraction from pumpkin seed oil is the one exception and consists mainly of Δ^7-sterols with small proportions of Δ^5-sterols. On the basis of the results of this and previous studies in this laboratory, the sterol fractions of higher plant oils may be classified broadly into two categories based upon the proportion of Δ^5-sterols to Δ^7-sterols in the sterol fractions. The sterol fractions of one category contain Δ^5-sterols in larger proportion than Δ^7-sterols, whereas the sterol fractions of the other category contain Δ^7-sterols in larger proportion than Δ^5-sterols. This classification, however, may not correlated with the taxonomical arrangement of plant family. Moreover, it does not always follow that the plants of one and the same family are alike in their sterol pattern. Thus, the sterol fractions from soybean and peanut (*Leguminosae*) are of the Δ^5-sterol type (1), while the sterol fraction from alfalfa (*Leguminosae*) is of the Δ^7-sterol type (2). The *Compositae* may be regarded as another instance of plant family in which plants with the sterol fraction of Δ^5-sterol type and also plants with the sterol fraction of Δ^7-sterol type are included. The sterol fractions of sunflower and safflower oils are of Δ^5-sterol type, whereas the sterol fraction from *Vernonia anthelmintica* seeds contains two major components, an unusual sterol $\Delta^{8,14,24(28)}$-stigmastatrienol and Δ^7-avenasterol, accounting for over 90% of the total with smaller amounts of other sterols, including Δ^5-sterols (15-17).

It is known that cholesterol occurs in the sterol fraction of many vegetable oils, generally in extremely minor proportions or in a few percent at the most, although the sterol fractions from several species of *Cruciferae* plants were reported to contain exceptionally high proportions, 15% at their highest in the case of *Cheiranthus Chiri* L., of cholesterol (18-19).

The present study on the sterol fraction from the seed oil of capsicum, a *Solanaceae* plant, revealed that it contained as much as 9% of *cholesterol*.

Seed oils from *Cruciferae* plants are known to contain brassicasterol in relatively high proportions in their sterol fractions. The results of the present study on mustard seed oil accord with previous studies in this respect, for the sterol fraction of this oil contained brassicasterol in a decidedly high proportion, 11%, as compared with other 19 oils as shown in Table III.

Finally, the results of the present study concerning 24-methylenecholesterol may be noted here. This sterol was isolated first from oysters and clams by Idler and Fagerlund (20) and has become known as a sterol widely distributed in marine organisms (21). Moreover, this sterol has been reported to occur in some tissues of higher plant (19, 22-24). The present study confirmed the presence of this sterol in akamegashiwa seed oil and also in capsicum and mustard seed oils, as described before, suggesting that this sterol is considerably widespread in vegetable oils.

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Composition of *Celastrus orbiculatus* Seed Oil[1]

ROGER W. MILLER, C.R. SMITH, JR., D. WEISLEDER, R. KLEIMAN, and **W.K. ROHWEDDER,** Northern Regional Research Laboratory,[2] Peoria, Illinois 61604

ABSTRACT

The seed oil of *Celastrus orbiculatus* (family Celastraceae) was reinvestigated to determine whether the benzoic acid that it contains is esterified with glycerol. Unlike acetic acid, benzoic acid is not incorporated in glycerides of this oil but, instead, is esterified with two sesquiterpenoid triols of the empirical formula $C_{15}H_{26}O_4$. *trans*-Cinnamic acid also is esterified with these triols. Acetic acid is a component of these nonglyceride esters, as well as of the triglycerides. We found no evidence for the occurrence of formic acid in this oil. By countercurrent fractionation, we found that this oil contains 8% "ordinary" triglycerides, 59% monoacetotriglycerides, and 33% nonglyceride substances.

INTRODUCTION

Although benzoic acid has been reported as a seed oil constituent of various species of Celastraceae (1-5), it has not been established as a glycerol ester. Gunde and Hilditch (2) reported the occurrence of formic, acetic, and benzoic acids in the seed oil of *Celastrus paniculatus*. They concluded that these three acids are not part of the glycerides but are esterified with a tetrahydroxylic compound, $C_{15}H_{26}O_5$. Later, Kleiman, et al., (6) showed that acetic acid is incorporated in glycerides of *Euonymus verrucosus* seed oil and is placed stereospecifically in position 3 of *sn*-glycerol. Their gas liquid chromatographic (GLC) results demonstrated that monoacetotriglycerides represent 68-98% of the seed oils from selected species in the Celastraceae. These authors also found UV absorption indicative of benzoate in the seed oil of *E. verrucosus* (6), but Bedi, et al., (7) observed no IR or UV evidence for benzoate in *E. hamiltonianus*.

Since acetic acid is incorporated in triglycerides of various celastraceous seed oils, we considered it important to determine whether or not benzoic acid also is part of these glycerides. For this study, we selected *Celastrus orbiculatus*, whose seed oil, in comparison with those of related species in our collection, has a particularly high UV absorption (274 nm) assigned to benzoate.

MATERIALS AND METHODS

Extraction of Seed

Coarsely ground seeds of *C. orbiculatus* (purchased from Harry Saier Co., Dimondale, Mich.) were extracted 6 hr with petroleum ether (bp 30-60 C) by the Soxhlet procedure. Solvent was removed from the extract with a rotating evaporator.

Chromatographic Methods

GLC analyses of the unfractionated oil, as well as fractions derived from oil, were conducted with an F & M model 5750 instrument which has a flame ionization detector. A 0.3 x 115 cm stainless steel column packed with 3% OV-1 on Gas Chrom Q (100-120 mesh) was used, temperature programed at 2 C/min 140-350 C. Free acids were analyzed with the same instrument, but with a thermal conductivity detector and a 0.3 x 212 cm stainless steel column packed with 20% FFAP on Chromosorb W (acid washed, silylated, 100-120 mesh). Analyses were temperature programed at 4 C/min, 120-220 C. Retention times of components were established by means of a reference mixture which contained formic, acetic, and benzoic acids as well as C_3-C_{12} normal aliphatic acids. (Unexpectedly, formic acid emerges *after* acetic acid under these conditions and tends to overlap propionic acid [6].)

Methyl esters were analyzed with a Packard model 7401 instrument, as described previously (8).

Thin layer chromatography (TLC) was performed on 20 x 20 cm glass plates coated with Silica Gel G F-254 to a thickness of 0.25 mm. Hexane-ethyl ether-acetic acid (85:15:1) was the solvent system for the intact oil or triglycerides; chloroform-ethyl ether (16:1) for nonglyceride esters, and chloroform-acetone (70:30) for alcohols. Visualization methods included: exposure to UV light or charring with sulfuric acid-dichromate for triglycerides and other esters, application of UV light, or 0.35% (w/v) potassium permanganate solution for alcohols.

Preparative TLC of unhydrolyzed fractions from the oil was conducted on 20 x 20 cm

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[1]Presented at the AOCS Meeting, Mexico City, April 1974.

[2]ARS, USDA.

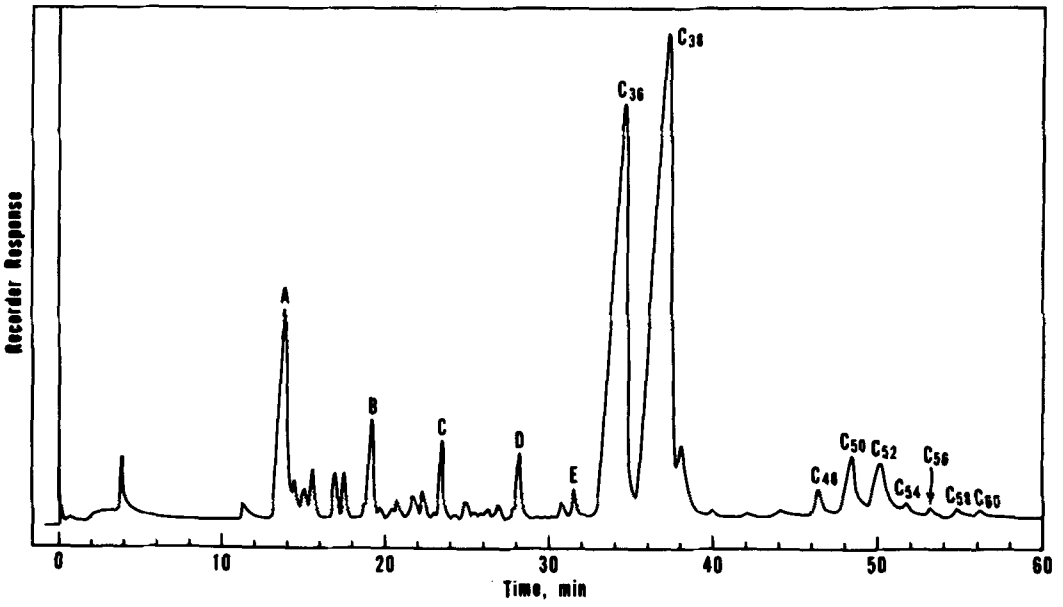

FIG. 1. Gas liquid chromatogram of *Celastrus orbiculatus* seed oil. Peaks A, B, C, D, and E are nonglyceride components discussed in the text. Triglyceride peaks are designated by carbon number.

plates precoated to a thickness of 2 mm with Silica Gel G F-254; chloroform-ether (16:1) was the developing solvent, and the components were detected with UV light. Alcohol fractions provided by hydrolysis were chromatographed on ordinary Silica Gel G plates with 1 mm layers developed with chloroform-acetone (75:25); dichlorofluorescein spray was applied for visualization of bands.

Spectrometric Methods

Perkin-Elmer models 137 and 337 spectrometers were used to determine IR spectra with 1% chloroform solutions.

UV absorption spectra were recorded with a Beckman model DK-2A spectrophotometer; 0.1% cyclohexane or anhydrous ethanol solutions were employed.

NMR spectra were recorded with a Varian HA-100 instrument. Samples were dissolved in deuterochloroform with tetramethylsilane as the internal standard.

Gas chromatography-mass spectrometry (GC-MS) was carried out with a Packard model 7401 gas chromatograph and a Du Pont (CEC) 21-492-1 mass spectrometer by the published procedure of Kleiman and Spencer (9). A Nuclide 12-90-DF double focusing mass spectrometer was used for high resolution MS.

Countercurrent Fractionation

The countercurrent distribution (CCD) procedure (10-12) was conducted in a 200 tube Craig-Post apparatus. Seed oil (10 g) was separated using hexane-nitroethane as the solvent system at temperatures no higher than 22 C (11,13). The tubes contained 40 ml of lower phase and 10 ml of upper phase throughout the first 1000 transfers. After 200 transfers, upper phases were decanted into a fraction collector, where 2 transfers/tube were combined. The volume of upper phase was changed to 40 ml after 1000 transfers; and, after 1200 transfers, upper phases were collected individually for 200 more transfers. Samples were isolated by evaporating solvent, including nitroethane, with a rotating evaporator.

Hydrolysis and Esterification Procedures

For isolation of alcohol moieties, the method of choice was hydrolysis with 0.2 M methanolic barium hydroxide (14). From 10 to 50 mg sample was refluxed 2 hr with 5 ml methanolic base; a proportionately bigger volume was used to hydrolyze larger samples. The methanolic solutions were concentrated almost to dryness with a rotating evaporator, and the methanol was replaced with ca. the same volume of water. After the aqueous suspensions were centrifuged to remove barium soaps of the long chain fatty acids, the supernatants were extracted with chloroform. The combined chloroform extracts were dried over sodium sulfate and evaporated with a rotating evaporator to give an alcohol fraction.

After extraction of the basic supernatant

TABLE I

Fatty Acid Composition of *Celastrus orbiculatus* Seed Oil and Selected Countercurrent Distribution Fractions[a]

Methyl ester	Original oil	Ordinary triglycerides, transfer 442	Acetotriglycerides			
			Transfer 862	Transfer 950	Transfer 1062	Transfer 1211
14:0	0.2	-	-	Trace	0.1	0.2
15:0	Trace	0.1	-	Trace	Trace	0.1
15:1	Trace	-	-	-	Trace	Trace
16:0	21.1	26.9	33.6	33.4	29.8	16.1
16:1	0.2	0.8	0.2	0.3	0.3	0.9
17:0	0.1	0.3	0.2	0.2	0.1	0.1
17:1	Trace	-	Trace	0.1	0.1	0.1
18:0	4.1	8.0	9.0	8.4	3.1	1.9
18:1	8.8	27.3	10.4	10.4	6.1	4.6
18:2	31.4	9.8	40.1	31.4	30.0	44.5
18:3	29.5	2.8	5.5	15.0	29.4	31.2
20:0	0.5	0.8	0.3	0.1	0.1	0.1
20:1	0.6	5.9	0.2	0.1	0.2	Trace
20:2	Trace	0.2	-	-	0.1	Trace
20:3	Trace	0.3	-	-	Trace	Trace
22:0	Trace	0.1	Trace	-	-	-
22:1	1.6	9.9	0.2	-	0.2	0.1
22:2	-	0.5	-	-	-	-
24:0	0.2	0.6	-	-	-	-
24:1	-	0.4	-	-	-	-

[a]Area percent by gas liquid chromatography of methyl esters.

with chloroform, low mol wt acids remained in solution as barium salts; these were extracted with ethyl ether after acidification with sulfuric acid. Combined extracts were dried over sodium sulfate, then cautiously distilled with a Vigreux column to remove solvent and provide a concentrate of free acids.

Methyl esters were prepared from triglycerides by the boron trifluoride-methanol method of Metcalfe, et al., (15) as modified by Dill (16).

Miscellaneous

Melting points were determined on a Fisher-Johns apparatus.

RESULTS AND DISCUSSION

This investigation demonstrated that, unlike acetic acid, benzoic acid is not incorporated into the triglycerides of *C. orbiculatus* seed oil. Both GLC and CCD results confirmed earlier conclusions (6) that monoacetotriglycerides are the major constituents of the oil. GLC analysis revealed a series of nonglyceride components which are more volatile than the triglycerides. By CCD, the oil was separated into three main classes of constituents—"ordinary" triglycerides, acetotriglycerides, and nonglyceride substances which contain all of the benzoate groups that occur in this oil. Further fractionation of the nonglyceride portion by TLC revealed a group of esters derived from isomeric sesquiterpenoid triols. By a combination of NMR and GC-MS techniques, we ascertained that these sesquiterpenoid alcohols are esterified with different combinations of acetic, benzoic, and *trans*-cinnamic acids. We found no evidence for the occurrence of esterified formic acid in *C. orbiculatus* seed oil.

GLC Analysis of *C. orbiculatus* Oil

GLC analysis of the intact oil revealed a series of apparently related peaks (A, B, C, D, and E in Fig. 1), comprising 13% of the sample, that eluted before acetotriglycerides. The acetotriglycerides appeared as two large peaks—27% C_{36}, with the fatty acid composition $C_2C_{16}C_{18}$ according to the triglyceride notation of Harlow, et al., (17) and 39% C_{38} with $C_2C_{18}C_{18}$. Ordinary triglycerides (9% by GLC) ranged C_{48}-C_{60}. Results of the GC-MS peaks A, B, C, D, and E are discussed in a subsequent section.

Identification of Low Mol Wt Acids

The low mol wt acid mixture (7.2 g) isolated after barium hydroxide hydrolysis of the unfractionated oil (39.0 g) contained the following components according to GLC (amounts expressed as area percent): acetic, 19.2; butyric, 1.6; hexanoic, 3.7; lauric, 1.8; benzoic, 68.3; and unidentified, 2.7, as well as traces of propionic, valeric, octanoic, and decanoic acids.

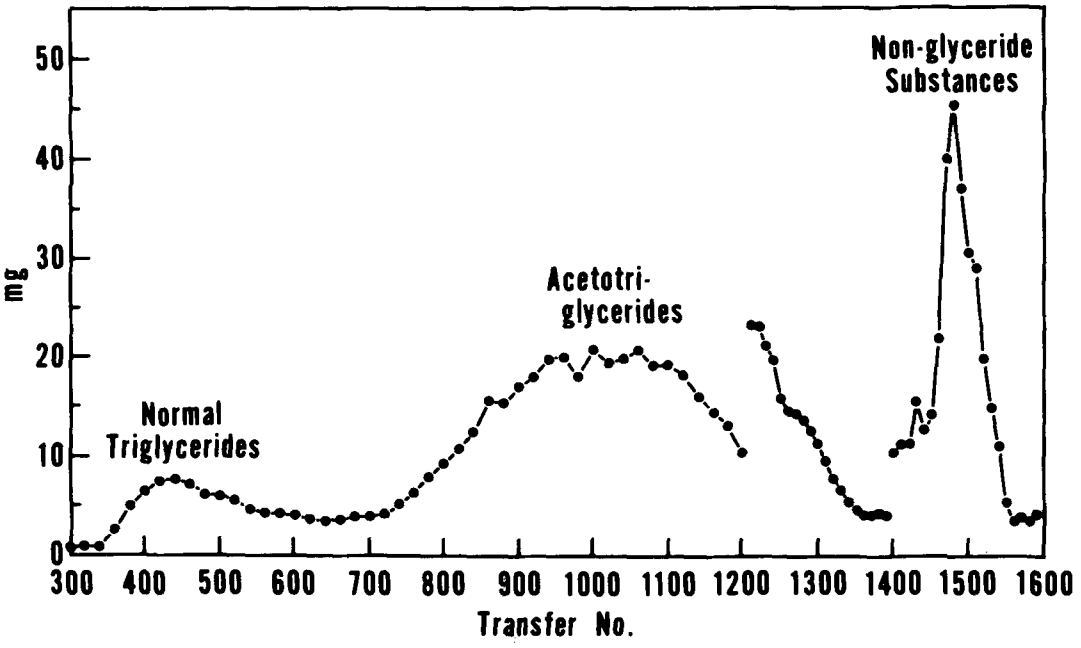

FIG. 2. Countercurrent distribution of *Celastrus orbiculatus* seed oil. Solvent system, hexane-nitroethane; 10 ml upper phase decanted, transfers 300-1200; 40 ml upper phase decanted, transfers 1200-1400; fundamental series, transfers 1400-1600.

Formic acid was not detected. Because of its long retention time, *trans*-cinnamic acid would not have been detected in this mixture.

Countercurrent Distribution and Triglyceride Composition

The wt distribution achieved by CCD of 10 g of *C. orbiculatus* seed oil is indicated in Figure 2. Monitoring of selected tubes by TLC revealed that the first peak collected (transfers 300-650, 8% by wt) consists mainly of ordinary triglycerides—i.e., those that incorporate 3 long chain fatty acids. The material from transfers 650-1400, totaling 59% by wt, was mostly acetotriglycerides. The remaining peak in the CCD curve, 33% by wt, is a complex mixture of polar compounds. In addition to TLC and GLC examination, the identity of acetotriglycerides was confirmed by their NMR spectra, which were marked by a strong singlet at δ 2.10 associated with acetate groups and a multiplet (δ 4.2) due to protons in the glycerol moiety (18,19). There was no benzoate in the triglyceride region, as shown by NMR and UV spectra (no aromatic signals in δ 7.3-8.2 region, NMR; no absorption maxima near 274 nm, UV). Neither was there evidence for di- or triacetins. The percentages of the three main fractions that we separated by CCD are in general agreement with those recorded by Kleiman, et al., (6) for this same species.

In accord with previous observations (11,12), the least mobile triglycerides are those with the most unsaturation (Table I). Virtually all of the 20:1 and 22:1 acids are found in the "ordinary" triglyceride fraction, in accord with GLC analysis of the oil. In contrast, values for 18:2 and 18:3 are fairly low in the ordinary triglycerides but high in acetotriglycerides. The content of 16:0 is substantial and is fairly uniform throughout the various fractions. The presence of only one acetate group, and its placement in position 3 of the *sn*-glycerol moiety, have been noted previously in the case of *E. verrucosus* seed oil (6). These observations indicate considerable lack of randomness of fatty acid distribution in triglycerides of *C. orbiculatus* seed oil.

Gunde and Hilditch (2), as well as Cattaneo, et al. (3), and also Sengupta and Bhargava (5) used solvent partitioning with petroleum ether-methanol in their studies of celastraceous seed and fruit lipids, though with a less elaborate scheme than full-scale CCD.

Isolation of Nonglyceride Esters

NMR spectra of the most polar CCD fraction (transfers 1400-1600, Fig. 2) indicated that benzoate (aromatic protons at δ 7.5-8.2) is present. Faint NMR indications of protons associated with a glycerol moiety were found to be due entirely to a minor glyceride component (frac-

TABLE II

Tentative NMR Spectra of Thin Layer Chromatographic (TLC) Fractions and Derived Alcohol[a]

Protons[b]	TLC fractions U	M	L	Alcohol I
Acetate	δ 1.62, s, 3H	δ 1.58, 2.08, 2s, 6H	δ 2.01, 1.80, 2s, 6H	-
Aromatic	7.2-7.7, m, 10H 8.0-8.2, m	7.25-7.6, m 7.9-8.1, m, 5H	7.20-7.65, m 5H	-
Olefinic	-	-	6.33, 7.66, AB q, 2H (J = 16)	-
H-1	5.52, dd, 1H (J = 10, 5)	5.43, dd, 1H (J = 10, 4.5)	(?)	4.3, m, 1H
H-6	5.55, s, 1H	5.30, s, 1H		4.3, s, 1H
H-7		2.2-2.4, m, 1H		
H-8				
H-9	5.04, d, 1H (J = 5)	4.99, d, 1H (J = 6)	(?)	3.4, s, 1H
H-12	1.45, s, 6H	1.38, s, 6H	1.34, s, 3H	1.47, s, 6H
H-13			1.37, s, 3H	
H-14	1.05, d, 3H (J = 7.5)	0.98, d, 3H (J = 7.5)	1.28, d, 3H (J = 8)	1.13, d, 3H (J = 8)
H-15	1.40, s, 3H	1.30, s, 3H	1.20, s, 3H	1.05, s, 3H

[a]Chemical shifts are expressed in ppm from tetramethylsilane. Samples were dissolved in deuterochloroform. Values for J are expressed in herz (Hz).

[b]See Figure 4 for numbering of protons.

tion BL, discussed below). UV absorption of 7 transfers in this area indicates the strongest aromatic absorption (274 nm) at the peak of the wt curve (transfer 1480). Because of the presence of *trans*-cinnamate (noted below) with absorption at least 25 times that of benzoate in the 270-280 nm region (20,21), the concentration of benzoate was impossible to estimate by UV. Transfers 1475-1485 were shown by TLC to be similar in composition and were combined for further fractionation. Preparative TLC resolved this material into 5 fractions designated BL (10.8%), L (30.5%), M (45.5%), U (10.2%), and AU (3.0%).

Fraction BL possibly contains mono- or diglycerides, since its IR spectrum shows hydroxyl absorption at 3660 cm^{-1}, and NMR indicates glycerol protons (multiplets δ 4.2 and 5.3); however, signals associated with aromatic protons are absent.

The NMR spectra of the middle three fractions, L, M, and U (Fig. 3), show a close relationship; they do not exhibit any proton signals for glycerol (multiplets at δ 4.2 and 5.3 [18,19]), thus indicating that these fractions are not glycerides. Benzoate groupings in U and M are indicated by multiplets at δ 7.2-7.7 (*meta* and *para* protons on aromatic rings) and δ 8.0-8.2 (*ortho* protons). The *trans*-cinnamate moiety of L was detected by a multiplet at δ 7.2-7.65 combined with a distinctive AB quartet (J = 16 Hz) at δ 6.33 and 7.66 due to *trans*-olefinic protons. The *ortho* aromatic protons are shifted upfield from their position for benzoate so that they overlap signals due to the other aromatic protons; this portion of the spectrum of L is quite similar to that of a reference sample of methyl *trans*-cinnamate. Sharp singlet signals at δ 1.62 (U), δ 1.58 and 2.08 (M), and δ 2.01 and 1.80 (L) are ascribed to acetate groups; these assignments will be discussed later. IR spectra of U, M, and L show carbonyl peaks due to both aliphatic (1735 cm^{-1}) and aromatic (1710 cm^{-1}) ester groups but with different relative intensities.

Fraction AU, the least polar TLC fraction, has not been characterized; its NMR spectrum shows little similarity to those of U, M, and L.

Examination of Nonglyceride Alcohol Fraction

The alcohol fraction (1.5 g), isolated after barium hydroxide hydrolysis of the unfractionated seed oil (39.0 g), was a white solid. GC-MS examination of this mixture revealed two major components—Alcohol I (51%) and Alcohol II (35%)—both of which had a molecular ion of m/e 270. Other ions present in the spectrum of I indicated apparent losses of CH_3 (M - 15),

TABLE III

Probable Composition of Several Gas Liquid Chromatographic Peaks from Seed Oil of *Celastrus orbiculatus*

Peak[a]	Area %	M^+, m/e[b]	M-15, m/e[b]	Base peak, m/e	$C_{15}H_{26}O_4$ Alcohols esterified with the following acyl groups:
A	7.8	(458)	443	105	2 Acetates + 1 benzoate
B	2.2	484	(469)	131	2 Acetates + 1 cinnamate
C	1.5	520	505	105	1 Acetate + 2 benzoates
D	1.3	546	(531)	131	1 Acetate + 1 benzoate + 1 cinnamate
E	0.6	(608)	(593)	105	2 Benzoates + 1 cinnamate

[a]As designated in Figure 1.

[b]Values in parentheses were not observed but are inferred from other ions.

TABLE IV

Composition of Thin Layer Chromatographic (TLC) Fractions by Gas Liquid Chromatography-Mass Spectrometry

TLC fraction	Major component			Minor components		
	Amount, %	M^+, m/e[a]	Corresponds to GLC peak[b]	Amount, %	M^+, m/e[a]	Corresponds to GLC peak[b]
U	90	520	C	8	546	D
M	69	(458)	A	15	(546)	D
				5	(484)	B
				4.5	(520)	C
L	49	484	B	13	516	
				7	458	A
				5	502	
				3	504	

[a]Values in parentheses were not observed but are inferred from other ions.

[b]As designated in Figure 1; see also Table III. GLC = gas liquid chromatography.

H_2O (M - 18), 2 H_2O (M - 36), CH_3 plus 2 H_2O (M - 51), C_4H_9 (M - 57), and CH_3 plus 3 H_2O (M - 69), thus suggesting the presence of 3 OH groups. High resolution MS of Alcohol I gave a value for the parent ion of 270.1821 and so established $C_{15}H_{26}O_4$ as its empirical formula. The MS of Alcohol II is similar to that of I and indicates that the two compounds probably are closely related isomers.

Preparative TLC of this same alcohol fraction separated Alcohols I and II, as well as some minor components which may be unrelated to I and II. In the solvent system employed, Alcohol I has R_f 0.5, whereas II has R_f 0.3. Alcohols I and II were isolated as high melting, crystalline solids (I, mp 222-223 C; II, mp 240-241 C). The NMR spectrum of I (Table II) suggested structural features similar to those of the tetrahydroxy sesquiterpene malkanguniol (Fig. 4) recently characterized by den Hertog, et al. (22). Malkanguniol was isolated after hydrolysis of *C. paniculatus* seed oil and was shown to have the empirical formula $C_{15}H_{26}O_5$—a striking verification of Hilditch's early proposal (2). den Hertog, et al., mentions two important ions at m/e 137 and 124 or 125 in the MS of malkanguniol, both derived from the 5 membered oxygen containing ring. The prominence of these same ions in the MS of I reinforces the structural relationship between the two, although I has one less hydroxyl function.

Malkanguniol is related structurally to other alcohols that occur in *C. paniculatus* seed (22), presumably in esterified form, and also to a group of sesquiterpenoid polyols that occur as components of alkaloidal esters in various genera in the Celastraceae. Members of the latter group of alcohols are more highly oxygenated than malkanguniol but have the same ring system; these include maytol from *Maytenus ovatus* (23), as well as euonyminol and isoeuonyminol, derived from *Euonymus* species (24,25).

GC-MS Examination of Nonglyceride Esters

After unfractionated *C. orbiculatus* seed oil had been examined by GC-MS with particular attention to peaks A, B, C, D, and E (Fig. 1), TLC fractions U, M, and L likewise were analyzed by GC-MS. After correlating these mass spectral results with the mass of the principal

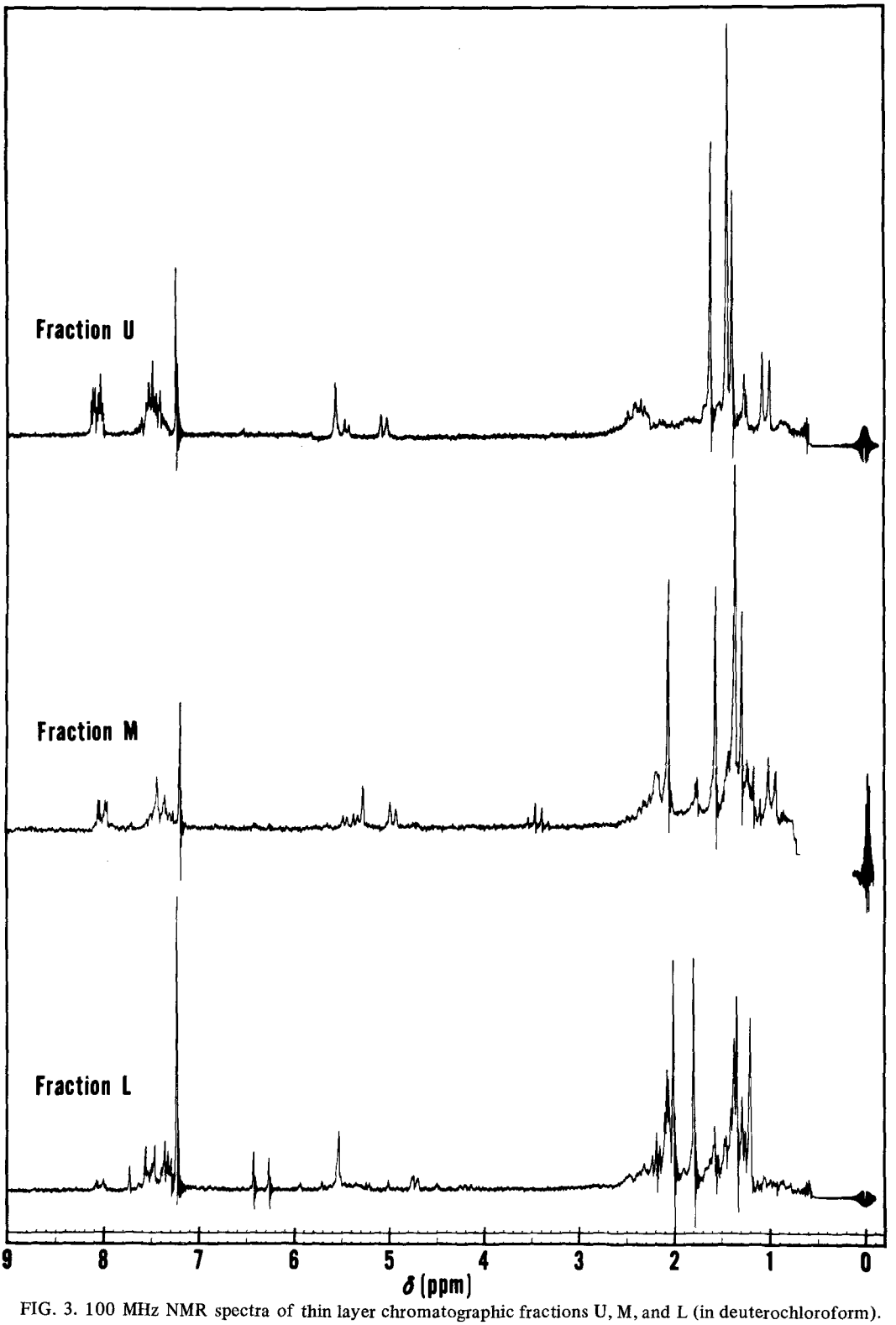

FIG. 3. 100 MHz NMR spectra of thin layer chromatographic fractions U, M, and L (in deuterochloroform).

nonglyceridic alcohols (established by high resolution MS) and significant features of the NMR spectra of U, M, and L, we postulated certain combinations of alcohols with the three acids whose presence was indicated (Table III). GC-MS of fractions U, M, and L revealed that one of the five major nonglyceride GLC peaks (Fig. 1) predominated in each and was accompanied by lesser amounts of other components of this group (Table IV). The spectrum for peak A apparently showed no molecular ion but did have an M-CH_3 peak at m/e 443. The base peak at m/e 105 was indicative of $C_6H_5^{\oplus}C{=}O$ and a peak at m/e 77 resulted from $C_6H_5^{\oplus}$.

MS of peak B contained a molecular ion at m/e 484. The base peak, m/e 131, indicated the $C_6H_5CH{=}CHC{=}O^{\oplus}$ ion. Also associated with a cinnamic acid moiety were peaks at m/e 103 (from $C_6H_5CH{=}\overset{\oplus}{C}H$ ion) and m/e 77 ($C_6H_5^{\oplus}$).

The spectrum of peak C had a molecular ion at m/e 520 and an M-CH_3 ion at m/e 505. The base peak at m/e 105 and another at m/e 77 both denoted the presence of benzoic acid.

MS of peak D exhibited a molecular ion at m/e 546 with a base peak at m/e 131 and another peak at m/e 105 that was almost as intense. These ions pointed to an ester containing equal numbers of benzoate and cinnamate groups.

The spectrum of peak E apparently shows neither a molecular ion nor an M-CH_3 peak. The relative intensities at m/e 131 and the base peak at m/e 105 suggested two benzoic acid moieties for every cinnamic acid group present.

NMR Spectra of TLC Fractions

In addition to conclusions that are summarized in Tables III and IV, certain features of the NMR spectra of U, M, and L (Fig. 3) are worthy of comment. The more distinct signals in the NMR spectra of U, M, and L are summarized in Table II, and tentative assignments are made within the framework of the ring system assigned to malkanguniol (Fig. 4) by den Hertog, et al. (22). Although each TLC fraction is a mixture, the major component apparently determines the characteristic features of its NMR spectrum. In the spectrum of malkanguniol triacetate, den Hertog, et al., (22) observed an AB quartet at δ 4.22 and 4.63 which they assigned to the geminal protons at C-15 (Fig. 4). Similar AB quartets were noted in the NMR spectra of the ketotriacetate and tetraacetate derived from malkanguniol and also in spectra of some closely related derivatives of euonyminol examined by Shizuri, et al., (24) and by Pailer, et al. (25). Since this quartet is

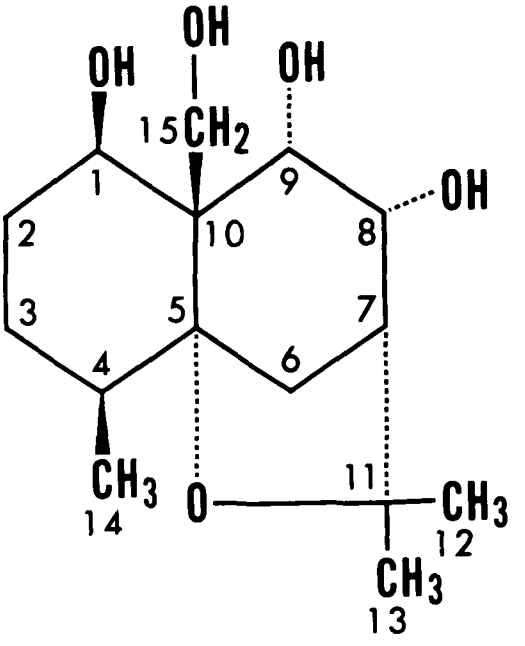

FIG. 4. Structure of malkanguniol, $C_{15}H_{26}O_5$, according to den Hertog and coworkers (22 and personal communication from H.J. den Hertog, Jr.).

absent from the spectra of our esters, they must have no primary hydroxy function at C-15 or elsewhere in the molecule. Accordingly, there should be, instead, a singlet associated with a tertiary methyl group; to this, we assign a high field singlet at δ 1.30 for M with similar chemical shifts for the related esters (Table II).

A doublet near δ 1.0 (J = 7-8) appears fairly consistently in the spectra summarized in Table II (in the case of L, it is shifted downfield to ca. δ 1.28 and is somewhat obscured by other signals). This doublet dovetails with one observed for malkanguniol derivatives which was assigned to the secondary methyl group at C-14 by den Hertog, et al. (22).

The NMR spectra of U and M (Fig. 3), as well as the predominant alcohol, show a 6 proton singlet near δ 1.4 which we assign to the gem-dimethyl grouping (C-12 and C-13, Fig. 4) of the alcohol moiety. In contrast, these methyl groups are not equivalent in the spectra of malkanguniol acetates (22) and appear as two 3 proton singlets. The spectrum of L resembles those of the malkanguniol acetates in this respect. A molecular model of malkanguniol reveals that oxygen functions at C-8 or C-9 may be in proximity to C-13 or C-14, depending upon the conformation adopted, and that these two methyl groups might be deshielded differently as a result. Accordingly, some structural

differences among these compounds at C-8 or C-9 may be suspected.

Correlations between GC-MS data and NMR spectra for U, M, and L have been pointed out (Tables III and IV). However, the combinations shown in Table III require the assignment of NMR signals (Table II) at δ 1.62 (fraction U) and at δ 1.58 (fraction M) to acetate methyls—positions that are so unusually far upfield for this grouping as to invite some skepticism. Nevertheless, these assignments are supported by certain observations of Shizuri, et al., (26) regarding evonine, an alkaloid with an alcohol moiety (evoninol) which has the same ring system as malkanguniol but has more hydroxyl functions. These Japanese workers prepared a series of four different derivatives of evoninol, each with one of five acetate groupings replaced with benzoate. Among these monobenzoates was one with a signal at δ 1.52 ascribed to an acetyl group. In addition, it appears that one of the acetate methyls of maytoline (an alkaloid ester of maytol) has a signal at δ 1.65 (23). Evidently, this anomalous upfield shift of acetate resonances is caused by a shielding influence of aromatic acyl groups (27) and need not be expected in fully acetylated counterparts of our esters.

Formate ester groups were not detected by either NMR or MS spectra of any fractions we examined. However, the formate proton would be expected as a singlet at δ 8.01 and, consequently, might be obscured by multiplets from aromatic protons.

We believe that the sesquiterpenoid alcohols from *C. orbiculatus* are trihydroxylic and closely related to alcohols which occur as esters in other species of the plant family Celastraceae. Evidently, they possess the same ring system. We are continuing our studies of these unusual esters and hope to elucidate fully the structure of the alcohols.

In retrospect, we must express admiration for the careful work of T.P. Hilditch in investigating *C. paniculatus* seed oil, especially considering the techniques available to his group in the 1930s. Prior to the 1973 communication of den Hertog, et al. (22), further investigations of this and related celastraceous seed oils added surprisingly little to the findings of the Hilditch group that were published in 1938 (2).

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SHORT COMMUNICATIONS

Stereospecific Analysis of Human Plasma Triglycerides

ABSTRACT

Brockerhoff-type procedures were used to determine the amounts of each fatty acid at each position of plasma triglycerides from normal and hyperlipemic humans. Statistically significant differences between both groups are found for the 2 and 3 positions.

INTRODUCTION

Brockerhoff investigated the distribution of fatty acids in the three distinct positions of triglycerides by a combination of partial hydrolysis of triglycerides by pancreatic lipase, phosphorylation of the formed diglycerides, and stereospecific action of phospholipase A on phosphatides (1). The positions 1, 2, and 3 of the glycerol moeity are numbered stereospecifically according to the proposals of the International Union of Pure and Applied Chemistry - International Union of Biochemistry Commission on Biochemical Nomenclature (2,3).

METHODS

Plasma is extracted by a modified Folch-Pi method. Triglycerides are isolated by preparative thin layer chromatography (TLC). Monoglycerides are formed by pancreatic lipase hydrolysis of triglycerides as described by Luddy, et al. (4). Phosphatides are prepared by a modified pancreatic lipase hydrolysis of triglycerides and phosphorylation of the formed diglycerides by phenyl dichlorophosphate (5). Phosphatides are converted to lysophosphatides (1-monoacyl-sn-glycerol-3-phosphorylphenol) by phospholipase A (6).

The fatty acid composition at each position can be determined by gas liquid chromatography (GLC) of fatty acids in the triglycerides, monoglycerides, and lysophosphatides. Since the 3 position is determined only by calculation, large relative errors occur for minor or trace components. Even negative concentrations may result from the calculations. The p value represents the significance of differences in means, as assessed by Student's t-test using standard formulae.

Table I shows the means and standard deviations for each fatty acid at each position of pooled plasma triglycerides from clinically normal individuals and of triglycerides from hypertriglyceridemic patients with pronounced

TABLE I

Stereospecific Distribution of Fatty Acids in Plasma Triglycerides of Normals and Hyperlipemics[a]

	Normals (n = 4)				Hyperlipemics (n = 8)			
Position	1 + 2 + 3	1	2	3	1 + 2 + 3	1	2	3
14:0	2.1 ± 0.7	2.3 ± 0.8	1.3 ± 0.8	2.8 ± 1.7	3.0 ± 1.2	2.5 ± 1.9	2.2 ± 1.8	4.2 ± 1.6
14:1	0.1 ± 0.2	0 ± 0	0.2 ± 0.2	0.2 ± 0.3	0.1 ± 0.2	0.1 ± 0.2	0.2 ± 0.3	0.1 ± 0.2
15:0	0.5 ± 0.3	0.7 ± 0.3	0.2 ± 0.2	0.6 ± 0.5	0.6 ± 0.3	0.6 ± 0.3	0.6 ± 0.5	0.6 ± 0.4
16:0	27.1 ± 1.2	55.0 ± 8.5	12.6 ± 1.8	13.6 ± 6.7	29.8 ± 2.1	49.9 ± 2.8	19.8 ± 4.9	19.7 ± 4.2
16:1	6.2 ± 0.4	7.0 ± 0.7	6.7 ± 0.5	4.9 ± 1.3	7.1 ± 2.4	6.4 ± 1.7	8.3 ± 3.1	6.6 ± 3.1
16:2	0.6 ± 0.3	0.6 ± 0.4	0.7 ± 0.3	0.5 ± 0.5	0.7 ± 0.3	0.8 ± 0.5	0.7 ± 0.3	0.7 ± 0.4
17:0	0.6 ± 0.4	1.0 ± 0.6	0.3 ± 0.3	0.4 ± 0.5	0.6 ± 0.3	1.1 ± 0.6	0.3 ± 0.3	0.3 ± 0.4
18:0	4.0 ± 0.6	6.3 ± 1.2	2.9 ± 1.6	3.0 ± 1.0	5.8 ± 1.3	8.9 ± 1.9	3.6 ± 1.1	5.0 ± 1.8
18:1	38.9 ± 2.0	19.0 ± 2.9	46.0 ± 2.4	51.7 ± 6.5	35.9 ± 3.8	21.2 ± 3.3	42.9 ± 6.3	43.7 ± 3.7
18:2	16.7 ± 1.4	6.2 ± 2.6	25.6 ± 2.3	18.2 ± 2.3	12.0 ± 1.3	5.0 ± 1.9	16.9 ± 2.9	14.3 ± 3.3
18:3	1.5 ± 0.4	0.8 ± 0.5	1.1 ± 0.3	2.5 ± 0.7	1.9 ± 0.9	1.2 ± 0.8	1.7 ± 1.5	2.7 ± 1.2
20:0	0.3 ± 0.3	0.4 ± 0.4	0.3 ± 0.2	0.4 ± 0.3	0.5 ± 0.2	0.7 ± 0.5	0.4 ± 0.4	0.6 ± 0.4
20:2	0.2 ± 0.3	0.2 ± 0.3	0.1 ± 0.2	0.3 ± 0.4	0.3 ± 0.3	0.3 ± 0.4	0.3 ± 0.3	0.2 ± 0.4
20:4	1.2 ± 0.4	0.8 ± 0.7	1.9 ± 0.4	0.8 ± 0.5	1.3 ± 0.4	1.1 ± 0.7	1.8 ± 0.8	1.0 ± 0.8
22:0	0.2 ± 0.3	0.2 ± 0.2	0.2 ± 0.2	0.3 ± 0.5	0.4 ± 0.3	0.3 ± 0.2	0.4 ± 0.3	0.5 ± 0.7

[a]Results expressed in mole percentage, mean ± standard deviation.

Frederickson type IV hyperlipoproteinemia (7). All normals had cholesterol levels between 150-250 mg %, triglycerides between 40-180 mg %, phospholipids between 180-310 mg %, and a normal lipoprotein electrophoresis pattern on cellulose-acetate strips.

RESULTS

As expected, human plasma triglycerides are highly asymmetric. Ca. half of the saturated fatty acids are esterified at the 1 position which also is occupied largely by monoenoic components.

In normals, the concentration of 18:2 is significantly higher at the 2 position than at the 3 position ($p<0.005$). No statistically significant difference was found between the 2 and 3 positions for 16:0 and 18:1 in normals, and for 16:0, 18:1, and 18:2 in hyperlipemics.

For the 1 position, no difference is found between normals and hyperlipemics ($p>0.10$).

At the 2 position, saturated fatty acids occupy 14-24 moles % in normals and 18-40 moles % in hyperlipemics. Both normals and hyperlipemics show a high concentration of 18:1 and 18:2, but hyperlipemics have more 16:0 and less 18:2 than normals ($p<0.005$).

Concentration of both 18:1 ($p<0.05$) and 18:2 ($p<0.025$) are significantly lower at the 3 position of hyperlipemics than of normals.

Christie, et al., (8) analyzed triglycerides from human atherosclerotic plaques, liver, heart muscle, and adipose tissue. None of the results agree with those from plasma triglycerides. The marked difference between plasma triglycerides of patients suffering from type IV hyperlipoproteinemia and liver triglycerides remains unexplained. Transesterification reactions between fatty acids of other classes of lipids and triglycerides, as well as between fatty acids of tissues and plasma, must occur on large scale. The nonrandom distribution of fatty acids between the 1 and the 3 position can be explained by different specificities of the diglyceride: acyl-CoA acyl-transferases and the enzymes which acylate the 1 position or by different pools of acyl-CoAs on which these enzymes operate.

J. PARIJS
G.A. DE WEERDT
R. BEKE
F. BARBIER
Department of Gastroenterology
State University of Ghent
Ghent, Belgium

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Convenient Apparatus for Extraction of Tissue Lipids

ABSTRACT

An all glass apparatus has been designed for rapid, convenient extraction of lipids from small samples of tissue. Samples are handled in vacuo or under nitrogen atmosphere throughout the extraction procedure.

INTRODUCTION

Extraction of tissue lipids by the Folch procedure (1) involves homogenizing the tissue with solvent, removal of tissue residue by filtration, washing of extract with water or salt solution, centrifugation, removal of aqueous phase, drying over sodium sulfate, filtration, and re-

Frederickson type IV hyperlipoproteinemia (7). All normals had cholesterol levels between 150-250 mg %, triglycerides between 40-180 mg %, phospholipids between 180-310 mg %, and a normal lipoprotein electrophoresis pattern on cellulose-acetate strips.

RESULTS

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J. PARIJS
G.A. DE WEERDT
R. BEKE
F. BARBIER
Department of Gastroenterology
State University of Ghent
Ghent, Belgium

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Convenient Apparatus for Extraction of Tissue Lipids

ABSTRACT

An all glass apparatus has been designed for rapid, convenient extraction of lipids from small samples of tissue. Samples are handled in vacuo or under nitrogen atmosphere throughout the extraction procedure.

INTRODUCTION

Extraction of tissue lipids by the Folch procedure (1) involves homogenizing the tissue with solvent, removal of tissue residue by filtration, washing of extract with water or salt solution, centrifugation, removal of aqueous phase, drying over sodium sulfate, filtration, and re-

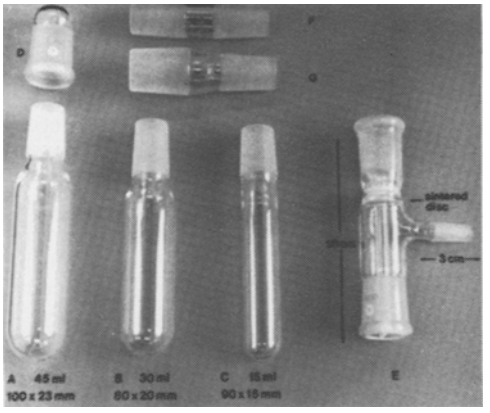

FIG. 1. Extraction tubes (A-C), cap (D), filtration tube (E), and adapters (F and G) for attaching standard glassware to filtration tube.

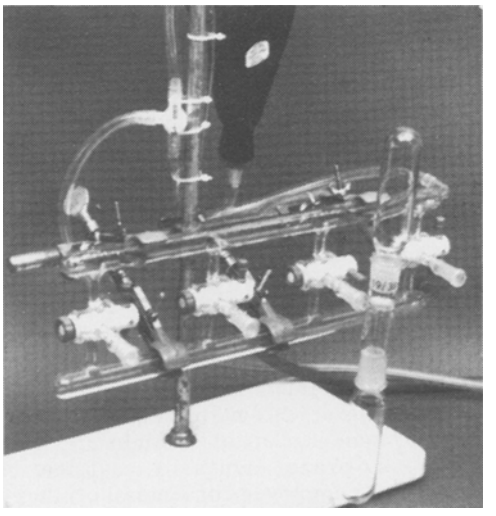

FIG. 2. Vacuum-nitrogen manifold with filtration unit in place.

moval of solvent under vacuum. Additional filtration may be carried out after dissolution of extract in fresh solvent. All of these operations should be carried out with minimum exposure of the extract to air, and contamination by lipid-soluble components of filter papers must be avoided. The equipment described has been designed to provide rapid, convenient extraction of lipids from small samples of tissue (up to 2 g) with minimal contamination, transferral of solutions, and exposure to air.

EQUIPMENT

The apparatus is depicted in Figures 1 and 2. Extraction tubes (Fig. 1A-C), fitted with 19/26 male standard taper ground glass joints, were constructed in 3 sizes, with capacities of ca. 45, 30, and 15 ml (100 x 23 mm, 80 x 20 mm, and 90 x 15 mm [inside diameter] respectively). The caps consist of sealed female standard taper joints (Fig. 1D). Homogenization is affected by means of a Polytron PT 10 homogenizer (Brinkman Instruments, Toronto, Ontario) which operates on a mechanical-ultrasonic principle. Similar equipment is available from other manufacturers (Tekmar Co., Cincinnati, Ohio). The diameter of the probe on this unit is 12 mm which establishes the minimum internal diameter of the extraction tube.

Filtration is accomplished on sintered glass under vacuum. The filtration tube (Fig. 1E) is 10 cm long and is constructed with a 19/38 female standard taper joint at the upper end and a 19/26 female joint at the lower end, with a coarse sintered glass disc sealed below the upper joint. A drain tube 6 mm in diameter, mounted below the sinter by means of a ring seal, terminates flush with the outer end of the lower standard taper joint. The unit is equipped with a side-arm terminating in a 10/19 male standard taper joint.

The vacuum-nitrogen manifold (Fig. 2) consists of two 18 mm glass tubes, 35 cm long, joined by 4 three-way stop cocks, each of which terminates in a 10/19 female standard taper joint. One side of the double manifold is connected to a water aspirator, the second side to a supply of nitrogen. A 3 liter gas bag acts as a reservoir on the nitrogen line.

TECHNIQUE

After homogenizing the sample in the appropriate volume of solvent, a filtration tube is attached to the top of the extraction tube, and a similar tube is inserted into the other end of the filtration tube as a receiver. The system is inverted, attached to the manifold, and vacuum applied. When filtration is complete, the vacuum is turned off and nitrogen introduced into the filtration unit by means of the three-way stop cock.

After removal of the unit from the manifold, water or salt solution is added to the organic extract, the tube capped, and the contents shaken. Separation of the phases is affected by centrifugation. The large extraction tubes were designed to fit the standard 50 ml buckets used in most common laboratory centrifuges; foam rubber cushioning is required for the smaller tubes. After removal of the aqueous phase by means of a pasteur pipette, the extract is dried over sodium sulfate which then is removed by repeating the filtration procedure. The receiver

may be attached to a standard rotary evaporator by means of a suitable adapter (19/26-24/40 double female joints, not illustrated) and the solvent evaporated under vacuum. After dissolving the lipid in fresh solvent, any insoluble residue is removed by filtration. For the final filtration step, a round bottomed flask equipped with a female standard taper joint (19/38 or 24/40) may be used as a receiver and attached to the filtration tube by means of a suitable adapter (Fig. 1F and G). The flask then may be used directly on the rotary evaporator for the final evaporation.

The apparatus may be employed in the extraction of lipid fractions from silica gel scraped from thin layer chromatographic plates. The vacuum zone collection (Brinkman Instruments, Toronto, Ontario; catalog. no. 25 09 800-5) employed for removal of silica gel from the plate fits into the filtration tube, permitting direct transfer of the gel to the sintered glass filter for elution of the lipid. Alternatively, the gel may be scraped into an extraction tube, extracted with solvent, and removed by the standard filtration procedure.

The initial design of this equipment employed a filtration tube with male standard taper joints and extraction tubes with female standard taper joints, permitting direct connection of these tubes to the rotary evaporator. The sintered glass filter was mounted in the lumen of a male standard taper joint. With this design, the rim of the male joint presented an obstacle to efficient drainage of the solvent from the walls of the extraction tube. The current design provides better drainage of the contents of the extraction tube onto the filter which completely fills the bore of the filtration tube. The use of similar joints on both ends of the filtration unit permits extraction tubes to be used interchangeably as filtrate receivers and avoids unnecessary transferral of solutions.

The use of standard taper joints of different sizes from those employed in the present equipment may be desirable depending upon the capacities of the extraction tubes employed and requirements of the particular laboratory. The basic design lends itself readily to modification.

The equipment was constructed by the Emerald Glass Co., Toronto, Ontario, to the specifications of the author.

BRIAN L. WALKER
Department of Nutrition
University of Guelph
Guelph, Ontario, Canada N1G 2W1

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Lack of Dietary Adaptation of Intestinal 2-Monoglyceride Acyl Transferase

ABSTRACT

Acylation of 2-monopalmitin with (1-^{14}C) palmityl-coenzyme A was studied with microsomal fractions isolated from the intestinal mucosa of both hamsters and rats. Microsomes from hamster intestinal mucosa yielded equal molar amounts of 1,2-diglycerides and triglycerides, while those from rats synthesized more 1,2-diglycerides (70%) than triglycerides (30%). Although the enzyme system from the hamster was almost twice as active (55 nmoles monoglyceride acylated/min/mg protein) as that from the rat (31 nmoles monoglyceride/acylated/min/mg protein), there was essentially no difference in the rates of acylation when animals were (A) fasted for 3 days, (B) fed a high fat diet (15% safflower oil), or (C) fed a high carbohydrate diet for 5 days.

INTRODUCTION

Synthesis of triglycerides in the intestinal mucosa occurs predominantly by the monoglyceride pathway (1). This pathway operates

may be attached to a standard rotary evaporator by means of a suitable adapter (19/26-24/40 double female joints, not illustrated) and the solvent evaporated under vacuum. After dissolving the lipid in fresh solvent, any insoluble residue is removed by filtration. For the final filtration step, a round bottomed flask equipped with a female standard taper joint (19/38 or 24/40) may be used as a receiver and attached to the filtration tube by means of a suitable adapter (Fig. 1F and G). The flask then may be used directly on the rotary evaporator for the final evaporation.

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BRIAN L. WALKER
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University of Guelph
Guelph, Ontario, Canada N1G 2W1

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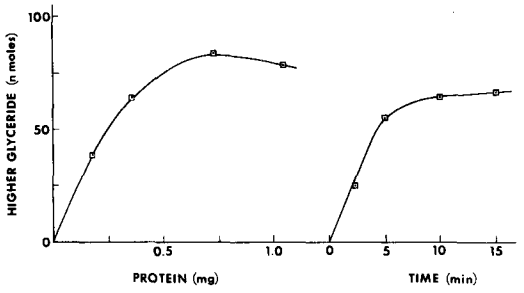

FIG. 1. In the studies on the effect of enzyme concentration, the reaction mixtures contained: 2-monopalmitin (0.1 mM); (1-^{14}C) palmityl-coenzyme A (0.2 mM); potassium fluoride (10 mM); crystalline bovine plasma albumin (4 mg); 0.25 ml 0.5 M Tris-maleate buffer (pH 7.0); 0.1 ml 5% Tween 80; and microsomal protein (isolated from intestinal mucosa of rats maintained on a stock diet) in the amounts indicated, all in a total volume of 1 ml. Incubation was carried out for 5 min at 37 C with shaking. Reaction was stopped by the addition of 5 ml chloroform:methanol (2:1 v/v); total lipids were extracted, and the different lipid classes were separated by thin layer chromatography (5). The radioactivity in the di- and triglycerides was determined, and the amount of higher glyceride synthesis was calculated. In the studies to determine the optimal incubation period, higher glyceride synthesis was determined with 0.5 mg microsomal protein at all time periods.

in the intestinal mucosa only during fat absorption, for, during periods of nonabsorption, the substrates (2-monoglycerides and fatty acids) are not available to the tissue. A question, therefore, arises as to whether the level of enzymes concerned with such synthesis is adaptable to the diet.

With 1-monoglyceride as substrate and using an indirect spectrophotometric method, Clark, et al., (2) observed a modest (30%) decrease in the monoglyceride acyl transferase activity in the microsomal fractions of intestinal mucosa of rats which were made deficient in essential fatty acids by feeding a fat-free diet supplemented with 4% tripalmitin for 8-12 weeks. Since lipolytic activity in the intestinal lumen yields 2-monoglycerides from triglycerides during fat absorption, it seems more appropriate to use this isomer as substrate for the assay of monoglyceride acyl transferase. In addition, whereas the 2-monoglyceride is converted to triglyceride, the 1- isomer is acylated mostly to 1,3- diglyceride by microsomal preparations from rat intestinal mucosa (3). Thus, to test the possible dietary regulation of the monoglyceride acyl transferase activity with a more natural substrate, one that will allow for the synthesis of triglycerides, we investigated the conversion of 2-monopalmitin and (1-^{14}C) palmityl-coenzyme A (CoA) to 1,2- di- and triglycerides.

TABLE I

2-Monoglyceride Acyltransferase Activity in Microsomal Fractions of Intestinal Mucosa from rats and Hamsters under Different Dietary Conditions[a]

Dietary condition	Enzyme activity in tissue from: Rat	Hamster
Stock diet	31.1 ± 2.3	55.2 ± 3.1
High fat diet	32.5 ± 2.6	57.4 ± 3.2
Fat-free diet	28.9 ± 1.6	51.7 ± 2.5
Fasted	30.7 ± 1.9	54.6 ± 2.9

[a]Incubations were carried out for 2 min as described in Figure 1 with 0.2-0.4 mg protein (rat) or 0.1-0.2 mg protein (hamster). The amount of 2-monoglyceride acylated/min/mg protein was calculated from the synthesis of 1,2- di- and triglycerides. Results were presented as means ± standard error of 4 experiments. Each experiment was performed in duplicate with microsomal fractions isolated from the pooled mucosa (upper half of intestine) of three animals.

EXPERIMENTAL PROCEDURES

Male rats (Holtzman strain) and golden hamsters maintained on a stock diet (Purina rat chow containing 5% fat) were used in this study. The animals were divided into 3 groups. Some were fed a high fat diet (15% safflower oil) and others a fat-free diet (Nutritional Biochemicals Corp., Cleveland, Ohio), for 5 days and still others fasted for 3 days prior to sacrifice. The procedures used for isolation of the microsomal fraction from the intestinal mucosa, preparation of 2-monopalmitin and (1-^{14}C) palmityl-CoA, and estimation of monoglyceride acyl transferase activity have been described earlier (4).

RESULTS AND DISCUSSION

Results of experiments to determine the dependence of the monoglyceride acyl transferase reaction upon the amount of microsomal protein and the linearity of the reaction with respect to incubation time are given in Figure 1.

The specific activity of 2-monoglyceride acyl transferase in the microsomal fractions from rat intestinal mucosa was ca. half that found with enzyme preparations from hamsters (Table I). Regardless of the previous nutritional status of the animals, acyl transferase activity remained unchanged. Under all dietary conditions used, the microsomal fractions from hamster intestinal mucosa yielded equal molar amounts of 1,2-di- and triglycerides, while those from rat intestinal mucosa gave rise to more 1,2-diglyceride (70%) than triglyceride (30%).

Activities of some enzymes of the intestinal mucosa of rats adapt to changes in the diet even after short periods of time. For example, a 2 or

3 day fast results in a considerable decrease in the acetyl-CoA carboxylase activity in the intestinal mucosa (6,7). On the other hand, depending upon the age of the rats, feeding a fat-free diet for 5 days increases the acetyl-CoA carboxylase activity from 2- to 8-fold (6). Furthermore, feeding a 5% cholestyramine diet for 7 days induces a 2-fold increase in the activity of β-hydroxy-β-methylglutaryl-CoA reductase in the microsomal fraction of intestinal mucosa (8). Unlike these enzymes, 2-monoglyceride acyl transferase in the microsomal fractions of intestinal mucosa of rats and hamsters remained unaltered when the animals were fasted for 3 days or fed either a high fat diet or a fat-free diet for 5 days (Table I). However, these results do not preclude the possibility that dietary adaptation of the acyl transferase activity in the intestinal mucosa may occur in other species or under other conditions, such as longer periods of a given dietary regimen.

G. ANANDA RAO
S. ABRAHAM
Veterans Administration Hospital
Martinez, California 94553
Bruce Lyon Memorial Research
Laboratory
Children's Hospital Medical Center
of Northern California
Oakland, California 94609

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LETTER TO THE EDITOR

Δ- and E-Hydroxylation of Keto Acid by Mushrooms

Sir: The ability of actively fermenting yeast to reduce α- and β-keto acids to hydroxy acids has been known for sometime (1,2). More recently, it was demonstrated that a variety of yeasts, bacteria, and molds were capable of extending the reductive process to a series of alkyl monocarboxylic acids containing carbonyl functions at either the γ- or δ-positions of the carbon chain (3).

As it had been observed previously that higher fungi of the class Basidiomycetes could affect a variety of transformations on the steroid nucleus (4,5), it became of interest to see if these more highly differentiated organisms also contained enzymes which could affect changes in alkyl mono and dicarboxylic acids containing a carbonyl function located at various positions on the carbon chain.

Mushroom cultures for these studies were obtained from J.J. Ellis, Northern Utilization Research and Development Laboratories, Peoria, Ill. The following cultures were studied: NRRL 2366 *Pleurotus ostreatus*, NRRL 2367 *Collybia velutipes*, NRRL 2368 *Lepiota naucina*, NRRL 2369 *Morchella crassipes*, NRRL 2603 *Morchella esculenta*, NRRL 2370 *Cantharellus cibarius*, NRRL 2372 *Lycoperdon umbrinum*, and NRRL 2775 *Agaricus bisporus*. Stock cultures were maintained on 4% malt agar plates at 4 C. Mycelia for the bioreduction studies were obtained from dense mycelial mats grown in 2 liter Fernbach flasks containing 200 g glass beads and enough nutrient broth, plus 2% glucose, to cover the glass beads. Incubation at 25 C without agitation for 7-10 days produced a heavy mat. The mycelium was dispersed in a sterile blender (30 sec) and used at a 10-20% inoculum level.

Δ-keto-hexanoic acid was used in initial screening tests to determine which mushroom species could carry out the bioreduction process. Mycelium was inoculated into 250 ml Erlenmeyer flasks containing 50 ml nutrient broth and 2% glucose and incubated for 72 hr at 25 C on a rotary shaker. Cold-sterilized 5% δ-keto-hexanoate (1 ml) was added, and the incubation was continued for 30 min. An additional 1.0 ml substrate and 5.0 ml 30% sterile glucose then were added, and the fermentation was continued for 4 days. The medium was clarified by filtration, acidified to pH 2.0 with sulfuric acid, saturated with sodium chloride, and extracted with 50 ml diethyl ether (3x). The pooled ether fractions were washed with saturated sodium chloride (3x) and dried over anhydrous sodium sulfate crystals. The ether extracts then were evaporated to dryness with nitrogen.

The presence of δ-C_6-lactone was detected by its characteristic smell in fermentation extracts of four cultures. Strong aroma was produced by NRRL 2366, NRRL 2369, and NRRL 2603. Faint aroma was produced by NRRL 2370. No aroma was detected in NRRL 2367, NRRL 2368, NRRL 2372, and NRRL 2775 or in uninoculated keto-acid controls. Δ-C_6-lactone was purified further by dissolving the ether extract in 50 ml 1N potassium hydroxide and washing the caustic solution with 50 ml diethyl ether (3x). The potassium hydroxide solution was saturated with sodium chloride, acidified with sulfuric acid to pH 2.0, and reextracted into ether as before. Methyl esters of this acid fraction were prepared for definitive detection of keto- and hydroxy-acids.

Methylated fermentation extracts were analyzed on a 2 ft column of UCW-98 10%, held 2 min at 80 C, followed by temperature programing at 4 C/min to 200 C using a Beckman GC-5 gas chromatograph. Improved resolution of the lactone and keto-ester was obtained using a 6 ft., 20% Carbowax 20 M column, temperature programed at 4 C/min from 100-190 C. Peaks from the gas chromatogram were passed into a Hitachi RMU-6E single-focusing mass spectrometer operated at 70 eV using a 6 sec scan. Mass spectra confirmed the presence of δ-C_6-lactone and unreduced δ-keto-hexanoic acid.

The ability of *M. crassipes* NRRL 2369 to reduce various other medium chain keto-mono- and keto-dicarboxylic acids was investigated further. These fermentations, extractions, and analyses were conducted as previously described, except that 2 liter flasks containing 500 ml nutrient broth with 2% glucose were

TABLE I

Reduction of Keto-Acids by *Morchella crassipes* NRRL 2369 at 25 C for 96 hr

Keto acids	Mycelium dry wt (g)	Ether extract (mg)	Keto-acid (mg)	Hydroxy-acid (mg)	Lactone (mg)
Δ -C_5	1.80	160	56	—[a]	—
Δ -C_6	2.10	200	150	—	40
ϵ -C_7	2.20	233	70	11	—
α -C_8	0.60	172	—	—	—
α -C_9	0.70	68	25	5	—
α -C_7 (dioic)	1.80	48	13	—	—
Control	1.60	—	—	—	—

[a]— = No keto acid or reduction product detected.

used. Results of one experiment in which the sodium salts of keto-acids (0.5 g; except for 2-keto-nonanoic- 0.19 g) were added to actively metabolizing mushroom mycelium are presented in Table I. The formation of 6-hydroxy-heptanoic acid from its corresponding 6-keto-acid extends the specificity of the mushroom keto-reductase system to the ϵ- carbon in the chain. The inability of mushroom mycelia to reduce the keto function in 5-keto-valeric could be due to one of the following reasons: (A) an alkyl group on the omega side of the keto group may be necessary, (B) there may be a steric hindrance associated with the branched chain, or (C) an adjacent methylene group may be required. Interestingly, two of the keto compounds which can be reduced readily to the corresponding lactone (δ-C_6-lactone) or hydroxy-acid (6-hydroxy-heptanoic acid) stimulated mushroom growth, as evidence by increased mycelial wt. Conversely, both the C_8 and C_9 α-keto-acids may have been inhibitory to the mushrooms, as mycelial wt yields were reduced sharply in these flasks. Nevertheless, reduction to the hydroxy-acid readily occurred with the C_9-keto-acid substrate. The α-keto-C_5-dicarboxylic acid was not converted into a product; no hydroxy compound could be detected by gas chromatography.

Similar results to those described above were obtained when *P. ostreatus* NRRL 2366 was tested with the same substrates.

From the data provided it is evident that several mushroom species contain enzyme complements which enable them to reduce some alkyl-keto-acids to the corresponding hydroxy acids.

R.J. WARGEL
W.L. ALM
J.F. ROLAND, JR.
Kraftco Corporation, Research and Development
Glenview, Illinois 60025

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Bile Acid Composition of Rainbow Trout, *Salmo gairdneri*

J.E. DENTON and **M.K. YOUSEF**, Department of Biological Sciences, University of Nevada, Las Vegas, Nevada 89154, and Laboratory of Environmental Pathophysiology, Desert Research Institute, University of Nevada, Boulder City, Nevada 89005, **I.M. YOUSEF**, Department of Pathology, University of Toronto, Toronto, Canada, and **A. KUKSIS**, Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada M5G 1L6

ABSTRACT

The bile acid composition and metabolism of rainbow trout *Salmo gairdneri* has been investigated by thin layer chromatography, gas liquid chromatography, and radio gas liquid chromatography methods. For this purpose gallbladder bile was collected from fed fish at 6 and 13 months and from starved fish at 12 months of age. Cholic acid was found to be the main component and constituted over 85% of total. Chenodeoxycholic acid accounted for 14% or less and the 3α,12α-7-keto- and 7α,12α-3-keto-5β-cholanoates for 1% or less of total. The bile acids were conjugated mainly with taurine, only small amounts of glycocholic acid being detected. Ca. 5% of the taurocholate was sulfated, as were trace amounts of cholic and glycocholic acids. The size of the bile acid pool was found to increase in the older fish and to decrease in starved fish. Unlike mammalian livers, the livers of the trout converted radioactive chenodeoxycholic acid into cholic acid.

INTRODUCTION

In the past, bile acid composition has been studied primarily in mammalian species. Studies on nonmammalian vertebrates have been limited. Haslewood (1) has summarized the types of bile salts found in nature as alcohol sulfates, taurine conjugates of 27-carbon acids, taurine conjugates of 24-carbon acids, and glycine conjugates and has claimed that their distribution bears a close relationship to the evolutionary position of the animal. This idea is interesting and can be developed further as data on bile acid composition of a wider range of vertebrates becomes available.

In the following study, we have examined the composition of the bile acids of the gallbladder bile of the rainbow trout, *Salmo gairdneri*, under normal conditions and as influenced by age and starvation.

MATERIALS AND METHODS

Standard cholic (CA), deoxycholic, chenodeoxycholic, and lithocholic acids of 99% purity were obtained from Supelco (Bellefonte, Pa.) and standard hyodeoxycholic acid of 99% purity from Applied Science Laboratories (State College, Pa.). The glycine and taurine conjugates of the common bile acids of 99% purity were purchased from Supelco. The 3-sulphate esters of cholic, deoxycholic, chenodeoxycholic, and lithocholic acids and of their glycine and taurine conjugates were prepared in the laboratory using sulfur trioxide by method 3 of Jenkins and Sandberg (2). Cholic acid-24-^{14}C (50 mCi/mMole) was obtained from New England Nuclear, Boston, Mass., and chenodeoxycholic-24-^{14}C, deoxycholic-24-^{14}C, and lithocholic-24-^{14}C (35.8 mCi/mMole) were purchased from Tracerlab, Waltham, Mass.

Rainbow trout were obtained from Willow Beach National Fish Hatchery, Willow Beach, Ariz. Three groups of fish were used in the study: group 1 included 6 fish which were 6 months old and fed ad libitum; group 2 had 6 fish which were 13 months old and fed ad libitum; and group 3 had 9 fish which were 12 months old and were fasted for 45 days. The gallbladder of each fish was dissected, removed, immediately frozen, and kept at -20 C until analyzed.

Extraction of Bile Acids

The bile (0.2-0.5 ml) was extracted by the addition of 10 ml hot ethanol-methanol (95:5 v/v) and shaking (3). The precipitated protein was removed by centrifugation at 2000 g at 4 C for 15 min. The alcoholic extract was decanted off, the precipitate washed with 2 ml hot methanol and filtered off. The extracts were combined, diluted to 40% alcohol with distilled water, and delipidated with petroleum ether. The aqueous alcohol solution then was evaporated to dryness under nitrogen in vacuo. The residue was dissolved in 1 ml methanol, and 0.5 ml was used for analysis of the conjugated bile acids, while the other 0.5 ml was saved for total bile acid determination and identification.

TLC of Conjugated Bile Acids

An aliquot of the bile acid extract was applied as a band to a thin layer chromatographic (TLC) plate (20 x 20 cm) coated with Silica Gel G (Merck, Darmstadt, Germany) in a

0.25 mm thick layer. The plates were developed in n-butanol-glacial acetic acid-water (10:1:1, v/v/v) for 4 hr (4). After evaporation of the solvents, the bile acids were located by iodine vapor and the relative R_f values of any bands compared to those of authentic standards of CA and CDCA, their taurine and glycine conjugates, and the conjugate sulfates for identification. Each band was scraped off and the bile acid conjugates extracted with 0.05 N HCl in 75% ethanol. Each conjugate group was subjected to further analysis, as described below. The recovery of the bile salts from the TLC plates was 95 ± 2%, as indicated by analysis of radioactive glycocholic acid.

Solvolysis of Sulfate Esters

The TLC fractions corresponding to sulfated bile acid standards were subjected to solvolysis (5). The sulfate esters (1 mg or less) were hydrolyzed at room temperature (24 hrs) with acetone-ethanol (9:1, v/v) adjusted to pH 1 with 2 N HCl. The solvents then were evaporated and residue subjected to TLC, as described above. Any free bile acids or their taurine and glycine conjugates were recovered and analyzed further.

Hydrolysis of Taurine and Glycine Conjugates

The TLC fractions (1 mg or less) corresponding to standard taurine and glycine conjugates of bile acids were subjected to saponification with 2.5 N NaOH (2.5 ml) at 115 C for 12-15 hr in a sealed glass tube (3). After cooling, the reaction mixture was acidified with dilute HCl and the bile acids extracted with diethyl ether. The recovery of radioactive bile acids from the taurine conjugates under the above conditions averaged 90 ± 5%.

Gas Liquid Chromatography (GLC) and Radio-GLC

For this purpose, the bile acids were converted into the methyl esters by methanolic 2,2-dimethoxypropane (Aldrich Chemical Co., Milwaukee, Wisc.) and concentrated HCl (6). Prior to GLC, the methyl esters were converted into the trifluoroacetates by reaction with 0.3 ml trifluoroacetic anhydride at 40 C for 40 min (3). The GLC analyses were performed on a Packard 7401 gas chromatograph system equipped with dual glass columns (4 ft x 2 mm inside diameter) containing a mixture of 3% QF-1 and 3% OV-17 (5:1, w/w) both on 100-120 mesh Gas Chrom Q (Applied Science Laboratories, State College, Pa.). The separations were made at 225 C isothermally using helium (30 ml/min) as the carrier gas. The GLC system was calibrated (3) by means of a standard mixture made up of equal wt proportions of lithocholic, deoxycholic, chenodeoxycholic, hyodeoxycholic, cholic, and 7-ketolithocholic acids. Unknown bile acids were quantitated using 5β-cholanoic acid as internal standard. The radio-GLC system was similar to that described by Swell (7).

The column effluent was passed through a combustion furnace containing copper oxide where it was converted into CO_2 and any radioactivity monitored in the proportional radioactive gas counter. The efficiency of the latter system was ca. 80% for ^{14}C, and any acids yielding more than 1000 dpm were measured with a relative error of 10% or less.

The quantitative GLC data were subjected to t-test between groups, and $P>0.05$ was considered not significant. The estimates from total bile acid analyses were compared to those obtained by combined TLC-GLC analysis following summation and normalization of the data (8).

TLC of Bile Acid Methyl Esters

The bile acid methyl esters were separated according to the number of hydroxyl and keto groups by TLC on Silica Gel G using chloroform-acetone-methanol (70:25:5, v/v/v) as developing solvent (9). The bands of the bile acids were located by briefly exposing the plates to iodine vapor. The areas corresponding to standard mono-, di-, and tri-hydroxy and keto bile acids were cleared of silica gel, and the scrapings were extracted with 10-20 ml methanol-acetone (1:9, v/v).

Part of the eluate was evaporated to dryness on the direct probe attachment of the mass spectrometer and the spectrum obtained. The rest of the sample was evaporated to dryness and the residue trifluoracetylated or trimethylsilylated prior to gas liquid chromatography-mass spectrometry (GLC-MS), as described below.

GLC-MS of Bile Acids

Combined GLC-MS analysis was performed with a Varian Mat CH-5 single focusing mass spectrometer coupled to a Varian Mat computer (10). For this purpose, the bile acid methyl esters were converted into the trimethylsilyl ethers using trimethylsilyl-chloride, hexamethyldisilazane and dry pyridine as described by Elliott, et al. (11). The GLC separations were obtained on a Varian model 2700 moduline gas chromatograph equipped with a 180 cm x 2 mm inside diameter glass column containing 3% OV-210 on 100-120 mesh Gas Chrom Q. The bile acids were resolved isothermally at 250 C, passed through a transfer line and a Watson-Bieman separator into an ion source, all oper-

ated at 270 C. The ionization voltage was 70 ev, the accelerating voltage 3000 volts, and the electron emission energy 100 μA. Scanning was done at 5 sec/decade at a resolution of 800-1000. All spectra taken over the GLC peaks were corrected for total ion current variation.

Incubations

The biochemical conversion of CDCA into CA was demonstrated by incubating radioactive CDCA with the liver homogenates (12). Liver tissue (1 g) was homogenized in 0.1 M phosphate buffer (pH 7.6) containing 0.25 M sucrose, 0.01 M $MgCl_2$, and 0.03 M nicotinamide. Aliquots of the homogenate were incubated with a 0.1% lecithin emulsion of 100 nmoles CDCA containing 0.1 μCi CDCA-24-^{14}C for 1 hr at 37 C. The purity of labeled CDCA was determined by TLC and radio-GLC to be better than 99%. At the end of the incubation, 19 ml ethanol containing 0.01% ammonium hydroxide was added along with 100 μg unlabeled CDCA and CA as carriers. The contents of the tubes were mixed thoroughly, heated at 60 C for 30 min, centrifuged, and the supernatant removed and evaporated to dryness under nitrogen. The bile acids were recovered following saponification of the extracts, as described above. After methylation, the bile acids were separated by TLC (13) using isooctane-isopropyl ether-acetic acid (50:25:40, v/v/v) and the bands corresponding to standard CA and CDCA recovered and the radioactivity measured by radio-GLC, as described above, and by scintillation counting. For the latter purpose, 15 ml Aquasol (New England Nuclear, Boston, Mass.) was added and the radioactivity determined in a Packard liquid scintillation spectrometer equipped with an automatic external standard. Each fraction was counted for 10 min in triplicate.

RESULTS

Identification of Bile Acids

Figure 1 shows the TLC separation of the bile acid samples. The various fractions were identified by reference to standards and the relative R_f values recorded in the literature. In most cases, strong spots were seen corresponding to taurocholic, sulfated taurocholic, sulfated cholic, glycocholic, and sulfated glycocholic acids. The various R_f values of the standards corresponded closely to those reported by Palmer (5,14) in a similar TLC system. Table I gives the mole percentages of each conjugate in the total bile acid mixture of each group of fish as determined by GLC following hydrolysis of the conjugates. It is evident that all groups are similar in their conjugation patterns and that differences in age

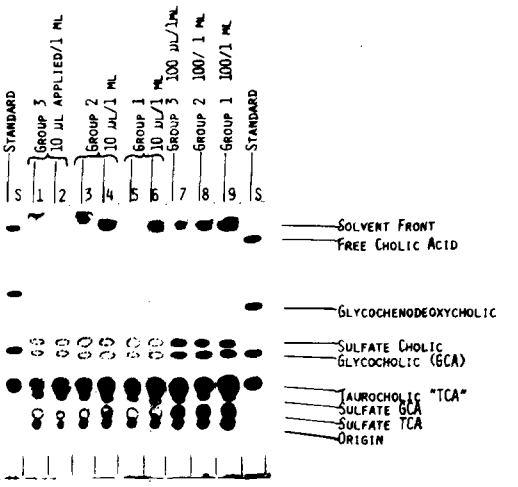

FIG. 1. Thin layer chromatogram of biliary bile acid conjugates of rainbow trout. Chromatography conditions: Silica Gel G; butanol-acetic acid-water (10:1:1, v/v/v) as developing solvent; phosphomolybdic acid spray. Lanes S, standard bile acids and conjugates as shown on the right of the figure. Lanes 1 and 2, 10 μliter applications of 1 ml solutions of total bile salts from two fish in group 3; lanes 3 and 4, 10 μliter applications of 1 ml solutions of total bile salts from two fish in group 2; lanes 5 and 6, 10 μliter applications of 1 ml solutions of total bile salts from two fish in group 1; lanes 7, 8, and 9, 100 μliter applications of 1 ml solutions of total bile salts from one fish each in group 3, 2, and 1, respectively.

TABLE I

Mole Percentage of Various Conjugates in Total Bile Acids of Rainbow Trout[a]

Type of conjugate	6 Months old	13 Months old	Starved fish 12 Months old
Sulfated tauro cholic acid	4 ± 0.4	3 ± 0.2	4 ± 0.2
Sulfated glyco cholic acid	3 ± 0.2	3 ± 0.2	5 ± 0.4
Sulfated cholic acid	2 ± 0.2	3 ± 0.3	1 ± 0.01
Tauro cholic + chenodeoxycholic acid	89 ± 2.1	89 ± 3.4	88 ± 1.5
Glyco cholic acid	2 ± 0.1	2 ± 0.2	2 ± 0.2

[a]Corrected gas liquid chromatographic estimates ± standard error (see text).

or starvation had no significant effect upon the conjugation type. In all instances, the bulk of the bile acid was conjugated with taurine, although sulfated bile acids occasionally made up as much as 10% of the total bile acid pool.

TLC of the bile acid methyl esters derived from the various conjugate classes revealed the presence of large amounts of CA and smaller amounts of CDCA, along with traces of keto bile acids. GLC of the trifluoroacetates of the bile acid methyl esters confirmed that, in all groups of fish, the peak corresponding to CA was the major component. The peak corresponding to CDCA was much smaller, especially in the adult fish (groups B and C). In addition, a minor peak of variable size emerged in the region corresponding to the trifluoroacetyl ester of methyl deoxycholate.

A GLC-MS examination of the mixed bile acid methyl esters, as the trifluoroacetyl derivatives using a QF-1 column, revealed that the major peak was indeed due to CA (15). The mass spectrum of this derivative showed a molecular ion at m/e 710 and a base peak at 367, which corresponded to a loss of 2 trifluoroacetyl groups and the side chain, M − (2 x 114 + 115). A major fragment also was seen at m/e 253, which was due to the steroid nucleus of a trihydroxy bile acid. A correct mass spectrum also was obtained for the smaller peak identified as CDCA (15). It had a molecular ion at 598 and a base peak at 369, which corresponded to M − (114 + 115). It also had major fragments at m/e 255 and at m/e 484. The fragment at m/e 255 corresponded to that anticipated for the steroid nucleus of a dihydroxy bile acid.

The GLC peak corresponding ca. to the trifluoroacetyl ester of methyl deoxycholate was identified as a degradation product of the trifluoroacetyl ester of methyl cholate (10). It had a molecular ion at 596, corresponding to the di-trifluoroacetate of the methyl ester of a monounsaturated dihydroxy bile acid. The base peak was at m/e 367, which corresponded to M − (114 + 115). Other major fragments were seen at m/e 482, which corresponded to M − 114, and at m/e 253, which was due to the steroid nucleus of a trihydroxy bile acid. Since the retention time of the peak was slightly lower than that of deoxycholic acid, it was concluded that the loss of the trifluoroacetyl ester group had occurred at carbon 7, resulting in the formation of a monounsaturated deoxycholic acid. The presumed origin of this bile acid was confirmed by GLC-MS analysis of the high temperature degradation products of the trifluoroacetate of pure methyl cholate. Likewise, two other minor GLC peaks eluted in the monohydroxy bile acid region were identified as the degradation products of the trifluoroacetate of methyl cholate. Their mass spectra corresponded to that of lithocholic acid, except for a discrepancy of 4 hydrogens in the fragment corresponding to the steroid nucleus. The identification of unsaturated bile acids as artifacts of GLC of trifluoroacetates has been described elsewhere (10). No evidence of the occurrence of unsaturated bile acids in the trout was obtained when the analyses were made with the methyl esters or the methyl ester trimethylsilyl ethers.

The identification of the bile acids was completed by a direct probe MS of the TLC fractions of the bile acid methyl esters. The most polar band corresponded to trihydroxy bile acids and gave a correct spectrum for methyl cholate (16) with a base peak at m/e 386 and a large fragment at m/e 253, which is characteristic of trihydroxy bile acid. The next most polar TLC band gave no recognizable spectra when examined as the methyl ester, but, after trimethylsilyaltion, it was possible to identify it as the 3α,12α-dihydroxy-7-keto-5β-cholanoate, which is a known compound with a published spectrum (15). This trimethylsilyl ether showed a molecular ion at m/e 564, and a base peak at m/e 341, corresponding to the loss of the side chain and one trimethylsilyl ether group and one molecule of water. Other major peaks were seen at m/e 251, corresponding to the steroid nucleus of a dihydroxy ketone; at m/e 269, which represents the steroid nucleus of a dihydroxy bile acid plus an oxygen; at m/e 366, corresponding to M − (2 x 90 + 18); at m/e 474, corresponding to M − 90; and at m/e 549, corresponding to M − 15, as well as other smaller fragments characteristic of the overall structure of this acid. The third TLC band corresponded to CDCA, and, on direct probe mass spectrometry, the appropriate spectrum was obtained (15). It had a molecular ion at m/e 406, a base peak at m/e 370, as well as a large fragment at m/e 255, corresponding to the steroid nucleus of a dihydroxy bile acid. The fourth TLC band corresponded to 3-keto,7α, 12α-dihydroxy bile acid, which could be identified without trimethylsilylation. It is a known bile acid with a published mass spectrum (14). This methyl ester had a molecular ion at m/e 420, a base peak at m/e 269, and a large fragment at m/e 251 corresponding to the steroid nucleus of a dihydroxy monoketo bile acid. The solvent front contained small amounts of a steroid material of low polarity which could not be immediately identified when analyzed as the methyl ester by direct probe.

Table II gives the mole percentages of the individiual bile acids in the total bile acid mixtures from the various groups of fish. Ca. 14% of the total bile acid in the 6 month old fish was CDCA and 80% CA. In 13 month old fish, the percentage of CDCA had decreased to ca. 6% and in starving 12 months old fish to ca. 1%, with corresponding increases in the proportion of CA. Table II also shows that the pool size of the bile acids was ca. 20 times as large in the 12 month old as in the 6 month old fed fish ($P>0.001$), and twice as large as in the starved 12 month old fish ($P>0.002$). The bile acid pool size measured by direct GLC and by combined TLC-GLC methods showed good agreement for all groups.

Metabolism of Bile Acids

Figure 2 shows the elution patterns of mass and radioactivity as obtained by radio-GLC of the trimethylsilylethers of the bile acid methyl esters recovered from the incubation of chenodeoxycholic-24-^{14}C with the liver homogenate of 12 month old fish. Ca. two-thirds of the added chenodeoxycholate has been converted into cholate. Table III gives the distribution of radioactivity among the bile acids of the trout liver following incubation of radioactive chenodeoxycholate with the various trout liver homogenates. It is seen that the livers of both 6 and 12 month old fish were capable of converting CDCA into CA, but that the older livers accomplished it ca. 3 times more rapidly. No conversion was observed when boiled homogenates were employed. This interconversion of the bile acids may explain the lower molar percentage of chenodeoxycholate in the older group of fish. There was no significant radioactivity found in any other bile acid band recovered by TLC of the bile acid methyl esters.

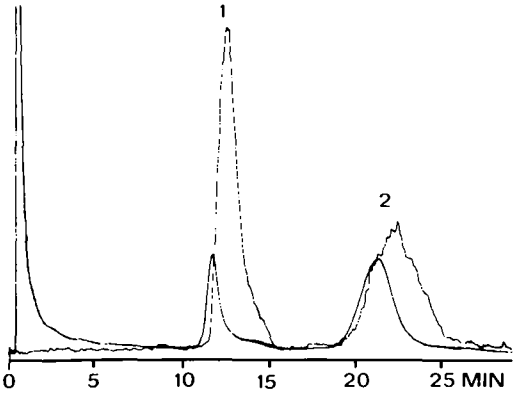

FIG. 2. Radio-gas liquid chromatography of bile acids of fish liver homogenates following incubation with chenodeoxycholic acid-24-^{14}C. Upper tracing, radioactivity; lower tracing, mass. Peaks 1 and 2 represent cholic and chenodeoxycholic acids, respectively. Instrument: Packard model 7401 gas chromatograph equipped with model 1894 proportional radioactivity monitor and a copper oxide furnace. Column: 120 cm x 2 mm inside diameter glass tube packed with 19:1 (w/w) mixture of 3% HI-EFF-8BP and 3% OV-210 on 100-120 mesh Gas Chrom Q, respectively. Carrier gas, argon, 55 ml/min. Temperatures: column, 225 C; injector, 225 C; detector, 240 C. Proportional counter conditions: range, 3000 cpm; time constant, 10 sec.; high voltage, 1650 v; quench gas:propane, 5 ml/min. Combustion furnace, 750 C. Flame ionization mass detector with 10:1 stream splitter. Sample: 2 μliter 1% solution of the bile acid methyl ester trimethylsilylethers in silylation mixture. Total radioactivity, 9000 dpm.

DISCUSSION

In general, our findings on the rainbow trout agree with the overall evolutionary pattern proposed by Haslewood (1). The bile salts of many fishes, including some bony fishes of fresh water contain chiefly either sulfated or taurine conjugated bile acids (17). Since the sulfates constitute only 5-10% of the total bile

TABLE II

Bile Acid Composition of Rainbow Trout[a]

Bile acids	6 Months old	13 Months old	Starved fish 12 months old
Total bile acid pool (μmoles)	0.63 ± 0.13	14.17 ± 0.46	7.91 ± 1.30
Total bile acid pool (μmoles)	0.60 ± 0.13	13.99 ± 0.57	7.11 ± 1.45
Cholic acid (mole % total)	85.35 ± 0.85	93.62 ± 0.31	98.58 ± 1.19
Chenodeoxycholic acid (mole % total)	14.67 ± 0.85	5.95 ± 0.29	1.40 ± 0.63
3α,12α-Dihydroxy-7-keto-cholanoic acid (mole % total)	Trace	Trace	Trace
7α,12α-Dihydroxy-7-keto-cholanoic acid (mole % total)	Trace	Trace	Trace

[a]Corrected gas liquid chromatographic-thin layer chromatographic analyses ± standard error (see text).

TABLE III

Metabolism of Labeled Chenodeoxycholic Acid in Liver Homogenate of Rainbow Trout[a]

cpm of:	6 Months old[b] cpm x 10^3	13 Months old[b] cpm x 10^3
cpm of chenodeoxycholic acid-24-^{14}C added	125	125
cpm of chenodeoxycholic acid-24-^{14}C after incubation	95 ± 4	31 ± 4
cpm of cholic acid-24-^{14}C after incubation	23 ± 3	87 ± 6
cpm of chenodeoxycholic acid-24-^{14}C after incubation with boiled homogenate	120 ± 5	118 ± 5
Total recovery of ^{14}C	118 ± 5	118 ± 7

[a]The substrate 100 nmole chenodeoxycholic acid-24-^{14}C (125 x 10^3 dm) was incubated with an amount of homogenate equivalent to 500 mg liver at 37 C in air for 1 hr in 5 ml 0.1 M phosphate buffer (pH 7.6) containing 5 mM $MgCl_2$, 1 mM nicotinamide, 0.5 mM glutathione, and NADPH generating system (1 μmole glucose-6 phosphate, 0.5 kornberg unit glucose-6 phosphate).

[b]Number of incubations were 6/group.

salt, the rainbow trout may be ranked in an advanced position among the *Teleostei*. Although sulfated bile acids are theoretically characteristic of primitive vertebrates (1), recent evidence indicates that higher vertebrates, i.e. man and laboratory rat, also can form sulfated bile acids under certain conditions (5,14,18). The chief bile acid in rainbow trout is CA, but CDCA is present in small amounts. Both of these acids are conjugated mainly with taurine.

Unlike that of the mammals (19), rainbow trout liver appears to convert efficiently CDCA into CA. A hydroxylation of CDCA to CA also is accomplished in other nonmammalian species, such as python (20), eel (21), and chicken (22). The higher ratio of CA to CDCA in the year old, as compared to the 6 month old fish may be related to hydroxylation of the CDCA to CA. The year old trout could be shown to affect this conversion at a considerably higher rate than the 6 month old trout.

Starvation of trout resulted in a reduction in the size of the bile acid pool and an increase in the ratio of CA to CDCA acid. It is difficult to explain the reduction in the pool size, since no information is available on the number of enterohepatic circulations of the bile acid pool of rainbow trout. However, in Rhesus monkey fasting is known to decrease the enterohepatic circulation and the volume of bile (23). The reduced secretion of the bile salts in starvation also would lead to decreased synthesis of bile acids as a result of a feed-back inhibition (24) which also could contribute to reduced bile acid pool.

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Lipid and Alkali Extractable Fatty Acids from *Mucor rouxii*: Effect of Thermal Changes in Growth Environment and Age of Cells

S. SAFE, Atlantic Regional Laboratory, National Research Council of Canada, Halifax, Nova Scotia[1]

ABSTRACT

The lipid levels of the fungus *Mucor rouxii* are affected markedly by both the growth temperature and the age of the cells. Both chloroform-methanol extractable (fraction I) and alkali extractable (fraction II) fatty acids are present in these cells, and the fraction II/fraction I ratio increased significantly with decreasing growth temperature. This ratio was, however, reasonably constant during the entire growth period at each specific growth temperature. In all cases, the unsaturation index of the fraction II fatty acids was significantly lower than the corresponding value for the fraction I fatty acids. Examination of the unsaturation indeces of the two fractions during the early log-phase growth period (48 hrs) indicated that the indeces decreased with increasing growth temperature; however, as the age of the cells increased, this relationship changed dramatically. At the end of 144 hrs growth, the unsaturation index for fraction II has reversed its temperature dependance completely, since the index is a maximum at the higher growth temperature. The degree of unsaturation for fraction I is a maximum at 25 C and is lower for cells grown at 15 and 35 C; this relationship is true between 96-144 hr after inoculation.

INTRODUCTION

Cellular fatty acids are primary metabolites which are extremely sensitive to environmental changes, particularly with respect to oxygen limitations (1-4) and temperatures (5-10). Growth of yeast and fungi under oxygen limiting conditions tends to cause a marked decrease in the production of unsaturated fatty acids with a corresponding increase in the shorter chained acids. It also has been observed that, with increasing growth temperature, there is a decrease in the unsaturation index of the cellular fatty acids. The reasons for these temperature induced changes have not been elucidated fully, but it has been suggested that they are associated with membrane stability factors (7). The present investigation was designed to determine the effect of temperature and the age of the cells upon the degree of fatty acid unsaturation in the fungus *Mucor rouxii* and also to determine whether these factors cause any changes in the fraction II/fraction I cellular fatty acid ratios.

MATERIALS AND METHODS

Organism and culture conditions: The isolation of *M. rouxii* (Calmette) Wehmer (HLX 1093) from sheep rumen contents has been described (11). Inocula of *M. rouxii* were prepared by adding sterile water to a culture maintained on a defined media to which agar (2% w/v) had been added. The spore suspension was filtered to remove cellular debris and a portion (ca. 10^7 spores) used to inoculate 600 ml medium contained in 2 liter conical flasks. The media was preincubated at 15, 25, or 35 C prior to inoculation of the flasks. The cells were harvested by filtration followed by immediate freeze-drying of the mycelium.

Extraction of free and bound fatty acids: The freeze-dried cells were macerated with chloroform-methanol (2:1) in a Waring blender and the mixture allowed to stand 12-18 hr followed by refluxing for 4 hr. The mixture was filtered and the filtrate concentrated to dryness and weighed to give the lipid extract. The lipid extract was treated with 10% methanolic potassium hydroxide solution for 2 hr, diluted with 3 volumes of water and extracted with ether. The basic extract then was acidified with hydrochloric acid and extracted with ether to give the chloroform-methanol extractable fatty acids (fraction I). The extracted cells were treated with 10% methanolic potassium hydroxide solution for 2 hr, and filtered to remove cellular debris, and the fatty acids (fraction II) could, thus, be isolated as described.

Fatty acid analysis: The two fractions were transmethylated by refluxing for 90 min in benzene/methanol/sulfuric acid (20:10:1). The resultant fatty acid methyl esters then were purified by thin layer chromatography (TLC) (petroleum spirit/ether (97:3) as solvent system) on silicic acid, weighed, and analyzed by

[1]Present address: Department of Chemistry, University of Guelph, Guelph, Ontario, Canada.

gas liquid chromatography (GLC) on a stainless steel column (6 ft x 1/8 in.) packed with 8% diethylene glycol succinate (HIEFF-IBP) on Gas Chrom Q (Applied Science Laboratories, State College, Pa.) at a temperature of 140 C.

RESULTS AND DISCUSSION

The effect of temperature upon the growth of the fungus *M. rouxii* is summarized in Figure 1. Maximum growth of the cells was observed at 25 C, although significant accumulation of cells also was obtained at 15 and 35 C. Early log-phase growth of the fungus at 15 C was significantly slower than at 25 or 35 C, however, after an initial lag period, the growth rate increased markedly between 72-120 hr after inoculation.

The relative amounts of chloroform-methanol extractable lipids synthesized by *M. rouxii* cells at 15, 25, and 35 C are summarized in Table I. There were some minor variations in lipid concentrations with respect to both the age of the cells and the growth temperature, but generally 7-10% of the dry wt of the cells were extractable using the above solvent system. The results are in contrast with data obtained from *Sacchromyces cerivisiae* (6) and *Candida lipolytica* (5) in which the highest lipid levels were obtained at the lower growth temperatures.

The amount of total extractable fatty acids

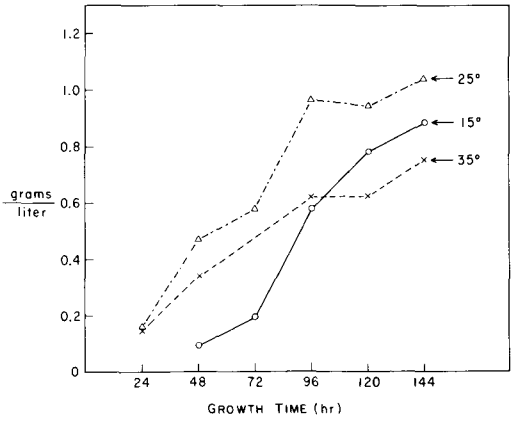

FIG. 1. A plot of mycelial wt of *Mucor rouxii* (g/liter) against age of the cells.

exhibited considerable variation with respect to both the age of the cells and the growth temperature (Table I). There was a general increase in the cellular fatty acid levels with increasing age of the cells at 15, 25, and 35 C, and the cells grown at the higher temperatures contained slightly higher fatty acid levels. The data shown in Table I clearly indicated that the fraction II/fraction I ratio was dependent upon the growth temperature. At 35 C, the value for this ratio was between 0.08-0.12 at the various time points from 0-144 hr after inoculation. Correspondingly, the ratio varied from

TABLE I

Relative Amounts of Lipid and Bound and Free Fatty Acids from *Mucor Rouxii* Grown at 15, 25, and 35 C

Temperature (C)	Age of cells (hr)	Percent lipid extract[a]	Percent total fatty acids[b]	[Fraction II][c] / [fraction I][d]
15	48	9.8	1.1	0.38
	72	8.6	1.3	0.42
	96	7.4	2.7	0.36
	120	8.3	2.9	0.36
	144	7.4	3.4	0.38
25	24	9.2	1.8	0.20
	48	7.8	2.5	0.17
	72	8.1	2.6	0.24
	96	7.1	3.8	0.21
	120	8.1	3.9	0.20
	144	7.9	3.7	0.20
35	24	10.0	2.2	0.10
	48	8.5	2.4	0.11
	72	7.2	2.6	0.08
	96	8.1	4.0	0.09
	120	9.0	3.9	0.12
	144	8.6	3.8	0.10

[a]Based upon mg chloroform-methanol extractable lipid/mg freeze-dried mycelium.

[b]Based upon mg fatty acid methyl esters/mg freeze-dried mycelium.

[c]Wt of base extractable fatty acid methyl esters.

[d]Wt of chloroform-methanol extractable fatty acid methyl esters.

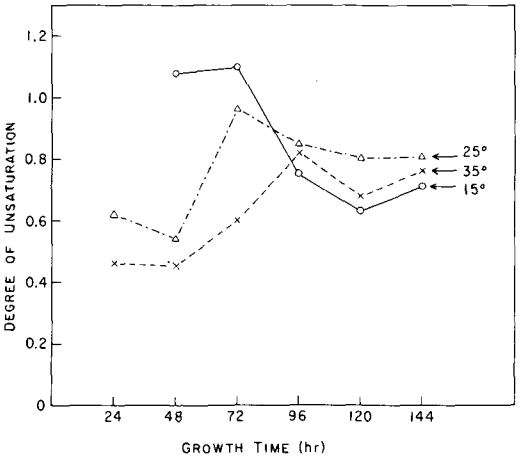

FIG. 2. A plot of degree of unsaturation of the chloroform-methanol extractable fatty acid fractions against age of the cells.

0.17-0.24 at 25 C and from 0.36-0.42 at 15 C, thus indicating that the ratios exhibited significant increases with decreasing growth temperature and are relatively constant with respect to the age of the cells. The results observed for *Mucor javanicus* showed that growth of the fungus in the absence of oxygen increased the fraction II/fraction I ratio, and recent results indicated a similar change in this ratio for *M. rouxii* fatty acids (S. Safe, unpublished results). The results reported herein thus add a second environmental parameter, i.e. temperature, which can affect a change in the relative unsaturation levels of the chloroform-methanol and alkali extractable cellular fatty acid fractions.

A summary of the fatty acid compositions and unsaturation indeces for the chloroform-methanol extractable fatty acid fractions is shown in Table II and Figure 2. Comparison of the fatty acids from the cells grown for 48 hr (early log-phase growth) indicated a familiar relationship between the degree of unsaturation and the growth temperature. The degree of unsaturation increased with decreasing growth temperature, and this was due primarily to the difference in the concentration of linolenic acid at the various temperatures. At a growth temperature of 15 C, the linolenic acid component was 20.1% of the total fatty acid fraction; this value decreased to 10.9 and 5.5% at 25 and 35 C, respectively. Examination of the unsaturation indeces during log-phase and late log-phase growth gave a completely different picture concerning the relationship between fatty acid unsaturation and growth temperature, as indicated in Figure 2. After 144 hr growth, the unsaturation index of the fatty acid fraction from the cells grown at 25 C was 0.81 whereas the corresponding indeces from the cells grown at 35 and 15 C was 0.78 and 0.71, respectively. Thus, during late log-phase growth, the degree of unsaturation was lower for the fatty acids synthesized in the cells grown at 15 C than for the cells grown at either 25 and 35 C. This result was almost a complete reversal of the data obtained during the early log-phase growth and shows that the unsaturation indeces for the chloroform-methanol extractable fractions were

TABLE II

Analysis of Fraction I[a] Fatty Acids from *Mucor Rouxii* Grown at 15, 25, and 35 C

Temperature (C)	Age of cells (hr)	Fatty acid composition 8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	Unsaturation index
15	48	0.6	7.2	8.1	9.3	14.4	13.5	1.8	15.9	9.3	20.1	1.08
	72	0.6	6.5	6.5	12.5	12.8	15.7	0.6	15.1	9.8	19.9	1.10
	96	1.8	12.3	12.3	14.1	11.9	11.0	3.1	15.6	5.5	12.3	0.75
	120	1.2	15.1	13.9	12.7	13.1	9.0	4.1	16.8	4.1	9.9	0.63
	144	1.5	12.1	14.7	14.9	10.6	7.5	3.4	17.2	7.1	10.8	0.71
25	24	1.2	13.2	14.4	17.6	14.1	9.7	2.9	11.7	4.1	10.9	0.62
	48	1.4	11.2	13.8	11.0	15.1	8.0	6.0	15.9	7.3	5.3	0.54
	72	0.4	3.3	7.7	12.5	14.2	11.3	2.7	22.1	12.7	13.0	0.96
	96	0.5	6.9	8.8	14.0	13.5	6.6	5.1	23.4	8.6	12.4	0.84
	120	0.5	12.6	14.7	13.6	10.2	7.2	3.7	16.3	7.8	12.8	0.80
	144	0.6	8.1	10.9	13.3	12.4	6.4	4.7	23.0	9.7	10.6	0.81
35	24	---	10.3	14.4	25.9	15.0	11.5	1.9	12.2	4.2	5.5	0.46
	48	0.4	9.3	13.6	22.7	14.9	8.7	4.3	19.2	3.2	3.5	0.45
	72	0.2	9.8	12.8	19.7	14.6	12.2	4.3	11.8	8.1	6.6	0.60
	96	0.1	4.5	7.5	12.2	13.7	10.0	4.2	31.0	8.8	7.9	0.82
	120	0.1	4.2	8.3	13.5	14.8	10.5	6.3	31.4	6.3	4.5	0.68
	144	0.2	5.9	7.2	11.4	14.2	9.8	6.9	30.0	8.4	6.1	0.78

[a]Chloroform-methanol extractable fatty acids.

dependent, not only upon the growth temperature, but, also upon the age of the cells. Comparison of the fatty acid compositions of these fractions indicated that the changes in the unsaturation indeces of the cells grown at 15 C were almost entirely due to decreases in the levels of the 18:3, 18:2, and 16:1 fatty acids as the age of the cells increased. In contrast, the concentration of the 18:1 acid showed an overall increase with increasing age of the cells. The 18:1 levels in the cells grown at 25 and 35 C also increased in a similar fashion, whereas the levels of the 18:3, 18:2, and 16:1 fatty acids exhibited irregular changes. Kates and Paradis (5) examined the fatty acid composition of *C. lipolytica* grown at 25 and 10 C, and their data contrasted with the results reported herein. The unsaturation indeces of the lipid extractable fatty acid fractions were similar at early and late log-phase growth; at intermediary points, the cells grown at the lower temperature exhibited the higher unsaturation index. The changes in the degree of unsaturation in the *C. lipolytica* cells were also due almost entirely to changes in the 18:2 and 18:1 levels.

There has been speculation that, with increasing cell growth temperature, the decrease in the unsaturated fatty acids is due to their replacement with saturated fatty acids which are thought to increase membrane thermal stability (7). Previous works (5-10) have shown that cells grown at lower temperatures contained more highly unsaturated fatty acids, and it was proposed that this was due to the presence of more dissolved oxygen which is required for fatty acid desaturation in yeast. The results for *M. rouxii* do not preclude the above possibilities, but there is an indication that both temperature and the age of the cells influence fatty acid composition and the influence of these parameters is currently being studied in a number of microorganisms.

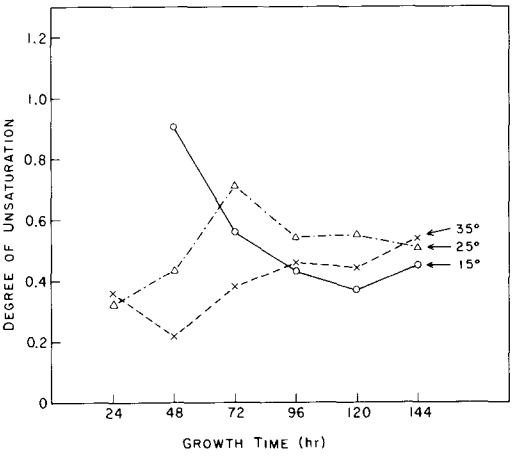

FIG. 3. A plot of degree of unsaturation of the alkali extractable fatty acid fractions against age of the cells.

The fatty acid compositions of the alkali extractable fatty acid fractions are summarized in Table III. The unsaturation indeces for these fractions were also dependent upon the growth temperature; the results after 48 hr showed that the unsaturation indeces increased with decreasing growth temperature, and this parallels the

TABLE III

Analysis of Fraction II[a] Fatty Acids from *Mucor Rouxii* Grown at 15, 25, and 35 C

Temperature (C)	Age of cells (hr)	Fatty acid composition										Unsaturation index
		8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	
15	48	---	8.8	11.9	17.1	12.4	18.6	1.0	8.3	5.2	17.6	0.90
	72	1.6	18.4	14.7	22.1	10.5	13.2	0.2	5.8	4.2	9.5	0.56
	96	2.5	21.6	19.0	20.3	9.2	16.2	1.0	7.0	2.2	7.0	0.43
	120	1.6	18.9	19.9	23.6	11.0	6.3	2.2	7.9	2.8	5.7	0.37
	144	1.6	16.4	15.3	17.5	13.7	6.9	4.8	14.3	2.1	7.4	0.45
25	24	1.9	23.2	21.0	22.9	10.9	7.2	1.1	4.0	2.4	5.3	0.32
	48	---	12.9	19.5	24.2	17.5	10.8	1.9	8.1	5.8	4.2	0.43
	72	0.4	6.3	12.3	20.9	15.0	15.0	2.1	12.9	1.6	13.2	0.71
	96	0.6	13.0	16.2	18.2	13.3	7.6	3.6	13.0	7.9	5.8	0.54
	120	0.9	14.0	20.5	22.2	10.0	6.5	1.3	9.2	6.1	9.2	0.55
	144	0.8	12.7	18.6	19.3	12.7	7.7	2.8	14.1	4.2	6.9	0.51
35	24	---	11.7	17.2	26.2	16.4	10.9	1.9	9.8	2.5	3.1	0.36
	48	---	13.8	25.5	29.3	10.3	9.0	1.4	8.6	1.4	0.7	0.22
	72	0.8	15.2	29.6	20.4	6.1	10.6	0.8	9.1	4.5	3.0	0.38
	96	0.3	14.7	23.7	22.4	6.6	11.2	1.0	9.8	4.9	5.2	0.46
	120	0.5	11.3	20.4	23.5	11.3	10.0	1.8	12.7	4.5	4.1	0.44
	144	0.5	10.0	20.4	19.6	10.0	11.9	1.0	15.9	5.5	5.2	0.54

[a]Alkali extractable fatty acids.

results obtained for the chloroform-methanol extractable free fatty acids. The unsaturation indeces for the alkali extractable fractions also changed markedly with the age of the cells, e.g. Figure 3; after 144 hr, the unsaturation index for the cells grown at 15 C was lower than the indeces for the cells grown at 25 or 35 C. The variations in the degree of unsaturation, as well as in the percent composition of the individual unsaturated fatty acids, were similar for both fractions I and II, although it should be noted that the degree of unsaturation of fraction II was always significantly lower than the values obtained for the corresponding fraction I at 15, 25, and 35 C.

Thus, it is clear that *M. rouxii* cells contain at least two cellular fatty acid fractions (I and II) which differ in both their fatty acid compositions and cellular concentrations and are also sensitive to the age of the cells and the cell growth temperature. Current work is in progress to determine what role the alkali extractable fatty acids play in the cell and what interrelationship exists between these two distinct fatty acid fractions.

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Brain Mitochondrial Incorporation of Elongated Fatty Acids into Phospholipids

S.A. MOSS and **F.M. YATSU**, Department of Neurology, University of California, San Francisco, California 94143

ABSTRACT

The characteristic patterns and asymmetric distribution of phospholipid fatty acids suggest precise control mechanisms. Our investigations were designed to assess mitochondrial fatty acid elongation and their pattern of incorporation into complex lipids. Fatty acid chain elongation in the total lipid fraction occurred primarily with the more abundant fatty acids present. Elongation patterns in free fatty acids were similar to the total lipid fraction except $C_{20:4}$ and $C_{22:4}$ were formed to slightly greater extent. Choline glycerophosphatide and ethanolamine glycerophosphatide displayed different patterns of elongation. Choline glycerophosphatide contained more elongated longer chain polyunsaturated fatty acids, while ethanolamine glycerophosphatide contained greater amounts of elongated shorter chain saturated and monounsaturated fatty acids. These results suggest that fatty acid elongation may play a specific role in fulfilling mitochondrial phospholipid fatty acid requirements.

INTRODUCTION

Each brain phospholipid has a characteristic fatty acid composition and relative proportion of saturated and unsaturated fatty acids (1-4). Fatty acids esterified to position 1 of phospholipids are predominantly saturated, while those esterified to position 2 are generally unsaturated (5,6). This asymmetric distribution of fatty acids may be important for their functional role in membrane physiology (7). The distribution of phospholipid fatty acids suggests that a mechanism exists to maintain the type and position of fatty acid esterification (4). The roles of phospholipase and lysophosphatide acylation enzymes have been implicated in determining the asymmetrical distribution of fatty acids esterified to phospholipids (8,9), but the investigations of Webster (10) do not confirm positional specificity for the acylating enzymes.

To assess the extent and specificity of mitochondrial chain elongation of fatty acids associated with phospholipids in vitro, rat brain mitochondria were incubated in a fortified system containing labeled acetyl Coenzyme A (CoA). The degree of incorporation of the elongated fatty acids into the various complex lipids was determined, as well as the specific fatty acid elongation patterns for the total lipid, free fatty acids (FFA), choline glycerophosphatide (CGP), and ethanolamine glycerophosphatide (EGP).

METHODS

Whole brains from normal heterozygous Gunn rats, 20 days of age, were removed after decapitation. The mitochondria were isolated according to the method of Aeberhard and Menkes (11) and Gray and Whittaker (12). The purity of the preparations was monitored by electron microscopy. Mitochondria protein was determined by the method of Lowry, et al., (13) using bovine serum albumin as the standard.

The incubation medium contained in 0.5 ml (14): 1 mg mitochondrial protein, 1 μmole nicotinamide adenine dinucleotide, reduced form (NADH), 1 μmole nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), 10 μmoles adenosine 5'-triphosphate (ATP), and 34 μmoles acetyl-1-(^{14}C)-CoA. Each sample was incubated at 38 C for 30 min in a Dubnoff shaker (2 Hz) in an atmosphere of N_2. The reaction was stopped by the addition of 19 volumes chloroform:methanol (2:1 v/v) and the lipids extracted. Unincubated control samples also were prepared and extracted immediately.

The lipids were extracted and purified by the technique of Folch-Pi, et al., (15). Portions of the purified total lipid fraction were dissolved in 15 ml diphenyloxazole-diphenyloxazole-benzene (PPO-POPOP) and counted in a Nuclear Chicago mark 1 liquid scintillation counter. The scintillation fluid was made by dissolving 5 g PPO and 300 mg dimethyl POPOP in 1 liter toluene. After incubation, the unincubated control samples incorporated less than 0.05% of the radioactivity added to the incubation medium.

The lipids were isolated and identified by the method of Rouser, et al., (16) and visualized with water vapor. The lipids for radioactive counting were dried and scraped into counting vials containing 15 ml scintillation solution. The CGP, EGP, and FFA fractions were saponi-

TABLE I

Percent Incorporation of Acetyl-1-(^{14}C)-Coenzyme A into Mitochondrial Lipids[a]

Phospholipids + galactolipids	Incubated mitochondria
Origin	0.5 ± 0.3
Serine glycerophosphatide	0.5 ± 0.5
Inositol glycerophosphatide	0.5 ± 0.6
Lyso-ethanolamine glycerophosphatide	10.6 ± 4.9
Sphingomyelin	4.3 ± 3.0
Lyso-choline glycerophosphatide	0.6 ± 0.5
Phosphatidic acid	0.0 ± 0.0
Choline glycerophosphatide	29.2 ± 1.9
Ethanolamine glycerophosphatide	19.7 ± 1.3
Sulfatide + diphosphatidylglycerol	0.8 ± 1.6
Cerebroside	3.5 ± 0.1
Total percent	71.0 ± 4.2
Free fatty acids	29.0 ± 3.1

[a]Rat brain mitochondria were incubated in the system detailed in the text. The lipids were isolated and their content of radioactivity determined by liquid scintillation spectrometry. The picomoles of acetate present and the percentages of radioactivity in each phospholipid were calculated from their specific activities. Mean of four samples ± standard deviation.

fied as described by Aeberhard and Menkes (11) and their fatty acids subjected to the Schmidt reaction (17). The ratio of the radioactivity in the total fatty acid to that in the carboxyl end was 1.0-1.2; 1-(^{14}C)-palmitic acid, which was run simultaneously as a control, gave similar values. These findings indicated that elongation was operational and not de novo synthesis. Lipids for fatty acid analysis were eluted from the silicic acid with chloroform:methanol (2:1 v/v) and washed by the technique of Folch-Pi, et al., (15) to free them from magnesium acetate. Their fatty acids were analyzed as previously described (14).

The total lipid, FFA, CGP, and EGP fractions were subjected to methanolysis. The ester linked fatty acids were methylated with 14% boron trifluoride methanol and purified by thin layer chromatography (TLC) as described by Morrison and Smith (18). Fatty acid separation, identification, and quantitation were performed as described by Yatsu and Moss (14). No attempt was made to isolate alkenyl and alkyl derivatives of EGP.

A Hewlett Packard model 402 gas chromatograph equipped with a flame ionization detector was used in this procedure. Both 6 ft glass columns contained 6% (w/v) diethylene glycol succinate polymer on 80-100 mesh diatoport S. Temperature programing from 150-200 C at a rate of 5 C/min gave a clear separation of peaks for individual fatty acids. Quantitation of fatty acids was achieved by an Infotronics model CRS100 digital integrator. The fatty acid peaks were identified by comparing their retention times relative to standards obtained from Applied Science Laboratories, State College, Pa. In addition, carbon numbers of fatty acids were assessed by hydrogenation, using platinum oxide as the catalyst, followed by rerunning the saturated products by gas liquid chromatography (GLC). Nine separate fatty acid peaks were identified by combined GLC and mass spectroscopy. These fatty acids were: $C_{18:0}$, $C_{18:1}$, $C_{20:0}$, $C_{20:1}$, $C_{20:2}$, $C_{20:3}$, $C_{20:4}$, $C_{22:4}$, and $C_{22:6}$. A stream splitter diverted a portion of the effluent carrier gas and permitted collection of 18 fatty acid fractions in capillary tubes. The methyl esters were washed from the collection tubes with 15 ml scintillation fluid, and the radioactivity was counted as already described. The recovery of radioactivity was ca. 80% on repeated checks of the procedure. Radioactive counts were considered significant when they were three times background. The relative specific activity (RSA) was assessed by dividing the percentage of the total radioactivity collected by the percentage of total fatty acids for each fraction (11).

MATERIALS

NADPH, NADH, and ATP were purchased from Sigma Chemical Co. (St. Louis, Mo.); acetyl-1-(^{14}C)-CoA 58 mCi/mmole and palmityl-1-(^{14}C)-CoA 52.8 mCi/mmole from New England Nuclear Corp. (Boston, Mass.); PPO-POPOP from Packard Instrument Co. (Downers Grove, Ill.); Silica Gel H and Silica Gel G from Brinkmann Instruments (Westbury, N.Y.); 14% boron trifluoride methanol from Applied Science Laboratories; 6% diethylene glycol succinate polymer on 80-100 mesh diatoport S from Hewlett Packard (Avondale, Pa.); and fatty acid standards KD and KF from National Institutes of Health (Bethesda, Md.). All other fatty acid standards used for GLC were obtained from Applied Science Laboratories.

RESULTS

Incorporation of Acetyl-1-(^{14}C)-CoA into Complex Lipids

The incorporation of acetyl-1-(^{14}C)-CoA into the total lipid fraction was 0.38 nmoles/mg protein/30 min. The percent incorporation of acetyl CoA into mitochondrial lipids and free fatty acids is presented in Table I. CGP, EGP, and FFA account for 77.9% of the radioactivity. Their percent activities are, respectively, 29.2, 19.7, and 29.0%.

Qualitative and Quantitative Analysis of Fatty Acids

The results of our investigations on the pattern of fatty acid elongation by acetyl CoA in the total lipid, FFA, CGP, and EGP fractions are presented in Table II. The percent composition of each fraction, its percent radioactivity, and its relative specific activity are shown. The total lipid fatty acid samples were obtained by methanolysis of all mitochondrial fatty acids without prior separation of the lipids. The most abundant fatty acids present are $C_{16:0}$, $C_{18:0}$, $C_{20:4}$, and $C_{22:6}$.

The samples described in the remainder of Table II were obtained by methylation of the fatty acids of individual lipids. The FFA reflects more nearly the pattern of CGP than EGP. The FFA has greater quantities of short chain saturated and monosaturated and less long chain polyunsaturated fatty acids (PUFA). CGP is similar since it also is decreased in long chain unsaturated fatty acids, $C_{22:4}$ and $C_{22:6}$. It contains, however, less $C_{18:0}$ and more $C_{16:0}$ and $C_{20:4}$ than the FFA. CGP and EGP are strikingly different in fatty acid composition. Generally, EGP contains more long chain PUFA, and CGP more of the shorter chain saturated and monosaturated fatty acids. EGP has less $C_{16:0}$, $C_{18:1}$, and $C_{18:2}$ and more $C_{18:0}$ and long chain PUFA ($C_{20:4}$, $C_{22:4}$, and $C_{22:6}$) than the other samples studied.

Patterns of Fatty Acid Chain Elongation

The mitochondrial total lipid fraction demonstrates the greatest incorporation of acetyl CoA into $C_{18:0}$, $C_{20:1}$, $C_{20:2}$, and $C_{22:4}$. These fatty acids represent the elongation of a portion of the more abundant fatty acids, namely $C_{16:0}$, $C_{18:1}$, $C_{18:2}$, and $C_{20:4}$. It is noteworthy that only 4.6% of the radioactivity is in $C_{20:0}$, yet its precursor, $C_{18:0}$, accounts for 18.9% of the fatty acid present. The high relative specific activity of $C_{20:1}$ is misleading due to the error inherent in quantitating a fatty acid representing less than 1% of the total mixture.

The pattern of elongation of FFA is similar to the total lipid fraction. The greatest incorporation of acetyl CoA occurs in the synthesis of $C_{20:1}$, $C_{20:2}$, and $C_{22:4}$. Stearic acid ($C_{18:0}$) in FFA is less than half as radioactive as that in the total lipid, yet the quantity of $C_{16:0}$, its precursor, is more abundant than in the total lipid. Arachidonic acid ($C_{20:4}$) and $C_{22:6}$ are found to a slightly greater extent in the FFA fraction than in the total lipid.

The elongation of CGP fatty acids resembles more closely the pattern found in the total lipid fatty acids than the other samples studied. Generally, the more abundant fatty acids are preferentially elongated. Stearic acid represents 10.0% of the fatty acids, yet is minimally elongated to $C_{20:0}$ which contains only 1.8% of the radioactivity.

EGP differs substantially in its pattern of elongation when compared to CGP. The greatest incorporation of acetyl CoA into EGP fatty acids occurs in the formation of $C_{18:0}$, $C_{18:1}$, $C_{20:1}$, $C_{20:2}$, and $C_{22:0}$. The elongation of $C_{16:0}$ which constitutes only 6.5% of the fatty acid content of EGP, to form $C_{18:0}$, is ca. three times as great as found in the total lipid or CGP and five times that in the FFA. Oleic acid and $C_{20:0}$ contain more radioactivity in EGP than CGP. Although $C_{20:0}$ is present in a smaller quantity in EGP, its elongation product, $C_{22:0}$, is almost five times as radioactive as in CGP. EGP shows a marked decrease in elongation to form $C_{20:2}$, $C_{20:4}$, and $C_{22:4}$ when compared to CGP. Although EGP has almost twice the quantity of $C_{20:4}$ and CGP, its elongation is less than one-quarter that found in CGP. Generally, the elongation of the longer chain PUFA is associated with CGP, while the shorter chain saturated and monosaturated fatty acids are associated with EGP.

DISCUSSION

Incorporation of Acetyl-1(^{14}C)-CoA into Complex Lipids

Phospholipids contain 71% of the radioactivity, while FFA accounts for 29%. Previous reports on mitochondrial fatty acid elongation have shown that elongated fatty acids are incorporated into phospholipids, but the pattern of incorporation has not been described. Quagliariello, et al., (19), using rat liver mitochondria, found 65% of the radioactivity in phospholipids, 29% in FFA, and 5% in cholesterol and neutral lipids. Harlan and Wakil (20) found 73% of the radioactivity in phospholipids, 19.4% in FFA, and 7.7% in cholesterol and neutral lipids. Our results show 71% in phospholipids and 29% in FFA. In contrast, Aeberhard and Menkes (11), using rat brain mitochondria, found only 29.5% of the radioactivity in phospholipids, while FFA had 61.0% and cholesterol and neutral lipids had 9.1%. They believe the low incorporation into phospholipids was due to their anaerobic conditions. Since our incubations were also anaerobic, the discrepancy of results is probably due to other differences in technique.

Qualitative and Quantitative Analysis of Fatty Acids

Bazan (21) found that, following decapitation, the brain FFA content increased from ca.

TABLE II

Fatty Acid Elongation by Rat Brain Mitochondria[a]

Carbon chain length	Total lipid fatty acids			Free fatty acids			Choline glycerophosphatide			Ethanolamine glycerophosphatide			Lyso-ethanolamine glycerophosphatide		
	Fatty acids	Radioactivity	RSA[b]	Fatty acids	Radioactivity	RSA	Fatty acids	Radioactivity	RSA	Fatty acids	Radioactivity	RSA	Fatty acids	Radioactivity	RSA
	Percent total			Percent total			Percent total			Percent total			Percent total		
16:0	16.3 ± 0.1[c]	1.7 ± 0.0	0.10	23.9 ± 2.9	1.9 ± 0.5	0.1	33.2 ± 0.6	2.7 ± 0.6	0.1	6.5 ± 0.2	0.6 ± 0.1	0.1	11.7 ± 2.7	No significant counts	No significant counts
16:0-18:0[d]	0.6 ± 0.2	1.3 ± 0.1	2.17	3.9 ± 1.4	1.6 ± 0.1	0.4	2.5 ± 0.8	1.9 ± 0.1	0.8	3.7 ± 0.4	0.9 ± 0.0	0.2	2.1 ± 1.2		
18:0	18.9 ± 0.6	11.6 ± 0.8	0.61	19.9 ± 0.8	4.7 ± 0.1	0.2	10.0 ± 0.6	10.4 ± 0.3	1.0	24.2 ± 0.5	31.2 ± 0.3	1.3	5.4 ± 0.1		
18:1	24.0 ± 0.2	5.4 ± 0.0	0.23	29.8 ± 1.5	3.6 ± 0.3	0.1	28.9 ± 0.4	6.9 ± 0.6	0.2	15.6 ± 0.2	8.4 ± 0.2	0.5	66.5 ± 4.5		
18:2	3.8 ± 0.1	0.7 ± 0.1	0.18	5.8 ± 0.1	1.3 ± 0.1	0.2	3.5 ± 0.3	1.4 ± 0.2	0.4	2.4 ± 0.1	1.1 ± 0.0	0.5	13.3 ± 0.5		
20:0	NI[e]	4.6 ± 1.8	-	0.6 ± 0.3	3.1 ± 1.0	5.2	0.1 ± 0.0	1.8 ± 0.1	18.0	0.1 ± 0.0	3.6 ± 0.4	36.0	NI		
20:1	0.2 ± 0.0	19.7 ± 0.1	98.50	0.4 ± 0.2	18.2 ± 1.0	45.5	0.5 ± 0.0	24.3 ± 0.1	48.6	0.3 ± 0.1	26.0 ± 2.1	86.7	NI		
20:2	0.4 ± 0.1	11.6 ± 1.2	29.00	0.5 ± 0.2	10.8 ± 0.1	21.6	0.4 ± 0.2	12.0 ± 0.3	30.0	0.4 ± 0.1	6.9 ± 1.1	17.3	0.8 ± 0.0		
22:0	0.2 ± 0.0	3.4 ± 0.7	17.00	1.3 ± 0.4	1.8 ± 0.3	1.4	0.5 ± 0.1	1.3 ± 0.2	2.6	0.2 ± 0.1	8.3 ± 1.2	41.5	NI		
20:4	21.6 ± 1.2	3.5 ± 0.0	0.16	7.5 ± 1.5	5.5 ± 0.8	0.7	13.1 ± 0.4	3.9 ± 0.1	0.3	23.0 ± 0.8	1.5 ± 0.1	0.1	1.2 ± 0.0		
Unidentified	0.1 ± 0.0	5.0 ± 1.8	50.00	2.1 ± 0.9	3.6 ± 0.1	1.7	0.1 ± 0.0	3.0 ± 0.1	30.0	0.1 ± 0.0	1.5 ± 0.3	15.0	0.4 ± 0.0		
22:4	2.8 ± 0.5	20.1 ± 2.1	7.18	0.8 ± 0.1	24.3 ± 1.1	30.4	1.3 ± 0.0	18.7 ± 0.7	14.4	5.4 ± 0.2	4.1 ± 0.3	0.8	NI		
Unidentified	1.8 ± 0.2	2.1 ± 0.3	1.17	NI	3.3 ± 0.4	-	1.2 ± 0.1	1.5 ± 0.2	1.3	3.4 ± 0.3	1.0 ± 0.1	0.3	NI		
Unidentified	NI	2.0 ± 0.3	-	NI	2.4 ± 0.3	-	0.1 ± 0.0	2.0 ± 0.3	20.0	0.5 ± 0.1	1.3 ± 0.5	2.6	NI		
22:6	9.7 ± 0.8	1.0 ± 0.1	0.10	3.1 ± 2.1	3.5 ± 0.6	1.1	4.9 ± 0.7	2.4 ± 0.1	0.5	14.4 ± 0.2	1.1 ± 0.1	0.1	NI		
After 22:6	NI	7.0 ± 0.7		0.7 ± 0.3	10.6 ± 0.5	15.1	0.3 ± 0.0	6.2 ± 0.6	20.7	0.5 ± 0.2	2.8 ± 0.2	5.6	NI		

[a]Rat brain mitochondria were incubated in the system detailed in the text. The fatty acids were isolated and identified by gas liquid chromatography, and their content of radioactivity was measured by liquid scintillation spectrometry.

[b]Ratio of the percentage of radioactivity collected to the percentage of total fatty acids in the sample. RSA = relative specific activity.

[c]Mean value ± standard error of the mean.

[d]This fraction collected between the two fatty acids listed.

[e]NI = peak too small to be integrated.

40 μg to 200 μg/g fresh tissue in 5 min. If the greatest source of FFA is due to a random deacylation of phospholipids, the fatty acid pattern of the total lipid and FFA fractions should be similar. This comparison discloses a similarity in the content of $C_{16:0}$, $C_{18:0}$, $C_{18:2}$, $C_{20:4}$, and $C_{22:6}$. A modest reduction in the quantity of $C_{20:4}$ and $C_{22:6}$ exists, however, in the FFA fraction. A strictly random deacylation process, therefore, does not appear to occur. Saturated fatty acids, such as $C_{18:0}$ are usually esterified to position 1, while PUFA, such as $C_{20:4}$ and $C_{22:6}$, are esterified to position 2 or phospholipids (5). The lower yield of PUFA in the FFA fraction suggests that the release of FFA following decapitation may preferentially affect the 1 position. This is in contrast with Bazan's conclusion (21) that the 2 position is primarily deacylated.

Similar results for the fatty acid distribution of CGP and EGP in rat brain mitochondria were obtained by Biran and Bartley (4). They found more $C_{16:0}$ and less $C_{18:0}$, $C_{20:4}$, and $C_{22:6}$ with CGP, while the reverse was true with EGP. Yabuuchi and O'Brien (5) reported similar results for CGP and EGP fatty acids in bovine grey matter.

Patterns of Fatty Acid Chain Elongation

The most abundant FFA are $C_{16:0}$, $C_{18:0}$, $C_{18:2}$, and $C_{20:4}$, and the greatest radioactivity is found in their 2-carbon elongation products with the exception of $C_{18:0}$. Pollett, et al., (22) proposed two different systems for fatty acid elongation: one producing $C_{18:0}$, the other, longer chain fatty acids. This mechanism might explain the minimal elongation of $C_{18:0}$ despite its high percent composition. An explanation for the labeling of fatty acids associated with phospholipid is acylation from the available FFA pool to lysoglycerophosphatides. A notable difference from the labeling pattern of FFA is seen with CGP and EGP. EGP shows a preponderant labeling of the shorter chain $C_{18:0}$ with 31.2% radioactivity as compared to 10.4% for CGP. Conversely, CGP has a preferential labeling of the longer chain $C_{22:4}$ with 18.7% compared to 4.1% for EGP. These findings suggest a specificity for the fatty acid elongation process and fatty acid requirements of phospholipids. In contradistinction to Webster's (10) conclusion, differential incorporation of elongated fatty acids into CGP and EGP also suggests a specificity for mitochondrial acyl transferase. Specific phospholipid fatty acid patterns may, thus, be maintained by a combination of fatty acid elongation and acyl transferase activity.

ACKNOWLEDGMENTS

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Hepatic Cholesterol Synthesis from Mevalonate and Squalene in Rats: Effect of Feeding Cholesterol Supplemented Diet During Weaning and Following Starvation

RONALD C. JOHNSON and **SHANTILAL N. SHAH**[1], Brain-Behavior Research Center, Sonoma State Hospital, Eldridge, California 95431

ABSTRACT

The conversion of squalene to digitonin precipitable sterols by microsomes and soluble fractions from liver and the incorporation of mevalonate into nonsaponifiable lipids and digitonin precipitable sterols by 5000 g liver supernatant was studied in suckling rats and rats weaned on diet (Purina chow) supplemented with varying levels of cholesterol. The results indicate that the conversion of squalene to digitonin precipitable sterols was low in suckling rats and high in rats weaned on Purina chow diet. Weaning rats on 1% cholesterol supplemented diet effectively eliminated the post-weaning increase in mevalonate incorporation into nonsaponifiable and digitonin precipitable sterols, and in the conversion of squalene to digitonin precipitable sterols. The incorporation of mevalonate into nonsaponifiable lipids and digitonin precipitable sterols, and of squalene into digitonin precipitable sterols also was studied in liver preparations from adult rats which were starved and refed control or cholesterol supplemented diet. It was observed that the conversion of squalene to digitonin precipitable sterols by liver fractions from fasted rats was low, while that by liver fractions from rats refed control diet was higher. Furthermore, the post-fasting increase in the conversion of mevalonate to nonsaponifiable lipids and digitonin precipitable sterols and in squalene to digitonin precipitable sterols conversion essentially was eliminated by refeeding a 1% cholesterol supplemented diet. The low conversion of squalene to digitonin precipitable sterols in suckling rats, rats weaned on cholesterol supplemented diet, and adult rats that were starved, or starved and refed cholesterol supplemented diet, was due to the reduced activity of microsomal enzymes. It is concluded from this study that dietary cholesterol prevents the increase in cholesterol synthesis observed in developing and regenerating liver by suppressing the activities of one or more enzymes between mevalonate and squalene and between squalene and cholesterol.

INTRODUCTION

The synthesis of cholesterol in the liver is sensitive to cholesterol in the diet. It generally is believed that hepatic cholesterol synthesis is regulated primarily at the 3-hydroxy 3-methyl glutaryl coenzyme A (HGGCoA) reductase step; however, results of several investigations (1-4) suggest that cholesterol synthesis in the liver also may be regulated beyond the mevalonic acid (MVA) step. Gould and Swyryd (1) have reported data indicating changes in the conversion of MVA to cholesterol in livers of adult rats fed dietary cholesterol. Slakey, et al., (2) showed a reduction in the conversion of MVA to cholesterol following starvation and increase in synthesis during refeeding of a cholesterol-free diet to adult rats. We recently reported (3) data indicating changes in the conversion of MVA to cholesterol by livers of suckling and weaned rats. Rao and Olsen (4) showed that refeeding adult rats a 5.0% cholesterol supplemented diet results in a marked inhibition of in vivo cholesterol synthesis from MVA. They suggested that dietary cholesterol also inhibits the cyclization of squalene.

In the present study, we have investigated the effect of dietary cholesterol upon sterol synthesis from MVA and squalene by cell-free preparations from livers of weaned rats to determine whether the post-weaning increase in hepatic cholesterol synthesis could be prevented by dietary cholesterol. We also examined hepatic cholesterol synthesis in adult rats which were starved for a period of 72 hr and refed a diet containing varying levels of cholesterol to test if the increase in cholesterol synthesis by preparations from livers of rats that are starved and refed a diet low in cholesterol also could be prevented by dietary cholesterol.

[1]Author to whom correspondence should be addressed.

EXPERIMENTAL PROCEDURES

Materials

DL-[2-^{14}C] MVA dibenzylethylenediamine (DBED) salt was purchased from New England Nuclear Corp., Boston, Mass., and diluted to a specific activity of 2.84 x 10^5 cpm/μmole with unlabeled MVA DBED salt purchased from Calbiochem, San Diego, Calif. [^{14}C] Squalene was prepared biosynthetically from DL-[2-^{14}C] MVA DBED salt by the method of Tchen (5). The biosynthetic squalene was checked for radiochemical purity by thin layer chromatography (TLC) and radioactivity scan. The specific activity of the labeled squalene was determined from the μmoles of MVA incorporated into squalene, as calculated from the amount of [^{14}C] recovered in the squalene fraction, and the specific activity of MVA used for the incubation. The number then was divided by 6 to give the μmoles of squalene synthesized. Total radioactivity in the squalene fraction was divided by μmoles of squalene synthesized, assuming that the amount of endogenous squalene was negligible. The specific activity of the synthesized squalene was found to be 2.52 x 10^4 cpm/μmole. Nictotinamide adenine dinucleotide phosphate reduced form (NADPH), nicotinamide adenine dinucleotide oxidized form (NAD^+), nicotinamide adenine dinucleotide phosphate oxidized form ($NADP^+$), glucose-6-phosphate, and nicotinamide were purchased from Sigma Chemical Co., St. Louis, Mo.; adenosine-5'-triphosphate (ATP) and glucose-6-phosphate dehydrogenase were purchased from Calbiochem, San Diego, Calif.; precoated Silica Gel G plates were purchased from Brinkman, Burlingame, Calif. Cholesterol, U.S.P., was purchased from Sigma Chemical Co. and recrystallized from 95% ethanol before use.

Diets

Diets containing 0.5, 1.0, and 2.0% cholesterol were prepared by dissolving cholesterol in acetone-ether 1:1 and adding to powdered laboratory chow (Ralston Purina, 0.07% cholesterol) and freed of solvent by evaporation (1). Suckling rats were 7 and 16 days postnatal age. Weanling rats were separated from the mother at 20 days after birth and fed lab chow or cholesterol supplemented lab chow. Adults rats, 3-4 months old, were deprived of food for 48-72 hr and refed control or cholesterol supplemented diets for a subsequent 72 hr interval.

Methods

For each experiment, 2-3 animals from the appropriate groups and of appropriate ages were sacrificed between 8-9 a.m. Livers were removed, perfused with saline, blotted dry and weighed, and homogenized in 2.5 volume of 0.1 M phosphate buffer (pH 7.4) containing 0.5 mM dithiothreitol (DTT). In studies with MVA as substrate, a 5000 g supernatant fraction prepared from the homogenate was used for incubation. Microsomal and 105,000 g supernatant (S_{105}) fractions were prepared from liver homogenates by differential centrifugation, washed, as previously described (6), and used for studying the conversion of squalene to sterols. Protein concentrations of 5000 g supernatant and microsomal and S_{105} fractions employed in these studies were determined by the biuret method (7). Incubations with MVA were carried out aerobically for 1 hr after the addition of cofactors: $NADP^+$, 1.2 mM; NAD^+, 0.6 mM; nicotinamide, 30 mM; glucose-6-phosphate, 4.0 mM; 1 unit, glucose-6-phosphate dehydrogenase; ATP, 1.2 mM; magnesium chloride, 5.0 mM; final volume in 0.1 M phosphate buffer (pH 7.4), 1.0 ml. Incubations with [^{14}C] squalene were carried out aerobically for 2 hr employing the same cofactors as used for MVA incubations, except for the substitution of 1.2 mM NADPH for $NADP^+$ and omitting ATP and magnesium chloride.

Labeled squalene was added in 5 μliter dioxane:propylene glycol (2:1) and preincubated with S_{105} for 15 min under N_2 at 37 C. Reactions were initiated by the addition of microsomes. Incubations were terminated after 2 hr by the addition of 15% KOH in 50% ethanol. A reference mixture of authentic unlabeled squalene, lanosterol, and cholesterol was added to each tube, and the contents were saponified for 1 hr at 80 C. Nonsaponifiable lipids (NSF) were extracted with two 5 ml portions of petroleum ether, washed once with water, dried over sodium sulfate, and the solvent was evaporated under a stream of nitrogen. The residue was dissolved in a small volume of chloroform-methanol (9:1), and a one-third aliquot was used for separation of squalene and sterols by TLC. Another one-third aliquot was precipitated by digitonin to estimate radioactivity incorporated into digitonin precipitable sterols (DPS). The samples were spotted on 20 x 20 cm Silica Gel G thin layer plates and were developed in unlined tanks containing benzene-ethyl acetate (95:5). R_f values in this system for squalene, lanosterol, and cholesterol were 0.90, 0.45, and 0.25, respectively. Individual spots corresponding to squalene, lanotsterol, and cholesterol regions were scraped into scintillation vials for determination of the radioactivity content. In all cases,

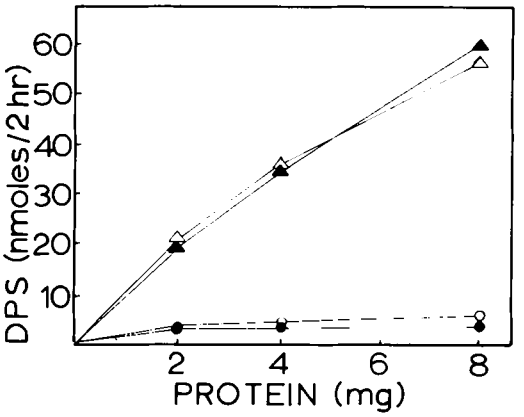

FIG. 1. Incorporation of squalene into digitonin precipitable sterols (DPS) by microsomal (MS) and S_{105} fractions from livers of suckling and weanling rats. Varying levels of total protein (S_{105} + MS, 5:1) were incubated with [^{14}C] squalene (2.5 x 10^4 cpm/μmole, 15,000 cpm); nicotinamide adenine dinucleotide phosphate reduced form, 1.2 mM; nicotinamide adenine dinucleotide, oxidized form, 0.6 mM; nicotinamide, 30 mM; glucose-6-phosphate, 4.0 mM; glucose-6-phosphate dehydrogenase, 1 unit; phosphate buffer (0.1 M, pH 7.4). Reactions were terminated after 2 hr by the addition of 15% KOH in 50% ethanol. Details of the isolation and determination of reaction products are described in the text. Each point is the average of closely agreeing duplicate determinations. ●---● = 7 day old, ○---○ = 16 day old, △---△ = 24 day old, and ▲---▲ = 28 day old.

greater than 90% of the radioactivity recovered in DPS was associated with the C_{27} sterol (cholesterol) region of the TLC plate, as reported earlier (3). Moreover, the combined radioactivity recovered in the lanosterol and cholesterol regions of the thin layer chromatogram agreed well with the amount of [^{14}C] recovered in DPS. It is assumed that the [^{14}C] measured in DPS essentially measures incorporation into cholesterol.

RESULTS

Sterol synthesis from squalene by microsomal and soluble fractions from livers of suckling and weaned rats: The results of our earlier studies on hepatic cholesterol synthesis from MVA by liver preparations from suckling and weaned rats (3) suggested that the conversion of squalene to sterols is low in suckling rats and high in weaned animals. We tested this hypothesis in the present study by incubating microsomal (MS) and S_{105} fractions from livers of suckling and weaned rats with labeled squalene. An S_{105}:MS protein ratio of 5:1 was used throughout this study because we have found that, based upon preliminary observations, this ratio is optimal for microsomal conversion of squalene to sterols. The results in Figure 1 illustrate the conversion of squalene to cholesterol by microsomes, plus S_{105} fractions from suckling (7 and 16 day old) and weaned (24 and 28 day old) rats as a function of protein concentration. It is apparent that there was a significant amount of squalene converted to cholesterol by preparations from weaned rats and that the amounts of squalene incorporated into sterols by suckling rat preparations was negligible. Microsomal and soluble fractions from livers of suckling and weaned rats were cross-mixed and assayed to examine whether

TABLE I

Conversion of Squalene to Sterols by Microsomal and 105,000 g Supernatant Fractions from Livers of Suckling and Weaned Rats[a]

Cell fraction	Digitonin precipitable sterols (nmoles/mg/2 hr)		Cholesterol (nmoles/mg/2 hr)		Lanosterol (nmoles/mg/2 hr)	
	exp 1	exp 2	exp 1	exp 2	exp 1	exp 2
M_1S_1	1.31	0.98	1.22	0.87	0.40	0.16
M_2S_2	1.47	1.24	1.36	1.04	0.36	0.12
M_2S_1	4.22	5.26	4.36	5.02	0.76	0.58
M_2S_2	4.92	5.60	4.88	5.48	0.74	0.44

[a]Microsomes (M) and soluble fractions (S) were prepared from livers of suckling (15 day old experiment (exp) 1; 16 day old, exp 2) and weanling (26 day old, exp 1; 27 day old, exp 2) rats. Each incubation contained 1.5 mg microsomal and 7.5 mg soluble protein and included: [^{14}C] squalene (2.5 x 10^4 cpm/μmole, 15,000 cpm) added in dioxane:propylene glycol, 2:1; nicotinamide adenine dinucleotide phosphate, reduced form, 1.2 mM; nicotinamide adenine dinucleotide oxidized form, 0.6 mM; nicotinamide, 30 mM; glucose-6-phosphate, 4.0 mM; glucose-6-phosphate dehydrogenase, 1 unit; final volume, 1.0 ml phosphate buffer, 0.1 M (pH 7.4). Incubations were carried out for 2 hr and terminated by the addition of 15% KOH in 50% ethanol. Details of the isolation and identification of reaction products are described in the text. Each value is the average of closely agreeing duplicate determinations. M_1, microsomes from suckling rats; M_2, microsomes from weanling rats; S_1, soluble fraction from suckling rats; S_2, soluble fraction from weanling rats.

the alteration in sterol synthesis from squalene is related to reduced activities of microsomal enzymes or is due to a reduction in the activator capacity of soluble fractions. The results in Table I indicate that, when liver microsomes from weaned rats were incubated with liver S_{105} fractions from either weaned or suckling rats, there was a significant conversion of squalene to sterols; however, when microsomes derived from livers of suckling rats were substituted for those from weaned rats, the level of incorporation was 20% of that observed in weaned microsomal preparations.

Sterol synthesis from MVA and squalene by liver preparations from rats weaned on diet containing varying levels of cholesterol: It has been suggested that the post-weaning increase in hepatic sterol synthesis is associated both with a dietary change to a relatively cholesterol-free diet and with the requirement of growing liver for cholesterol. To determine if dietary cholesterol could prevent the post-weaning increase in the activities of enzymes catalyzing MVA incorporation into NSF and conversion of squalene to DPS, we compared hepatic cholesterol synthesis from MVA and squalene in rats weaned on Purina chow and those weaned on the diet supplemented with varying levels of cholesterol, 72 hr after weaning. The amount of MVA converted to NSF and DPS by liver preparations from rats fed control diet increased with increasing protein concentration (Fig. 2), while the amount of MVA incorporated into NSF and DPS by preparations from rats weaned on 1 and 2% cholesterol supplemented diet was minimal at all protein concentrations studied. MVA conversion to NSF and DPS by preparations from livers of rats fed 0.5% cholesterol showed a slight increase with protein concentration. However, the incorporation was significantly lower as compared to the preparations from rats weaned on control diet and was somewhat greater than that exhibited by liver fractions from rats weaned on diets containing 1 and 2% cholesterol. The results in Figure 2 are rearranged in Figure 3 to show the relationship between the levels of dietary cholesterol and the extent of inhibition. The amount of MVA incorporated into NSF was reduced by 57% when animals were weaned on 0.5% cholesterol supplemented diets, while weaning on 1 and 2% cholesterol supplemented diets demonstrated respectively 82 and 92% inhibition of MVA conversion to NSF (Fig. 3A). The incorporation of MVA into sterols (DPS) was inhibited ca. 65, 90, and 98%, respectively, by weaning rats on 0.5, 1, and 2% cholesterol enriched diets (Fig. 3B).

Since the post-weaning increase in hepatic

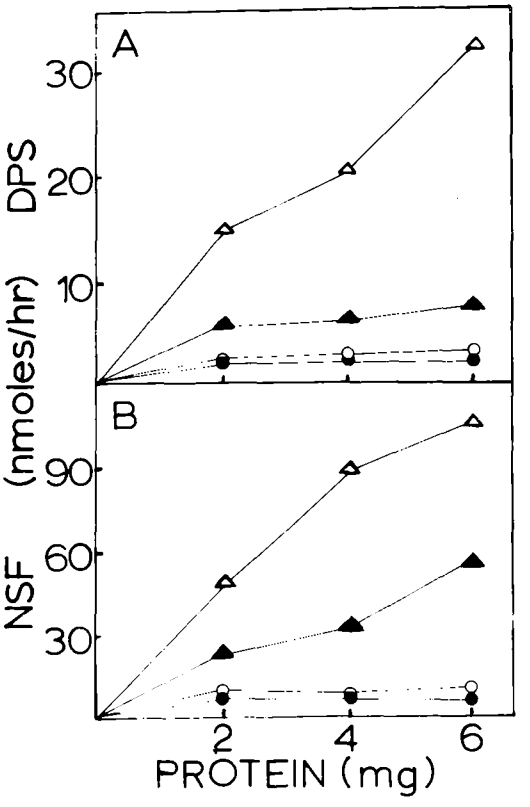

FIG. 2. Effect of dietary cholesterol upon digitonin precipitable sterols (A) and nonsaponifiable lipids (B) formation from mevalonic acid (MVA) in weanling rats. Varying amounts of hepatic 5,000 g supernatant fractions were incubated for 1 hr with labeled MVA (2.8 x 10^5 cpm/μmole, 150,000 cpm); adenosine 5'-triphosphate, 1.5 mM; magnesium chloride, 5.0 mM; nicotinamide adenine dinucleotide oxidized form, 1.0 mM; nicotinamide adenine dinucleotide phosphate, oxidized form, 1.0 mM; glucose-6-phosphate, 10 mM; glucose-6-phosphate, 1 unit, nicotinamide, 30 mM; final volume 1.0 ml. Details of the isolation and determination of reaction products are described in Figure 1 and in the text. △---△ = control, ▲---▲ = 0.5% cholesterol, ○---○ = 1.0% cholesterol, and ●---● = 2.0% cholesterol. Each point represents the average of closely agreeing duplicate determinations.

cholesterol synthesis from MVA essentially was eliminated by feeding a diet supplemented with 1% cholesterol for 72 hr, we investigated the conversion of MVA to NSF and DPS by liver preparations from animals weaned and maintained on a 1% cholesterol supplemented diet over a period of 9 days. Figure 4 indicates that the incorporation of MVA into NSF and DPS by animals weaned on 1% cholesterol supplemented diet was negligible throughout the interval studied.

The effects of dietary cholesterol upon the conversion of squalene to DPS by microsomal

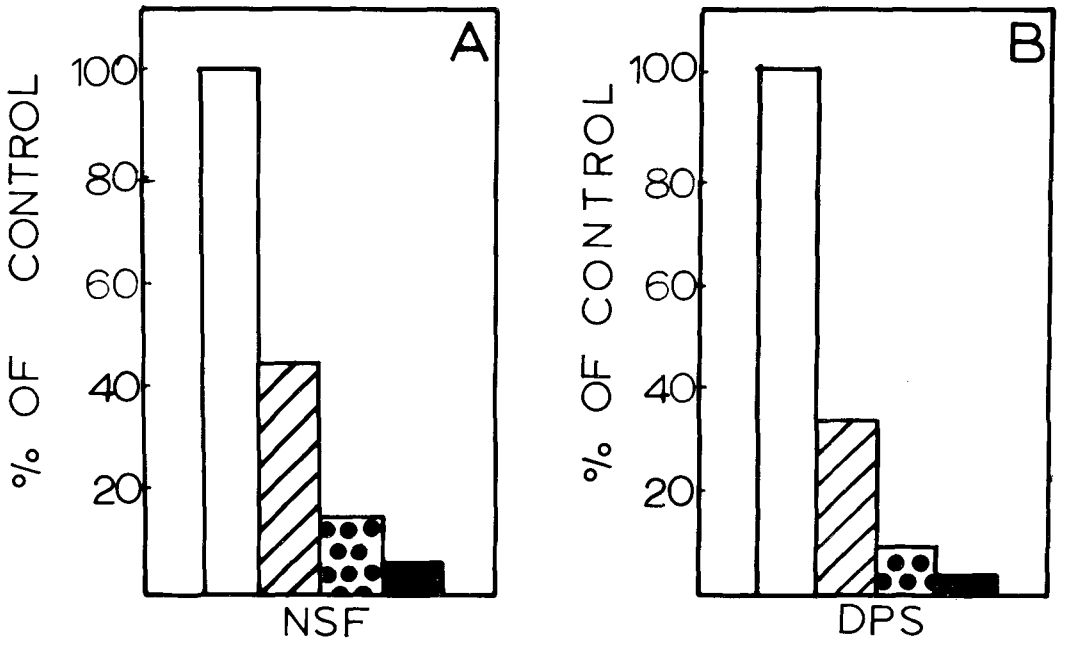

FIG. 3. Effect of dietary cholesterol upon nonsaponifiable lipids (NSF) (A) and digitonin precipitable sterols (DPS) (B) formation from mevalonic acid (MVA) in weaned rats. The data in Figure 2 have been rearranged to illustrate the relationship between the levels of dietary cholesterol and the extent of inhibition. □ = no supplement, ▨ = 0.5% cholesterol, ▩ = 1.0% cholesterol, and ■ = 2.0% cholesterol.

and S_{105} fractions from young rats weaned on control and cholesterol supplemented diets is shown in Table II. It was observed again that increased levels of cholesterol in the diet resulted in sharp decreases in DPS formation

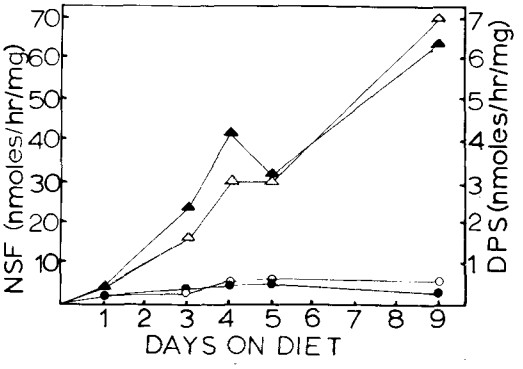

FIG. 4. Effect of prolonged feeding of cholesterol supplemented diet on hepatic cholesterol synthesis in young rats. Animals were weaned on control or 1.0% cholesterol diet for the period indicated and sacrificed. The livers were homogenized and 5000 g supernatant fractions were prepared for incubation with labeled mevalonic acid (MVA), as described in Figure 2 and in the text. Open symbols = digitonin precipitable sterols (DPS) formation, closed symbols = nonsaponifiable lipids (NSF) formation. ▲———▲ and △———△ = no supplement, and ●———● and ○———○ = 1.0% cholesterol.

from squalene. Preparations from rats weaned on 0.5, 1, and 2% cholesterol supplemented diet demonstrated 50, 75, and 80% inhibition respectively, of cholesterol synthesis from squalene. When liver microsomes from rats weaned on control diets were assayed in the presence of liver S_{105} from rats weaned on 0.5, 1, and 2% cholesterol supplemented diets, the conversion of squalene to cholesterol was reduced ca. 20% (Table II). When microsomes derived from livers of rats weaned on 0.5, 1, and 2% cholesterol supplemented diets were incubated with liver S_{105} fractions from control rats, instead of from cholesterol fed rats, only slight increases in the amount of squalene converted to sterols was observed.

Sterol synthesis from MVA and squalene by liver preparations from adult rats which were starved and refed diet containing varying levels of cholesterol: Refeeding a cholesterol-free diet after 48 hr starvation results in increased MVA incorporation into NSF and DPS, with a concomitant increase in liver wt (2). The effect of refeedng a diet containing added cholesterol to rats following starvation was examined to determine if the increased conversion of MVA and DPS is related to an increased need for cholesterol by "regenerating" liver. The results in Figure 5 illustrate that the preparations from rats refed control diet showed increases with

protein concentration in the amounts of MVA incorporated into NSF and DPS and that, at all protein concentrations studied, the fractions from rats refed cholesterol supplemented diets incorporated significantly less amounts of MVA into NSF and DPS. The data in Figure 5 are rearranged in Figure 6 to show percent inhibition vs the level of cholesterol in the diet. NSF formation was inhibited in rats refed 1 and 2% cholesterol enriched diet by 85 and 95%, respectively (Fig. 6A). The 0.5% cholesterol diet had a less pronounced effect upon this conversion (20% inhibition). Sterol formation from MVA (Fig. 6B) was inhibited almost completely (>97%) when rats were refed diets containing 1 and 2% added cholesterol. Moreover, the conversion of MVA to sterols by preparations from rats fed a 0.5% added cholesterol diet also was inhibited greatly (70%).

A decrease in the incorporation of MVA into NSF and DPS in the livers of starved animals has been reported (2). Results in Table III show that microsomes and soluble fractions from adult rats starved for 48 hr converted very little squalene to cholesterol. The conversion by microsomes from starved animals did not increase in the presence of S_{105} from nonstarved controls. The conversion of squalene to DPS by microsomal and soluble fractions from rats which had been starved and were refed diets containing varying levels of cholesterol is shown in Table IV. Preparations from rats refed 1 and 2% cholesterol supplemented diets demonstrated markedly lower (15 and 10% of control) ability to convert squalene to DPS, while in fractions from livers of rats refed 0.5% cholesterol supplemented diet, the conversion of squalene to DPS was 75% of control. When liver microsomes from control rats were assayed in the presence of S_{105} from livers of animals refed cholesterol supplemented diets, there was a slight decrease in squalene conversion to DPS. However, when microsomal fractions from livers of rats refed 0.5%, 1, and 2% cholesterol supplemented diets were substituted for those from control rats, the conversion was the same as that observed when homologous microsomal and soluble fractions were assayed.

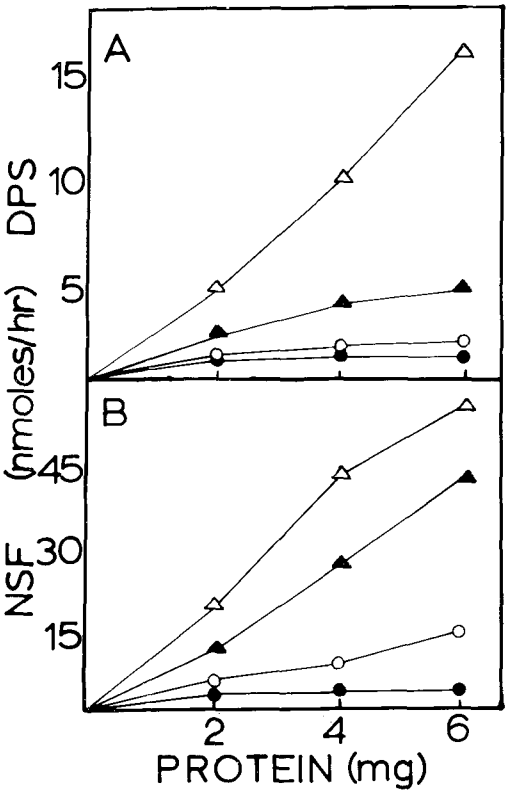

FIG. 5. Effect of dietary cholesterol upon digitonin precipitable sterols (A) and nonsaponifiable lipids (B) formation from mevalonic acid (MVA) in starved, refed adult rats. Animals were sacrificed 3 days after refeeding, and the livers were homogenized. Varying amounts of 5000 g supernatant fraction were incubated with labeled MVA as described in Figure 2 and in the text. △---△ = no supplement, ▲---▲ = 0.5% cholesterol, ○---○ = 1.0% cholesterol, and ●---● = 2.0% cholesterol.

DISCUSSION

The results of the present investigation indicate that: (A) the conversion of squalene to sterols is low in suckling rats and increases rapidly after weaning; (B) the post-weaning increase in the conversion of MVA to NSF (squalene) and squalene to cholesterol is prevented by weaning the animals on a cholesterol supplemented diet; (C) the conversion of squalene to sterols is reduced in starved rats; and (D) adult rats that have been starved and refed a cholesterol supplemented diet demonstrate a significantly diminished ability to convert MVA to NSF (squalene) and squalene to cholesterol, as compared with animals that have been starved and refed a diet low in cholesterol.

The low conversion of squalene to cholesterol by livers of suckling rats and the rapid increase in the rate by preparations from weaned rats is consistent with the observations of our earlier studies with MVA, in which we noted that the ratio of radioactivity recovered in DPS to the radioactivity recovered in NSF was low in preparations from suckling rats and higher in preparations from weaned rats (3). The change in the rate of conversion of squalene to cholesterol in preparations from livers of weaned rats is related to the alteration

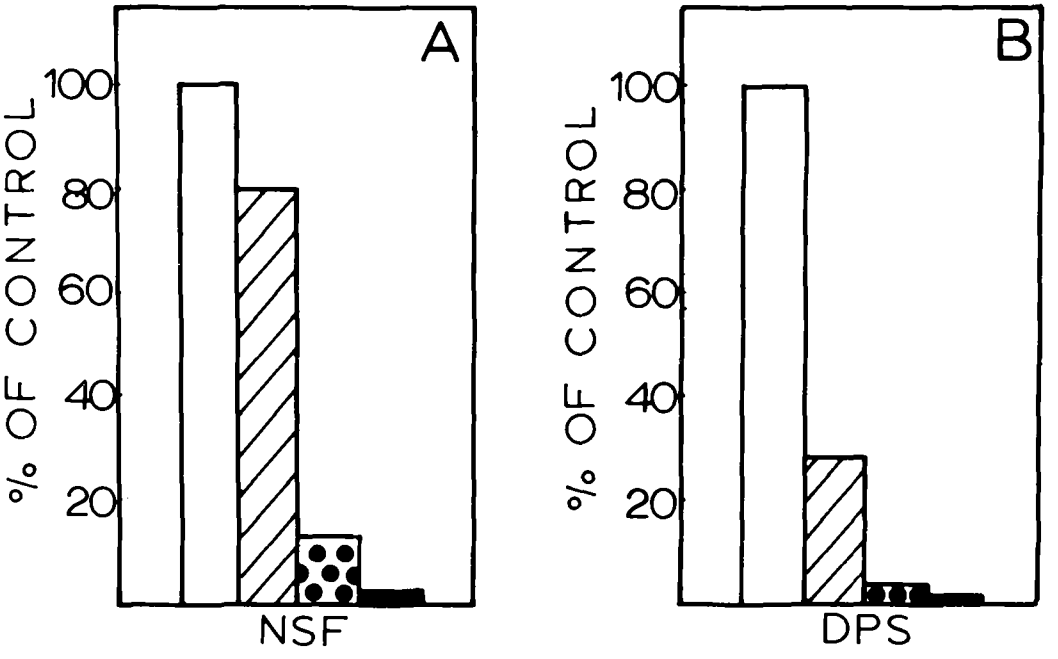

FIG. 6. Effect of dietary cholesterol upon nonsaponifiable lipids (NSF) (A) and digitonin precipitable sterols (DPS) (B) formation from mevalonic acid (MVA) in adult rats which have been starved and refed. The data in Figure 5 have been rearranged and presented here to illustrate the relative effects of refeeding control and cholesterol supplemented diets upon NSF and DPS formation from MVA. □ = no supplement, ▨ = 0.5% cholesterol, ▩ = 1.0% cholesterol, and ■ = 2.0% cholesterol.

TABLE II

Conversion of Squalene to Sterols by Microsomal and 105,000 g Supernatant Fractions from Livers of Rats Weaned on Diet Supplemented with Varying Levels of Cholesterol[a]

Diet of source rats (percent added cholesterol)		Digitonin precipitable sterols formation (nmoles/mg/2 hr)	Cholesterol formation[b] (nmoles/mg/2 hr)	Percent of control
M	S			
0.0	0.0	6.1 (5.9-6.5)	5.8 (5.2-6.2)	100
0.5	0.5	3.2 (3.0-3.3)	3.1 (2.9-3.3)	52.4
1.0	1.0	1.5 (1.2-1.6)	1.5 (1.5-1.7)	24.6
2.0	2.0	1.2 (1.0-1.4)	1.1 (0.9-1.2)	19.7
0.0	0.5	4.9 (4.6-5.2)	4.8 (4.4-5.2)	80.3
0.0	1.0	4.7 (4.3-4.8)	4.9 (4.3-5.2)	78.0
0.0	2.0	4.9 (4.6-5.3)	4.6 (4.0-5.1)	80.3
0.5	0.0	3.4 (3.2-3.6)	3.4 (3.3-3.4)	55.7
1.0	0.0	1.8 (1.2-2.8)	1.6 (1.0-2.3)	29.7
2.0	0.0	1.5 (0.9-2.5)	1.5 (0.8-2.2)	24.5

[a]Microsomes (M) and 105,000 g supernatant (S) fractions were prepared from livers of rats weaned on control or cholesterol supplemented diets, as indicated above. Each incubation mixture contained 1.5 mg microsomal plus 7.5 mg supernatant protein and included [^{14}C] squalene (2.5 x 10^4 cpm/μmole, 15,000 cpm) added in dioxane:propylene glycol (2:1); nicotinamide adenine dinucleotide phosphate, reduced form, 1.2 mM; nicotinamide adenine dinucleotide, oxidized form, 0.6 mM; nicotinamide, 30 mM; glucose-6-phosphate, 4.0 mM; glucose-6-phosphate dehydrogenase, 1 unit; final volume, 1.0 ml. Incubations were carried out for 2 hr. Details of the incubation and determination of radioactive products are described in the text. Values are the means of 2-4 separate experiments performed in duplicate. The range is given in parentheses.

[b]Radioactive product migrating with authentic cholesterol on thin layer chromatographic plate.

TABLE III

Conversion of Squalene to Sterols by Liver Fractions from Starved and Nonstarved Adult Rats[a]

Cell fraction	Digitonin precipitable sterols (nmoles/mg/2 hr)		Cholesterol (nmoles/mg/2 hr)		Lanosterol (nmoles/mg/2 hr)	
	exp 1	exp 2	exp 1	exp 2	exp 1	exp 2
M_1S_1	6.7	6.4	5.7	5.9	0.92	0.62
M_1S_2	5.9	5.8	5.2	5.4	0.68	0.56
M_2S_1	0.26	0.84	0.32	0.74	0.14	0.42
M_2S_2	0.18	0.46	0.12	0.32	0.15	0.23

[a]Microsomes and S_{105} fractions were prepared from livers of rats starved for 48 hr, and from nonstarved rats. Each incubation contained 1.2 mg microsomal plus 6.0 mg soluble protein (experiment [exp] 1) or 1.5 mg microsomal plus 7.5 mg soluble protein (exp 2). Details of the assay are described in Table I and in the text. Values are the means of closely agreeing duplicate determinations. M_1, microsomes from livers of nonstarved rats; M_2, microsomes from livers of starved rats; S_1, soluble fraction from livers of non-starved rats; S_1, soluble fraction from livers of nonstarved rats; S_2, soluble fraction from livers of starved rats.

TABLE IV

Conversion of Squalene to Sterols by Microsomal and 105,000 g Supernatant Fractions from Livers of Adult Rats Starved and Refed Diet Supplemented with Varying Levels of Cholesterol[a]

Diet of source rats (percent added cholesterol)		Digitonin precipitable sterols formation (nmoles/mg/2 hr)	Cholesterol formation[b] (nmoles/mg/2 hr)	Percent of control
M	S			
0.0	0.0	5.8 (5.0-6.1)	5.5 (4.7-6.0)	100
0.5	0.5	4.4 (4.1-4.6)	4.2 (4.0-4.4)	75.9
1.0	1.0	0.9 (0.6-1.8)	0.9 (0.5-1.4)	15.9
2.0	2.0	0.5 (0.4-0.7)	0.5 (0.4-0.6)	9.3
0.0	0.5	5.6 (5.4-5.8)	5.3 (4.9-5.6)	96.6
0.0	1.0	4.7 (4.3-4.8)	4.7 (4.6-5.1)	81.0
0.0	2.0	4.2 (3.9-4.4)	4.0 (3.8-4.1)	72.4
0.5	0.0	4.8 (4.8-4.8)	4.6 (4.3-4.9)	82.8
1.0	0.0	0.9 (0.8-1.3)	0.9 (0.9-1.0)	15.9
2.0	0.0	0.4 (0.2-0.5)	0.4 (0.2-0.5)	6.9

[a]Microsomes (M) and 105,000 g supernatant (S) fractions were prepared from livers of adult rats which had been starved for 72 hr and subsequently refed the control or cholesterol supplemented diets indicated above. Details of the assay are described in Table II and in the text. Values are the means of 2-4 separate experiments performed in duplicate. The range is given in parentheses.

[b]Radioactive product migrating with authentic cholesterol on thin layer chromatographic plate.

in activities of one or more microsomal enzymes. This is demonstrated by the facts that the capacity of microsomes from livers of suckling rats to convert squalene to DPS is low, that the conversion did not increase in the presence of S_{105} fractions from livers of weaned rats, and that microsomes from livers of weaned rats showed a significant conversion of squalene to DPS in the presence of S_{105} fractions from either suckling or weaned rats. Wróbel (8), in his recent study, has reported that the activity of one of these enzymes, namely 7-dehydrocholesterol reductase, is low in liver microsomes from suckling rats and high in weaned rats.

The reduced synthesis of cholesterol in suckling rats and the coincidence of a marked increase in MVA incorporation into NSF and DPS and squalene conversion to DPS by weaned rat preparations, with the change to a low cholesterol diet at the time of weaning, suggest that the low levels of enzymes catalyzing the covnersion of MVA to squalene and of squalene to sterols in livers of suckling rats may be associated with the cholesterol present in the maternal milk. The rapid rise in NSF and sterol synthesis immediately following weaning may, thus, be due to the dietary change to a relatively cholesterol-free diet. This also is supported by the fact that weaning rats on a cholesterol supplemented diet prevents the post-weaning rise both in MVA conversion to

NSF and in squalene conversion to DPS. In addition, the developing liver may require cholesterol for cellular growth. Since, during the time of weaning, liver growth is rapid, the requirement for cholesterol may be high; hence, the sterol synthesis in liver may increase. In the present study, the 0.5% cholesterol supplemented diet partially prevented the post-weaning increase in hepatic cholesterol synthetic capability, while the 1.0% cholesterol supplemented diet essentially eliminated the liver's ability to synthesize cholesterol from MVA and squalene, suggesting that the degree of inhibition of NSF and DPS formation from MVA is related to the level of cholesterol in the diet and that 1.0% cholesterol satisfies the sterol requirement of growing liver.

A reduction in the conversion of MVA to cholesterol following starvation of adult animals and an increase in synthesis during refeeding a cholesterol-free diet have been observed by Slakey, et al. (2). These authors have reported that starvation of adult rats for 48-72 hr results in a significant loss of liver wt and is accompanied by a loss of protein which, upon refeeding, returns to normal within 72 hr. It is reasonable to suggest that liver growth during refeeding is comparable to liver growth during post-weaning. Thus, the increase in MVA incorporation into NSF and DPS during the post-fast inverval may be associated with a cholesterol requirement for the "regenerating" liver. This is consistent with the findings of our present study–that refeeding a cholesterol supplemented diet prevents the post-fasting increase in liver synthesis of sterols from MVA and squalene. Our data that conversion of squalene to sterols is reduced in starved rats are consistent with the observations of Slakey, et al., (2) and those of Inamdar and Ramasarma (9).

The results of the present study clearly indicate that the activities of enzymes involved in the conversion of MVA to squalene and of squalene to cholesterol change during development. It is also apparent that enzymes which normally are suppressed or absent in livers of suckling rats can remain suppressed in livers of rats weaned on cholesterol supplemented diets. Similarly, in adult rats, the suppression or reduction of enzyme activities during starvation can remain suppressed or low by refeeding a cholesterol supplemented diet. These findings suggest that cholesterol biosynthesis in developing and regenerating liver also may be regulated between MVA and squalene and between squalene and cholesterol.

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Ecdysone Metabolism: Ecdysone Dehydrogenase-Isomerase

H.N. NIGG[1], J.A. SVOBODA, M.J. THOMPSON, J.N. KAPLANIS, S.R. DUTKY, and **W.E. ROBBINS,** Insect Physiology Laboratory[2], Beltsville, Maryland 20705

ABSTRACT

An enzyme system that converts α-ecdysone to its hormonally less active 3α-epimer was detected only in the midgut of the tobacco hornworm, *Manduca sexta* (L.). This system appears to be specific for the ecdysones and may represent a metabolic control point for regulating molting hormone activity.

INTRODUCTION

Biochemical studies, both in vivo and in vitro, have shown that conjugation and increased hydroxylation represent major metabolic modifications for the molting hormones in insects (1-6). In addition, oxidation affected by a partially purified enzyme system from *Calliphora vicina* Robineau-Desvoidy recently has been shown to be a means for the metabolic modification of α-ecdysone and 20-hydroxyecdysone to their 3-dehydro derivatives (7). These findings are supported by in vivo studies in *Locusta migratoria* (L.) and *C. vicina* (8,9). We wish to report on yet another in vitro metabolic transformation, the isomerization of α-ecdysone to its 3α-epimer (Fig. 1), and on the partial characterization of the enzyme system responsible for this conversion, its tissue location, and on the occurrence of this system in certain of the developmental stages of the tobacco hornworm, *Manduca sexta* (L.).

EXPERIMENTAL PROCEDURES

Enzyme Assays

Tissues used in these experiments were obtained from tobacco hornworms reared as previously described (10). As an enzyme source, 20 midguts 0-24 hr old tobacco hornworm prepupae were excised, washed with distilled water to remove food contents and the peritrophic membrane, and ground in 15 ml 0.2 M phosphate buffer, pH 7.2, in an all glass tissue grinder. This homogenate was diluted to 50 ml with the same buffer. Incubations contained 5.0 ml enzyme preparation, 3.0 ml phosphate buffer, 1 unit glucose-6-phosphate dehydrogenase, 6.6 x 10^{-3} mmoles glucose-6-phosphate, 3.9 x 10^{-3} mmoles nicotinamide adenine dinucleotide phosphate, oxidized form (NADP), and 50 μg α-ecdysone (in 20 μliter methanol), the final volume being 8.6 ml. All preparations were incubated for 4 hr at 30 C on a Dubnoff metabolic shaker.

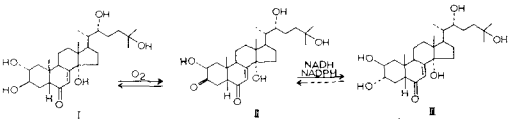

FIG. 1. Proposed metabolic scheme for the production of 3-epiecdysone. I = α-ecdysone; II = 3-dehydroecdysone; and III = 3-epiecdysone. NADH = nicotinamide adenine dinucleotide, reduced form and NADPH = nicotinamide adenine dinucleotide phosphate, reduced form.

Extraction of Products

Each assay was stopped by the addition of 40 ml methanol and centrifuged at 1000 g for 10 min. The methanol was removed, and the precipitate was extracted further with 40 ml methanol and recentrifuged. The methanolic supernatant fluid from five assays was combined, taken to dryness under vacuum, and the ecdysones and metabolites in the residue were extracted, fractionated, and isolated as in the isolation of ecdysones from homogenates of the tobacco hornworm (11).

Instrumentation

UV spectra were obtained with a Bausch and Lomb 505 spectrophotometer, and NMR spectra were recorded at 60 Mc with a Varian A-60A spectrometer using deuterated pyridine as the solvent and tetramethylsilane (TMS) as an internal standard. The mass spectra were obtained by using an LKB model 9000 gas chromatography-mass spectrometer (LKB Produkter AB, Stockholm, Sweden); the samples were introduced directly into the ionization chamber and the ionization energy was 70 ev.

High pressure liquid-solid chromatographic (HPLSC) and thin layer chromatographic (TLC) analyses were performed as previously described (12).

Materials

Corasil II (37-50μ) was purchased from Waters Associates, Framingham, Mass. Zorbax-Sil as a 1/4 m, 2.0 mm inside diameter

[1]NRC-ARS Postdoctoral Associate. Present address: Agricultural Research and Education Center, IFAS, University of Florida, P.O. Box 1088, Lake Alfred, Florida 33850.

[2]ARS, USDA.

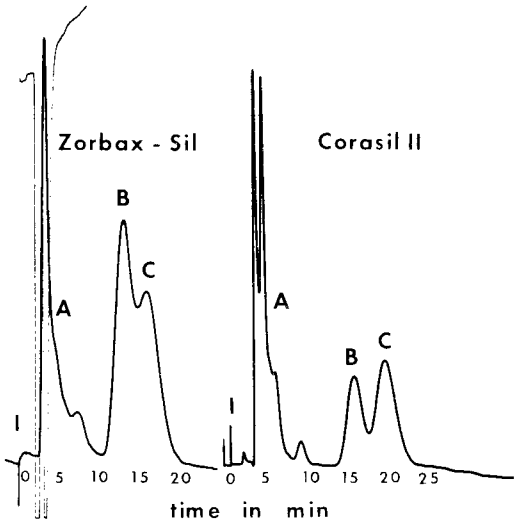

FIG. 2. High pressure liquid chromatography of reaction product. A Dupont model 830 high pressure liquid chromatography was used. $CHCl_3$: 95% ethanol (9:1). Flow rates: Zorbax-Sil, 0.5 ml/min; Corasil II, 1.5 ml/min. UV photometer with a fixed wavelength 254 nm (x 0.08 AU) ambient temperature. Chart speed was 10 min/in. I = injection, A = apolar unknowns, B = 3-epiecdysone, and C = α-ecdysone. Zorbax-Sil: 2.5 μg 3-epiecdysone, 2.5 μg α-ecdysone; Corasil II: 10 μg 3-epiecdysone, 10 μg α-ecdysone. One-tenth of the extraction of 5 combined incubations/injection. Corasil II assays: 3 day old prepupal midguts. Zorbax-Sil assays: 5 day old male pupal midguts. See text for other conditions.

prepacked column was from Dupont Instruments, Wilmington, Del. The glucose-6-phosphate dehydrogenase was Torula Yeast, Type XI, Sigma Chemical Co., St. Louis, Mo., and, unless otherwise indicated, all other biochemicals were from Sigma. 20-Hydroxyecdysone was purchased from Rohto Pharmaceutical Co., Osaka, Japan. The 2β,3α,14α-trihydroxy-5β-cholest-7-en-6-one was prepared by oxidation of cholest-2-en-6-one with peracetic acid and hydrolysis according to the procedure of Marker and Plambeck (13). The diol-ketone then was acetylated and converted to the final product as previously described for the preparation of the 2β,3β,14α-trihydroxy-5β-cholest-7-en-6-one and its 5α-epimer (14). 22-Deoxyecdysone was obtained from the tobacco hornworm (15).

All compounds used as substrates were shown to be chemically pure by TLC, HPLSC, and column chromatographic analyses. The radiochemical purity of 4-^{14}C-cholesterol (Amersham/Searle Corp., Arlington Heights, Ill.) was established by radio TLC on a Packard radiochromatogram scanner and also by column chromatography with liquid scintillation counting on a Packard Tri-Carb liquid scintillation spectrometer. All solvents used for extractions, TLC, HPLSC, and column chromatography were redistilled before use.

TLC analyses utilized Quanta/g precoated silica gel plates without indicator (Quantum Industries, Fairfield, N.J.).

RESULTS AND DISCUSSION

Chromatographic analyses of the partially purified incubation extract indicated an ca. 1:1 mixture of α-ecdysone and a compound that had a shorter retention time than α-ecdysone (Fig. 2). From 100 incubations, we accumulated and purified ca. 500 μg compound, as well as 500 μg unmetabolized α-ecdysone for structural characterization. TLC, HPLSC, and mass spectral analyses established the identity of the unmetabolized α-ecdysone. The molting hormone activity of the unknown ecdysone (mass is based upon UV and HPLSC) was one-fifth to one-tenth that of α-ecdysone in the housefly, *Musca domestica* L., assay (16). It gave an UV absorption maximum in methanol at 245 nm. By TLC analyses with single development in a chloroform-ethanol (4:1) solvent system, it has an R_f of 0.35 (α-ecdysone, R_f 0.31). Its mass spectrum gave a molecular ion peak at m/e 464, and its fragmentation pattern was similar to that of α-ecdysone. The NMR spectrum had methyl resonances at δ0.73 (18-H), 1.07 (19-H), 1.21, 1.30 (21-H), and 1.38 (26- and 27-H), which are identical to those of α-ecdysone and suggest that the environment of the methyl groups is similar to that of α-ecdysone. It was established that this compound was not 22-isoecdysone by comparative analyses using TLC and HPLSC, which showed that the metabolite was far less polar than 22-isoecdysone. The new ecdysone did not react with acetone in the presence of *p*-toluenesulfonic acid, though α-ecdysone under similar conditions readily gave a monoacetonide. Thus, the metabolite may differ from α-ecdysone in having either a 2β,3α- or 2α,3β-diol system. The 2β,3α-diol system was considered the more likely possibility due to the previously shown metabolic changes of the 3β-hydroxyl group of the ecdysones (7-9).

The support for the assignment of a 2β,3α-diol to compound III is as follows: Both synthetic 2β,3α,14α-trihydroxy-5β-cholest-7-en-6-one and the new ecdysone migrate slightly faster than their 3β-epimers upon TLC in a 4:1 chloroform-ethanol solvent system. The isomerization of the new ecdysone with 1% potassium carbonate in 90% methanol at 50 C for 30 min produced a more polar 5α-epimer. Synthetic

2β,3α,14α-trihydroxy-5β-cholest-7-en-6-one is also less polar than its 5α-epimer. On the other hand, α-ecdysone and 2β,3β,14α-trihydroxy-5β-cholest-7-en-6-one are more polar than their 5α-epimers. From the cited observations and results, we conclude that tobacco hornworm midgut homogenate converts α-ecdysone to a new ecdysone, 2β,3α,14α,22R,25-pentahydroxy-5β-cholest-7-en-6-one (3-epiecdysone).

Midgut preparations also were examined for the ability to isomerize other steroidal substrates at the 3 position. From mass spectral, TLC, and HPLSC analyses, 22-deoxyecdysone and 20-hydroxyecdysone are converted readily to their 3α-epimers. However, 22,25-dideoxyecdysone and 4-^{14}C-cholesterol were not altered at the 3 position. These results suggest a substrate requirement for an ecdysone nucleus and a 25-hydroxyl group on the side chain.

Further studies with crude homogenates of midgut tissue and crude homogenates of the combined remaining organs of 60-72 hr old fifth instar larvae, as well as 0-24 hr old prepupae, demonstrated the presence of 3β-ecdysone dehydrogenase-isomerase activity only in the midgut. This enzymic activity was destroyed by heating the midgut preparations at 65 C for 15 min. A series of assays of the isolated individual tissues (integument, blood, fat body, midgut, peritrophic membrane, gut contents, and combined remaining organs) of the same stages also showed activity only in the midgut.

The study of the enzymatic production of 3-epiecdysone was extended to include other stages of development of the tobacco hornworm. Incubation of crude homogenates of first-, second-, and third-instar larvae and of midguts of fourth-instar larvae (24 hr old in each instar) with α-ecdysone all showed production of 3-epiecdysone. Previous studies showed this activity in the midgut of 0-24 and 60-72 hr old fifth-instar larvae, of 0-24, 48, and 72 hr old prepupae and pupae, and, in fact, this activity remains at a substantial level in the 5 day old pupal midgut when this tissue has undergone extensive cytolysis. No production of 3-epiecdysone was demonstrated, however, in the midguts of 1 hr old adult males and females or in 24 hr old eggs.

For subcellular location and cofactor studies, the midgut preparations of 60-72 hr old fifth-instar larvae and a centrifugation routine of 1000 g for 15 min, 10,000 g for 30 min, 26,000 g for 30 min, and 85,000 g for 90 min were utilized. All pellets were resuspended once in buffer and recentrifuged before determinations were made. Only 6% of the total isomerase activity was sedimented in the 1000 g pellet; 94% was located in the 85,000 g supernatant fluid. All other cellular fractions were blank. Dialyzed high speed supernatant fluid preparations and twice washed 1000 g pellets consistently produced two to five times more 3-epiecdysone with nicotinamide adenine dinucleotide, reduced form (NADH), nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), or an NADPH generating system than with nicotinamide adenine dinucleotide, oxidized form (NAD), (NADP) or live enzyme blanks.

This is the first report of the in vitro production of 3-epiecdysone, and, presently, the metabolic significance of this enzymic conversion is unknown. However, our observations that 3-epiecdysone has one-fifth to one-tenth the molting hormone activity of α-ecdysone, plus the apparent specificity of the system that produces 3-epiecdysone, suggest that this conversion represents a metabolic control point for molting hormone activity. A key question, of course, is the relationship of the 3-epiecdysones to the 3-dehydroecdysones of Karlson, et al. (7). Their results plus the results of the present study suggest the relationship in Figure 1. The participation of the 3-keto intermediate in the conversion of α-ecdysone to 3-epiecdysone is not established, although mammalian studies indicate such a relationship exists for mammalian sex hormones (17,18). This also may prove to be the case with the insect molting hormones. Thus, the production of intermediates and α-ecdysone and 20-hydroxyecdysone could well take place through the 3α-epimers.

The production of unidentified apolar metabolites of α-ecdysone and 20-hydroxyecdysone has been reported to occur in several species of insects (19-25). A decision as to whether these apolar metabolites are the 3-dehydro-derivatives of α-ecdysone and 20-hydroxyecdysone or represent their corresponding 3α-epimers or both must await their isolation and identification. However, the 3α-epimers of α-ecdysone and 20-hydroxyecdysone were produced in vitro in the present study, and the 3α-epimer of 20-hydroxyecdysone recently was isolated and identified from the meconium fluid of the tobacco hornworm (26). Thus, these compounds may have hormonal functions of their own.

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Metabolism of Ether Linked Glycerolipids in Dogfish (*Squalus acanthias*) Serum: Evidence for Resistance of Ether Bond to Cleavage in 1-Alkyl-2,3-Diacylglycerols

DONALD C. MALINS and **PAUL A. ROBISCH**, Pioneer Research Unit—Northwest Fisheries Center[1], Seattle, Washington 98112

ABSTRACT

Rac-[1-^{14}C]-palmitylglycerol (chimyl alcohol), rac-[1-^{14}C]-palmityl-2,3-dioleoylglycerol, and rac-palmityl-2,3-[9, 10-^{3}H]-dioleoylglycerol were incubated with dogfish (*Squalus acanthias*) serum for periods of up to 15.0 hr. The ether bond of the carbon 14 labeled chimyl alcohol was cleaved readily, and radioactivity was incorporated into free fatty acids and the acyl chains of the major glycerolipids of the serum. In sharp contrast, the ether bond of the corresponding dioleoyl derivative remained virtually intact. However, the tritium from the acyl chains was incorporated into glycerolipids via intermolecular rearrangements of fatty acids. The findings are consistent with previous findings with rat liver microsomes showing that the ether linkages of alkylglycerolipids are resistant to oxidative cleavage when acyl groups are present on the glycerol moiety. However, substantial differences may exist between the conditions required for oxidative cleavage of the ether linkage of alkylglycerols in mammals and primative fish.

INTRODUCTION

Primative species of fish, such as the dogfish (*Squalus acanthias*) and the ratfish (*Hydrolagus colliei*), are a rich source of the 1-alkyl, 2,3-diacylglycerols (1). In studies on the metabolism of the ether linked lipids from *Squalus* liver, it was shown that [1-^{14}C]-hexadecylglycerol (chimyl alcohol) is cleaved readily (2); however, virtually no information exists on the metabolic fate of the large amounts of the 1-alkyl-2,3-diacylglycerols found throughout the body. It is noteworthy that the ether cleaving enzyme system in rat liver microsomes, which requires both Pte-H_4 (2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine) and molecular oxygen, does not utilize alkyl glycerolipids containing acyl substituents, such as rac-[1-^{14}C]-hexadecyldipalmitoylglycerol (3); however, cleavage occurs with the alkylglycerols, such as chimyl alcohol. Accordingly, it is of interest to determine whether the ether linkage of the 1-alkyl-2,3-diacylglycerols in the marine species is also resistant to cleavage in contrast to the alkylglycerols. Further, it is of interest to determine whether the substituent acyl groups are metabolized readily and whether the resulting fatty acids contribute to the formation of other lipid classes.

In the present work, we incubated rac-[1-^{14}C]-palmitylglycerol (chimyl alcohol), rac-[1-^{14}C]-palmityl-2,3-dioleoyglycerol, and rac-palmityl-2,3-[9, 10-^{3}H]-dioleoylglycerol with the serum of dogfish (*S. acanthias*) which is a rich source of the 1-alkyl-2,3-diacylglycerols, triacylglycerols, and phospholipids (4,5). We found that the ether bond of the carbon 14 labeled chimyl alcohol was cleaved rapidly, whereas the ether bond in the corresponding diester remained virtually intact. Interestingly, the tritium from the acyl chains was incorporated actively into the major glycerolipids, indicating that the fatty acids participated in intermolecular rearrangements.

MATERIALS AND METHODS

Preparation of Radioactively Labeled Alkylglycerols and 1-Alkyl-2,3-Diacylglycerols

Rac-[1-^{14}C]-palmitylglycerol (8.81 x 10^{11} dpm/mmole was prepared from palmitic-[1-^{14}C]-acid, using the method described by Baumann and Mangold (6). The purity of the carbon 14 labeled derivative was checked by thin layer chromatography (TLC) and radioautography, as described by Mangold et al. (7). Rac-[1-^{14}C]-palmityl-2,3-dioleoylglycerol was synthesized by reacting rac-[1-^{14}C]-palmitylglycerol with oleoylchloride in benzene-pyridine solution under reflux conditions for 4.0 hr. The final product was purified by TLC (2, 7-11), and the radiopurity (>99%) was established, as previously described (11). Rac-palmityl-2,3-[9, 10-^{3}H]-dioleoylglycerol (2.78 x 10^{10} dpm/mmole) was synthesized in a similar manner from 1-O-palmitylglycerol (chimyl alcohol) (Hormel Foundation, Austin, Minn. and oleoyl-[9, 10-^{3}H]-chloride. The latter

[1]National Marine Fisheries Service, NOAA, U.S. Department of Commerce.

TABLE I

Distribution of Radioactivity from Rac-[1-^{14}C]-Palmitylglycerol, Rac-[1-^{14}C]-Palmityl-2,3-Dioleoylglycerol, and Rac-Palmityl-2,3-[9, 10-^{3}H]-Dioleoylglycerol among Lipids of *Squalus acanthias* Serum[a]

Radioactively labeled alkylglycerolipids	Lipid class	0.5 hr		5.0 hr		10.0 hr		15.0 hr	
		Total dpm (x 10^3)	Percent administered dose	Total dpm (x 10^3)	Percent administered dose	Total dpm (x 10^3)	Percent administered dose	Total dpm (x 10^3)	Percent administered dose
Rac-[1-^{14}C]-palmitylglycerol	1-Alkyl-2,3-diacylglycerols	2,200	11.0	1,460	7.3				
	Acyl chains	1,110	5.5	600	3.0				
	Triacylglycerols	1,530	7.7	450	2.2				
	Free fatty acids	2,440	12.2	2,360	11.8				
	Phospholipids	390	1.9	308	1.5				
	Acyl chains	380	1.9	292	1.5				
Rac-[1-^{14}C]-palmityl-2,3-dioleoylglycerol	1-Alkyl-2,3-diacylglycerols	18,300	91.6	19,200	96.0	17,900	89.9	18,900	94.9
	Triacylglycerols	159	0.8	202	1.0	162	0.8	154	0.8
	Free fatty acids	Trace	---	Trace	---	Trace	---	Trace	---
	Phospholipids	Trace	---	Trace	---	Trace	---	Trace	---
Rac-palmityl-2,3-[9, 10-^{3}H]-dioleoylglycerol	1-Alkyl-2,3-diacylglycerols	16,800	84.0	12,000	60.0	13,900	69.5		
	Triacylglycerols	963	4.8	1,180	5.9	1,300	6.5		
	Free fatty acids	284	1.4	Trace	---	5	0.1		
	Phospholipids	443	2.2	7,490	37.5	1,220	6.1		

[a]Data normalized to administered dose of 20,000,000 dpm; experimental conditions are given in the text.

derivative was prepared from oleic-[9, 10-^{3}H]-acid (4.44 x 10^{12} dpm/mmole) and oxalyl chloride (Eastman Chemicals, Rochester, N.Y.). Purification by TLC (11) yielded a product of high radiopurity (>99%).

Preparation of Serum

Blood was taken from mature, freshly caught dogfish (*S. acanthias*) obtained from Puget Sound, Wash., during October 1973. The serum was obtained by centrifugation as previously described (5). The serum was fortified with 10 mM adenosine 5'-triphosphate (ATP), 10 mM $MgCl_2$, 0.1 mM coenzyme A, 2.5 mM glutathione, 200 mM sucrose, and 80 mM tris-HCl buffered at pH 7.4, as described in previous studies with *S. acanthias* (5,8). The serum contained 6.6 mg lipid/ml and 39.9 mg protein/ml (12).

Incubation Conditions

The serum (15 ml) was pipetted into 250 ml flasks and placed in a water bath at 14 C. Rac-[1-^{14}C]-palmitylglycerol, rac-[1-^{14}C]-palmityl-2,3-dioleoylglycerol, and rac-palmityl-2,3-[9, 10-^{3}H-dioleoylglycerol each were added to the serum as a suspension in 0.6 ml of 1% Trition X-100. The incubations were carried out under nitrogen with agitation for times ranging from 0.5-15.0 hr. After each incubation the reaction was terminated by rapid freezing in dry ice. Control experiments with serum boiled for 15 min at 100 C indicated that no more than 0.005% of the radioactivity in the specifically labeled compounds was incorporated into other lipid sites under these conditions.

Isolation and Analysis of Lipids

The isolations and characterizations of lipids were undertaken, as described previously (2, 8-11). The composition of the major lipids in the serum was as follows: triacylglycerols (7.9%), 1-alkyl-2,3-diacylglycerols (10.6%), free fatty acids (0.1%), and phospholipids (14.5%). The radioactivity was counted on a Packard Instruments Tri-Carb with counting efficiencies for tritium and carbon 14 at 20 and 55%, respectively.

RESULTS AND DISCUSSION

The present work involves a preliminary study of the metabolism of three radioactively labeled glycerolipids in the serum of *S. acanthias*: rac-[1-^{14}C]-palmitylglycerol, rac-[1-^{14}C]-palmityl-2,3-dioleoylglycerol, and rac-palmityl-2,3-[9, 10-^{3}H]-dioleoylglycerol. The fate of the radioactively labeled hydrocarbon chains was monitored with respect to their

incorporation into the glycerolipid fractions of the serum.

Rac-[1-^{14}C]-Palmitylglycerol

The ether bonds of alkylglycerols are cleaved readily in a variety of tissues (13), including *S. acanthias* liver (2). The mechanisms governing this cleavage, which account for the formation of aldehyde and ultimately acid, were described by Tietz, et al., (14) using a rat liver microsomal system. In the present work, substantial amounts of radioactivity from rac-[1-^{14}C]-palmitylglycerol appeared in free fatty acids and acyl chains of the major lipid classes of the serum (Table I). In 0.5 hr, 27.3% of the carbon 14 in the original rac-[1-^{14}C]-palmitylglycerol was incorporated into free fatty acid and acyl chains of triacylglycerols, 1-alkyl-2,3-diacylglycerols, and phospholipids. In both the 0.5 and 5.0 hr incubations, free fatty acid contained 11-12% of the carbon 14 in the original rac-[1-^{14}C]-palmitylglycerol, respectively. Accordingly, the findings indicate that alkylglycerols are cleaved extensively in *S. acanthias* serum yielding free fatty acids. These fatty acids serve as active precursors of the acyl chains of glycerolipids, particularly the triacylglycerols and 1-alkyl-2,3-diacylglycerols (Table I). The levels of alkylglycerols are low in the serum (less than 0.1% of the total lipid) (D.C. Malins and P.A. Robisch, unpublished results); however, their active catabolism suggests that their role in the formation of other lipids may be quite important.

Rac-[1-^{14}C]-Palmityl-2,3-Dioleoylglycerol

The fact that rac-[1-^{14}C]-palmitylglycerol was metabolized readily led us to investigate whether the 1-alkyl-2,3-diacylglycerols, which often comprise 10-30% of the total serum lipid (4), are metabolized under similar conditions through cleavage of the ether linkage. Rac-[1-^{14}C]-palmityl-2,3-dioleoylglycerol was incubated with serum under identical conditions to those employed with the carbon 14 labeled chimyl alcohol. The O-alkyl moieties remained virtually intact in incubations ranging from 0.5-15.0 hr, as indicated by the fact that, unlike the study with chimyl alcohol, the carbon 14 was not incorporated into other glycerolipid fractions to a significant extent (Table I), i.e. essentially all the carbon 14 was recovered as 1-alkyl-2,3-diacylglycerols. The data reveal, therefore, that the ether linkages of the diester derivatives of alkylglycerols are essentially metabolically inert under conditions which favor their oxidation in the alkylglycerols themselves. Thus, it appears that the catabolism of the O-alkyl moieties in *S. acanthias* serum takes place via the alkylglycerols or through a partially esterified neutral or phosphoglyceride structure.

The present findings afford an explanation for previous results obtained on fatty acid incorporations into the glycerolipids of *S. acanthias* liver (2,9,10,15). In studies with precursors, such as palmitic-[1-^{14}C]-acid, poor incorporation of carbon 14 into O-alkyl chains of 1-alkyl-2,3-diacylglycerols was obtained, whereas a substantial amount of the carbon 14 was incorporated into the acyl chains of these lipids, as well as into the triacylglycerols and phospholipids. Accordingly, it seems that, although oxidative cleavage of the ether bonds in 1-alkyl-2,3-diacylglycerols only occurs to a negligible degree, inter- and intramolecular rearrangements of the fatty acids take place to a significant extent (5).

It was shown that neoplasms containing high levels of the 1-alkyl-2,3-diacylglycerols do not have an active ether cleaving system (16). An interesting observation with the 7777 Morris hepatoma was the finding that the tissues from which the hepatoma was derived originally contained an active alkyl ether cleavage enzyme (17). The present findings point to the fact that such enzymes also may be inactive with respect to large pools of ether diesters that are found in sharks and other primative species, such as the ratfish (*H. colliei*) (1).

Rac-Palmityl-2,3-[9, 10-^{3}H]-Dioleoylglycerol

1,3-[9, 10-^{3}H]-Dioleoyl-2-palmitin is metabolized actively in the serum of *S. acanthias*, as previously described (5). The tritium labeled acids of this glyceride are incorporated preferentially into the acyl chains of the 1-alkyl-2,3-diacylglycerols at position 3 (5). These conversions, which appear to take place via acyl transfer reactions, raise the question whether the acids of the ether diesters are incorporated actively into the triacylglycerols and other glycerolipids of the serum.

The data (Table I) show that a substantial amount of tritium was, in fact, incorporated into triacylglycerols, phospholipids, and free fatty acids. Accordingly, it appears that the fatty acids of the 1-alkyl-2,3-diacylglycerols contribute significantly to the formation of other glycerolipids of the serum, whereas the alcohol portion of the molecule remains virtually unreactive.

The overall findings of the present work are consistent with those obtained with alkylglycerolipids in rat liver microsomes (3,18). In this system, substantial cleavage activity occurs with rac-[1-^{14}C]-hexadecylglycerol between pH 6.5 and 9.5 with maximum cleavage at pH 9 (18).

The present initial study (pH 7.4) did not include an examination of the influence of the conditions, e.g. O_2 requirements and cofactors required for oxidative cleavage of the ether linkage in rat liver microsomes (3,18). The fact that the ether linkage of chimyl alcohol was oxidized to a significant degree in our investigation in the absence of added cofactors suggests that substantial differences may exist between the mammalian enzyme system and those of the primitive fish, such as *S. acanthias*.

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Lipids of Cultured Hepatoma Cells: IV. Effect of Serum and Lipid upon Cellular and Media Neutral Lipids

RANDALL WOOD and **JOHN FALCH**, Division of Gastroenterology, Departments of Medicine and Biochemistry, University of Missouri School of Medicine, Columbia, Missouri 65201

ABSTRACT

Minimal deviation hepatoma 7288C cells were cultured in a modified Swim's medium supplemented with decreasing levels of serum, lipid-free serum, lipid-free serum plus fatty acids, and other additives. Cellular and media neutral lipid classes were quantitated, the fatty acids of triglycerides and sterol esters analyzed, and the carbon number distribution of triglycerides determined. Cellular triglyceride biosynthesis virtually was inhibited when the medium was supplemented with bovine serum alone. This inhibition was not observed when the medium was supplemented with fetal calf serum alone or mixtures of fetal calf serum and bovine serum. Cells cultivated on medium supplemented with lipid-free serum plus palmitic or linoleic acids had much lower levels of free and esterified cholesterol. The fatty acid composition of cellular triglycerides and cholesterol esters differed dramatically from the corresponding media lipid classes. Except when linoleic acid was added to the medium, changes in the media serum and lipid levels had only marginal effects upon the fatty acid composition of cellular triglycerides and cholesterol esters. These data, in conjunction with earlier data that showed the media neutral lipid levels did not decrease during cell growth, indicate that these hepatoma cells utilize little or no serum triglycerides and cholesterol esters. Linoleic acid added to the medium dramatically reduced the level of 18:1 acids in cellular triglycerides and cholesterol esters. Palmitic acid added to the medium did not change the fatty acid compositions significantly. Comparison of experimentally determined and calculated triglyceride carbon number percentages indicated a random distribution of fatty acids in this glyceride. The fatty acid composition of cellular triglycerides was similar to the composition of the cholesterol esters. The lack of characteristic and distinguishable compositions of these two classes that occur in most normal tissues suggests a loss of specificity in the lipid metabolism of this neoplasm at the class level.

INTRODUCTION

The importance of lipids and the advantages of using cultured minimal deviation hepatoma cells to study the changes that occur in the lipid metabolism of neoplasms has been discussed previously (1). Earlier studies of this series described the qualitative changes that occurred in cellular and media lipids and the quantitative changes observed in individual phospholipid class fatty acids from hepatoma cells cultured on media containing decreasing amounts of serum, lipid-free serum, and lipid-free serum containing added fatty acids (1,2). This report describes the quantitative analysis of the media and cellular neutral lipid classes and the detailed examination of the triglycerides and sterol esters from those experiments.

EXPERIMENTAL PROCEDURES

Minimal deviation hepatoma 7288C cells (HTC) were grown in roller cultures as monolayers on a modified Swim's 77 medium supplemented with various levels of serums and lipids (Table I), as previously described (1). Lipids were extracted from cells and media and separated into neutral lipid and polar lipid fractions (1).

The quantitation of the neutral lipid classes was determined by high temperature gas liquid chromatography (GLC) of the intact neutral lipid fraction, as described by Kuksis and colleagues (3,4). Modifications of the method and equipment that have proved advantageous in our hands are described. Neutral lipid aliquots (50-200 μg) were hydrogenated (5), diluted in 50 μliter N, O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) in a chromatography tube (Kontes), heated on the injector port of the chromatograph until the BSTFA refluxed, and an aliquot of the BSTFA solution analyzed. The rapid build-up of silicon on the detector of the instrument can be reduced greatly by evaporating the BSTFA and using chloroform as a solvent. A standard consisting of known quantities of free fatty acid, cholesterol, diglyceride, cholesterol ester, and triglyceride was analyzed daily to obtain a detector response factor for each lipid class, necessary for the quantitative

TABLE I

Media Containing Various Levels of Serum and Lipid Used To Culture Hepatoma Cells

Medium	Swim's 77 medium modifications			
	Percent bovine serum	Percent fetal calf serum	Percent lipid-free serum[a]	Other additions or deletions
A	20	5		
B	10	5		
C	5	5		
D		5		
E	5			
F		2.5		
G			5	
H			5	+ Palmitic acid (37.5 μg/ml)
I			5	+ Linoleic acid (37.5 μg/ml)
J		5		- Dextrose + Maltose
K	5	5		+ Sterculia foetida oil (50 μg/ml)
L	5	5		+ Insulin (1 unit/ml)

[a]Fetal calf serum.

calculations. Analyses were made on an Aerograph model 2100 gas chromatograph modified to accept a 3/16 in. column. A 75 cm x 4 mm (2.5 mm, inside diameter) glass column packed with 1% OV-17 coated on 100-120 mesh Gas Chrom-Q was fitted to the instrument with Pyrex to Kovar seals (6,7). Operating parameters were similar to those used previously (8). Peak areas were quantitated with a digital integrator.

Triglycerides and sterol esters were resolved by thin layer chromatography (TLC) on adsorbent layers of Silica Gel G developed in a solvent system of hexane-diethyl ether-acetic acid 80:20:1 (v/v/v). Resolved lipid bands were located by viewing chromatoplates sprayed with Rhodamine 6G under UV light. Adsorbent layers containing triglycerides or sterol esters were scraped directly into teflon lined screw cap culture tubes (16 x 100 mm) and converted to methyl esters by heating in a boiling water bath with 3 ml 2% sulfuric acid in anhydrous methanol. After 2-6 hr, an equal volume of water was added, the sulfuric acid neutralized with excess ammonium hydroxide and the methyl esters extracted thrice with hexane. Methyl esters were analyzed and quantitated on the same instrument using 180 cm x 2 mm (inside diameter) pyrex columns packed with 10% EGSS-X coated on 100-120 mesh Gas Chrom-P. Column oven temperature was programed 140-200 C at 2 C/min. Identities of fatty acid methyl esters were based upon analysis before and after hydrogenation and cochromatography with standards.

The source and quality of standards, solvents, reagents, etc., were the same as given previously (1).

RESULTS

Cell Growth

The growth rate and quantity of lipids obtained from HTC cells cultured on media A through I (Table I) have been described previously (1). The substitution of maltose for glucose (medium J) increased the doubling time to 4.2 days. The growth that did occur probably resulted from nonenzymatic hydrolysis of maltose and the glucose (0.56 mole %) contaminating the maltose. The poorly growing cells contained 47 and 32 $\mu g/10^6$ cells of phospholipid and neutral lipid, respectively, higher lipid levels than those cells growing more rapidly, as noted previously (1). The addition of insulin and sterculia foetida oil to medium containing 10% serum (media K & L, Table I) increased the cell doubling time moderately (1.8 and 2.3 days) when compared to media supplemented with only 10% serum (1). The quantity of lipid/million cells cultured on media K and L was ca. the same as medium C.

Quantitation of Neutral Lipid Classes

The percentage distribution of lipid classes in the neutral lipid fraction of cells grown on the various media is given in Table II. Free sterol, shown to be cholesterol by GLC and TLC analyses, and triglycerides were the major neutral lipids under most growth conditions.

TABLE II

Quantitative Distribution of Neutral Lipid Classes Derived from Minimal Deviation Hepatoma 7288C Cells and Media After Growth on Variety of Media

	Neutral lipid class percentages								
	HTC cells					Media[a]			
Medium[b]	Diglycerides	Free sterols	Free fatty acids	Triglycerides	Sterol esters	Free sterols	Free fatty acids	Triglycerides	Sterol esters
A	2.2	29.9	1.3	55.0	11.6	6.5	0.2	3.2	90.2
B	2.0	29.2	3.2	51.8	13.8	8.4	0.2	4.4	87.0
C	2.5	24.5	1.1	62.7	9.2	10.0	1.0	0.6	88.4
D	T[c]	27.5	T	60.8	11.7	15.0	1.6	1.3	82.1
E	4.7	43.0	21.1	8.6	22.5	9.1	2.4	0.4	88.1
F	3.5	40.5	6.3	40.1	9.7	13.7	7.6	1.0	77.6
G	11.5	22.8	27.3	23.1	15.3	18.5	34.9	1.3	45.3
H	13.4	10.2	30.0	42.8	2.6	19.3	8.2	1.1	72.0
I	10.6	17.9	16.1	52.1	3.4	8.8	67.4	4.2	19.6
J	6.7	24.6	8.7	44.9	15.2	12.0	2.6	2.3	83.1
K	9.6	26.2	16.4	40.5	7.3	10.3	0.4	0.2	89.1
L	12.0	25.9	18.0	38.4	5.5	9.3	0.3	1.2	89.2

[a]Analyses were performed on media collected from the last half of the growth period.

[b]Refer to Table I for complete description of growth media.

[c]T denotes detectable amounts less than 0.5%.

TABLE III

Fatty Acid Composition of Triglycerides and Sterol Esters Derived from Minimal Deviation Hepatoma 7288C Cells Cultured on Variety of Media

	Fatty acid percentages[a]														
	Triglycerides								Sterol esters						
Medium[b]	14:0	16:0	16:1	18:0	18:1	18:2	20:1	20:4	>20:4	14:0	16:0	16:1	18:0	18:1	18:2	20:1	20:4
A	0.8	7.6	6.3	6.0	63.5	8.2	4.5	1.4	0.9	1.1	12.9	8.3	7.5	57.5	10.3	2.4	T
B	1.4	9.3	7.2	5.6	60.5	7.6	6.0	0.7	1.8	3.2	11.2	9.1	5.1	56.2	9.3	5.9	T
C	1.6	10.7	8.9	5.1	62.9	4.7	4.4	0.9	T	4.9	13.8	10.3	7.5	49.2	2.3	8.8	2.0
D	1.9	10.9	8.4	5.4	65.2	2.0	4.7	1.0	T	3.4	15.6	9.2	7.9	59.6	T	6.4	T
E	1.2	8.7	6.3	5.9	62.8	8.5	4.9	1.5	T	2.0	10.4	7.8	3.6	63.5	11.1	1.5	1.5
F	1.5	7.5	10.6	3.5	70.2	2.2	4.4	T	T	2.1	10.5	12.1	4.5	63.2	1.3	6.3	T
G	1.9	9.3	8.7	4.4	71.6	T	4.1	T		3.6	8.0	10.4	3.0	65.8	1.3	4.8	T
H	1.3	10.1	7.2	9.3	62.2	4.4	3.3	1.0	0.4	2.6	19.9	11.6	10.1	53.8	1.8	T	T
I	1.4	7.7	3.3	5.1	16.4	50.2	0.6	5.1	3.0	2.1	16.0	7.9	6.0	22.1	34.7	T	6.8
J	1.8	11.4	4.7	6.7	61.6	5.1	3.8	1.5	2.4	4.0	11.8	6.6	5.9	53.9	3.3	6.3	2.1
K	1.9	10.6	4.7	8.2	59.9	5.9	5.3	1.3	0.9	2.5	13.6	5.5	7.9	58.4	5.9	4.6	1.5
L	1.6	8.9	6.2	5.2	62.2	5.3	5.7	0.8	T	3.3	14.9	6.8	6.4	59.1	4.7	4.8	1.5

[a]Percentages represent the mean of duplicate analysis of a single sample.
[b]Refer to Table I for complete description of growth media.

The removal of bovine serum from the medium had little effect upon the cellular neutral lipid composition (Table II); however, a change was observed when fetal calf serum was decreased 5-2.5% or by the use of lipid-free serum. Cells grown on medium containing only bovine serum showed more than an 85% reduction in triglycerides; this reduction resulted in an apparent rise in the level of the other classes. Cells grown on medium containing lipid-free serum contained elevated percentages of free fatty acids and diglycerides and decreased amounts of triglyceride. The addition of free fatty acids to medium containing lipid-free serum resulted in a decrease of cellular cholesterol and cholesterol esters. The replacement of dextrose with maltose, addition of sterculia foetida oil (triglycerides), and the addition of insulin to media containing serum had little effect upon the hepatoma neutral lipid composition, except for a moderate increase in free fatty acids.

The netural lipid composition of the media from the last half of the incubation period also is given in Table II. Sterol ester, the major neutral lipid of bovine and fetal calf serums, remained high in the media lipids. The growth medium obtained from cells grown in medium prepared with lipid-free serum contained elevated percentages of free sterol and free fatty acids and a decreased percentage of sterol ester. The addition of linoleic acid to medium containing lipid-free serum reduced the percentage of sterol ester growth as was observed in the cells. The increase in free fatty acids of this growth medium, probably resulted from unmetabolized linoleic acid, accounted for some of the apparent decrease in sterol ester percentage. Triglycerides, the major cell neutral lipid class, were present only in minor amounts in all the media after cell growth, regardless of media supplementation.

Cellular Triglycerides and Cholesterol Esters

The fatty acid composition of triglycerides and sterol esters derived from cells grown on various media is compared in Table III. Remarkably, the composition of the triglycerides remained relatively unchanged, except for two or three media. Linoleic acid replaced 18:1 in the triglycerides from cells cultured on medium supplemented with lipid-free serum plus linoleic acid. The other significant change observed was the increase of octadecenoic acids in cells cultured on medium supplemented with lipid-free serum or 2.5% fetal calf serum.

Cellular sterol esters, shown to be cholesterol esters by GLC and TLC, exhibited only slight changes in fatty acid composition, except

TABLE IV

Calculated and Determined Carbon Number Distribution of Triglycerides Derived from Minimal Deviation Hepatoma 7288C Cells Cultured on Variety of Media

Medium[b]	Origin of data[c]	Carbon number percentages[a]						
		48	50	52	54	56	58	60
A	Deter.	0.2	9.6	26.7	45.8	13.0	3.4	1.3
	Calc.	0.9	6.5	26.2	51.5	11.8	2.7	T
B	Deter.	2.8	14.3	28.7	36.8	12.2	4.2	1.0
	Calc.	1.1	7.4	27.2	45.8	13.2	4.5	0.7
C	Deter.	3.0	15.3	31.9	38.1	8.9	2.1	0.7
	Calc.	1.7	10.4	32.8	43.4	9.0	2.2	T
D	Deter.	3.8	17.3	31.6	34.5	9.0	2.4	0.9
	Calc.	2.4	11.2	31.6	43.9	9.8	0.8	0
E	Deter.		19.0	23.4	37.6	12.6	4.6	2.8
	Calc.	1.0	7.0	27.5	50.5	12.7	1.1	0
F	Deter.	1.5	12.2	28.5	40.0	12.8	3.8	1.2
	Calc.	2.2	10.9	31.9	47.2	7.0	T	0
G	Deter.		12.6	31.0	42.8	10.4	2.6	0.5
	Calc.	2.2	10.9	31.9	47.2	7.0	T	0
H	Deter.	0.8	11.4	31.0	43.5	9.1	2.3	2.0
	Calc.	1.3	8.4	30.1	47.9	9.6	2.4	T
I	Deter.	1.4	6.5	19.6	40.7	22.1	7.6	2.3
	Calc.	T	4.2	18.2	43.8	22.2	8.6	1.9
J	Deter.	1.4	14.8	28.2	38.2	11.4	4.8	1.1
	Calc.	1.8	8.9	26.6	43.6	11.9	5.8	0.8
K	Deter.	1.6	11.6	26.5	41.0	12.5	4.4	1.9
	Calc.	1.7	8.4	25.8	45.5	13.1	4.5	0.7
L	Deter.	1.1	10.6	27.6	42.1	13.0	4.1	1.4
	Calc.	1.6	8.2	25.1	43.8	12.7	4.4	0.7

[a]A carbon number represents the sum of carbon atoms in the three fatty acids esterified to glycerol.

[b]Refer to Table I for complete description of growth media.

[c]Deter. = Determined and Calc. = Calculated. The calculated values are for a random distribution of fatty acids among all three glycerol positions. Only the quantity of C-16, C-18, etc., acids (Table III) and the number of combinations of acids to give a particular carbon number dictate the value of the calculated carbon percentages.

when cells were grown on medium supplemented with lipid-free serum plus linoleic acid. Linoleic acid replaced 18:1 when linoleic acid was added to the medium. Cholesterol esters from cells grown on medium supplemented with only bovine serum contained the next highest percentage of linoleic acid. The percentage of linoleic acid in the cholesterol esters declined as the level of bovine serum in the media was reduced. Generally, the percentage of monoenoic acids increased as the level of serum in the medium decreased.

Determined triglyceride carbon number percentages are compared with calculated percentages for all growth conditions in Table IV. The calculated random distribution percentages generally agreed very well with the determined values. The most notable exception was triglycerides from cells grown on medium supplemented with only bovine serum. Some moderate differences also were noted in carbon numbers 50 and 54 when bovine serum supplemented the media at the 10 and 20% levels.

Media Triglycerides and Sterol Esters

Triglycerides derived from growth media after harvest of HTC cells differed dramatically in fatty acid composition (Table V) from the cellular triglyceride composition (Table III). Palmitate and octadecenoate percentages were ca. equal, whereas 18:1 concentrations were 6 times greater than 16:0 percentages in cellular triglycerides. Media triglycerides contained a high level of linoleic acid when free linoleic acid was added to the medium. Generally, the composition of the harvest media triglycerides agreed very well with the fatty acid composition of triglycerides derived from bovine and fetal calf serums shown at the bottom of Table V.

The fatty acid composition of sterol esters derived from media after the growth of HTC cells is shown in Table V along with the composition of sterol esters from bovine and fetal calf serums. When the media contained bovine serum in any proportion, the sterol esters exhibited a high percentage of linoleic acid similar to bovine serum sterol esters. The composition

TABLE V

Fatty Acid Composition of Triglycerides and Sterol Esters Derived from the Growth Media after Cell Cultivation and That of Serums

	Fatty acid percentages[a,b]															
	Triglycerides								Sterol esters							
Medium[c]	14:0	16:0	16:1	18:0	18:1	18:2	20:0	20:4	14:0	16:0	16:1	18:0	18:1	18:2	20:0	20:4
A	1.5	33.8	5.2	15.2	29.0	7.9	1.3	0.6	0.7	7.3	4.4	T	8.5	68.5	4.0	3.9
B	2.4	34.4	5.1	16.3	29.0	6.7	1.1	0.8	0.9	7.4	4.7	T	8.8	66.2	4.2	3.7
C	1.0	33.6	4.3	14.3	28.9	9.7	2.2	0.6	0.9	8.7	4.9	T	9.6	66.9	3.8	3.9
D	2.8	39.6	7.2	14.7	30.9	4.4	T	T	1.1	28.4	11.3	1.8	32.6	12.0		10.5
E	1.7	33.1	5.2	16.8	28.4	9.2	2.2	T	0.7	7.0	4.2	T	8.1	70.4	4.5	2.5
F	1.8	31.5	8.9	8.8	40.3	2.0	1.4	T	0.8	27.8	10.7	2.4	34.0	11.6		10.5
G				Sample exhausted								Sample exhausted				
H				Sample exhausted								Sample exhausted				
I	0.8	11.0	4.3	6.0	20.6	44.4	T	2.9	2.1	25.7	11.7	3.4	33.2	11.1		9.8
J	1.8	23.2	6.4	7.6	37.8	6.5	0.6	4.3	2.4	26.9	13.7	1.7	30.8	10.3		11.3
K	2.0	34.8	5.8	14.8	29.9	8.0	0.7	1.3	1.0	9.0	5.5	T	10.9	60.5	4.3	4.5
L	1.2	31.0	4.9	16.5	32.6	7.8	0.8	T	0.6	8.5	4.7	0.6	11.1	61.1	4.4	4.4
Bovine serum	1.0	32.1	4.2	24.9	30.3	6.0	T	T	0.6	5.6	3.7	T	7.5	68.9	5.0	3.7
Fetal calf serum	1.9	35.4	9.4	7.6	31.3	5.4	T	2.9	1.6	24.2	11.3	2.6	33.1	10.3	0.8	10.6

[a]Analyses were performed on triglycerides and sterol esters isolated from media collected from the last half of the growth period. T denotes less than 0.5%

[b]The difference between the sum of the percentages in any row and 100% represents the sum of minor acid percentages not included.

[c]*Refer to Table I for complete description of growth media.*

of sterol esters isolated from the media containing only fetal calf serum resembled the composition of fetal calf serum sterol esters. Unlike the effect upon cellular triglycerides, media triglycerides and cellular cholesterol esters, linoleic acid added to medium supplemented with lipid-free serum had no effect upon the composition of media sterol esters.

DISCUSSION

The substitution of maltose for glucose in the medium greatly inhibited the growth rate of HTC cells, presumably due to the lack of maltase, but had little effect upon the composition of cellular or media neutral lipids. Insulin, reported to stimulate cell growth (9) and fatty acid biosynthesis from glucose in fat cells (10,11), had no significant effect upon the growth rate or the neutral lipids of these hepatoma cells under the culture conditions employed. Likewise, the addition of sterculia foetida oil to the medium (a triglyceride that contains cyclopropene fatty acids known to alter oleic acid biosynthesis in the rat liver [12,13]) had little or no effect upon neutral lipid class composition. The specific effects of the various growth medium upon monoene biosynthesis in HTC cells are reported in a companion article (14).

A number of investigators (15-23) have shown with a variety of cultured cells that, when serum lipids were present in the media, de novo lipid biosynthesis was minimal and most cellular lipids were derived from the serum. A previous report from this laboratory demonstrated that the harvest media contained as much, and in most cases more, total neutral lipid and total phospholipid as was present originally in the media (1). The harvest media from the three additional growth conditions described in this report likewise contained more lipid than the media before cell cultivation. Analyses of individual phospholipid classes (2) and neutral lipid classes (this study) have shown that the fatty acid composition of cellular and media lipid classes differed dramatically and that the growth of HTC cells in the various media did not change its composition significantly. These data indicate that HTC cells, unlike most cultured cells, utilize serum cholesterol esters, triglycerides, and phosphoglycerides sparingly, if at all. On the other hand, free fatty acids (palmitic and linoleic) added to the medium were taken up and incorporated into neutral glycerides and phosphoglycerides. Medium triglycerides, but not cholesterol esters, contained an elevated percentage of linoleic acid when linoleic acid was added to the medium, indicating a release of triglycerides from the cell. The uptake of free fatty acids, incorporation into cellular neutral glycerides and phosphoglycerides, and release into the medium, by these cells also have been demonstrated with radioactive fatty acid (24).

The deletion of all bovine serum from the media resulted in only marginal changes in neutral lipid class and fatty acid compositions. However, bovine serum alone reduced cellular triglyceride content dramatically. Since bovine serum did not inhibit triglyceride synthesis in the presence of fetal calf serum, the latter must contain a factor that counteracts the inhibitory effect of bovine serum. The effect of bovine serum also was observed in the comparison of determined and calculated triglyceride carbon number percentages (Table IV). Calculated random distribution percentages showed less agreement with determined values when the triglycerides were from cells grown on media containing more bovine serum than fetal calf serum, or bovine serum alone. However, the degree of randomness in the distribution of fatty acids among the triglyceride positions in HTC cells is much greater than shown to occur in normal rat liver triglycerides (25). Ehrlich ascites cells show positional specificity of triglyceride fatty acids but do not exhibit the same preferential pairing of acids in the glyceride (5) as rat liver triglycerides exhibit (25). The loss of the ability to synthesize specific molecular species of glycerides, as opposed to all possible species, may be a metabolic defect of neoplasms. Linoleic acid in the growth medium reduced the percentage of monoenoic acids, especially 18:1, in cellular and medium triglycerides and cellular cholesterol esters 50-75%, whereas palmitic acid failed to show significant accumulation or effect. A similar effect of linoleic acid upon phospholipid monoenes has been reported. Media containing more bovine serum than fetal calf serum or only bovine serum, a rich source of esterified linoleic acid, gave rise to cellular triglycerides and cholesterol esters with a higher percentage of linoleic acid and lower percentage of monoenoic acids compared to cells grown on medium containing 2.5% fetal calf serum or lipid-free serum. These results suggest that the level of linoleic acid may regulate fatty acid biosynthesis, particularly monoene acid synthesis in these cultured hepatoma cells. This conclusion is consistent with data that suggests rat and mouse hepatic lipogenesis is influenced more by dietary linoleate than other acids (26-28). The specific effects of serum lipids and lipid-free serum upon the isomeric monoene fatty acid content of individual neutral lipids and phospholipids are reported in a companion

article (14).

Two additional points should be made regarding the composition of the neutral lipids. First, neither the triglycerides nor the cholesterol esters from the cells cultured on the lipid-free serum medium contained more than trace levels of 20:3 or 22:3 acids, fatty acids that become elevated in many cell lines grown under conditions where essential fatty acid deficiencies exist (29). This observation is in keeping with the low level of these acids found in the phospholipids of cells grown on this medium and supports the idea proposed earlier that these cells may lack the enzyme system required to desaturate further monoenoic acids effectively (2). Secondly, the fatty acid composition of cellular triglycerides shows remarkably close agreement with the composition of the cholesterol esters (Table III). This is in contrast to normal rat liver triglycerides and cholesterol esters, which exhibit characteristic and readily distinguishable compositions (30-32). This probably represents a further lack of specificity in lipid metabolism of neoplastic cells at the class level. The inability of this neoplastic cell to synthesize specific cholesterol ester species could be related to the apparent inability to synthesize specific species of triglycerides discussed earlier.

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Lipids of Cultured Hepatoma Cells: V. Distribution of Isomeric Monoene Fatty Acids in Individual Lipid Classes

RANDALL WOOD, JOHN FALCH, and **REX D. WIEGAND,** Division of Gastroenterology, Departments of Medicine and Biochemistry, University of Missouri School of Medicine, Columbia, Missouri 65201

ABSTRACT

Monoenoic acid fractions were isolated from phosphatidylcholines, phosphatidylethanolamines, triglycerides, and cholesterol esters derived from minimal deviation hepatoma 7288C cells cultured on 11 media containing varying levels of serums and lipids. Hexadecenoate (16:1), octadecenoate (18:1), and eicosenoate (20:1) fractions were subjected to ozonolysis and the isomeric composition of the monoene fractions determined quantitatively by gas liquid chromatography. The 16:1 fractions consisted of palmitoleic acid, the Δ9 isomer (85-90%), and the Δ11 isomer (10-15%) in most of the cases; growth media and lipid class origin had little effect upon composition. The predominate acids of the 20:1 fraction were the Δ13 and Δ11 isomers. Generally, the Δ13 isomer was present in the highest concentration, and this isomer was higher in phosphatidylcholines than the other classes. Vaccenic acid represented 33-66% of the 18:1 fraction, and the balance was oleic acid. Oleic acid concentrations decreased, and vaccenic acid levels increased as the growth medium serum and lipid levels decreased. Lipid classes did not exhibit any distinct preference for either isomer. These data represent the first quantitative isomeric analysis of monoenoic acids derived from individual lipid clases and are the first to show the occurrence of high levels of vaccenic acid in neoplastic cells. This study suggests that the elevated levels of oleic acid, one of the most frequently observed changes in tumor lipids, may, in fact, represent elevated levels of vaccenic acid.

INTRODUCTION

One of the most frequently reported abnormalities in the lipids of neoplasms is the elevation of oleic acid levels (1-6). Despite the fact that oleic acid represents one molecular species, *cis*-9-octadecenoic acid, the term often has been used without the double bond position having been established; the study of tumor lipids is no exception. Examination of the individual phospholipid (6) and neutral lipid classes (7) from cultured minimal deviation hepatoma cells recently revealed a high level of monoenoic fatty acids that increased further when the media contained low levels of lipids. Further studies with these cells showed that labeled palmitic acid was incorporated into 18:1 fatty acids (8), and more recent studies have indicated that a high percentage of the radioactivity was not in oleic acid (R.D. Wiegand and R. Wood, unpublished data). These studies provided the impetus for the present report, the quantitative determination of isomeric monoene fatty acids of individual lipid classes derived from hepatoma cells cultured on a variety of media.

EXPERIMENTAL PROCEDURES

Minimal deviation hepatoma 7288C (HTC) cells were cultured on a modified Swim's 77 medium supplemented with several levels of serums and lipids, as described previously (5,7). Individual neutral lipids and phospholipids were isolated by thin layer chromatography (TLC) and converted to methyl esters, and the monoene esters were resolved by preparative gas liquid chromatography (GLC) (6-8).

Unsaturated ester ozonides, prepared by a modification of the Beroza and Bierl procedure (9), were subjected to GLC and the resolved cleavage products quantitated. Ca. 1.0 ml carbon disulfide (-70 C) saturated with ozone was added to 0.25 ml carbon disulfide (-70 C) containing the sample (5-100 μg). Excess ozone was driven off immediately by bubbling dry nitrogen through the carbon disulfide. After the carbon disulfide had been evaporated to 10-50 μliter with nitrogen, the walls of the chromatographic tube (Kontes) were washed down and triphenylphosphine (100 mg/ml CS_2) added at the level of 20 μg/μg sample. Analyses were made with a Varian model 2100 chromatograph fitted with a 180 cm x 2 mm (inside diameter) pyrex column packed with 1% OV-17 coated on 100-120 mesh Gas Chrom-Q. Column oven temperature was programed 50-220 C at 6 C/min. Peak identities were determined by cochromatography with ozonides prepared from commercially available monoene esters of

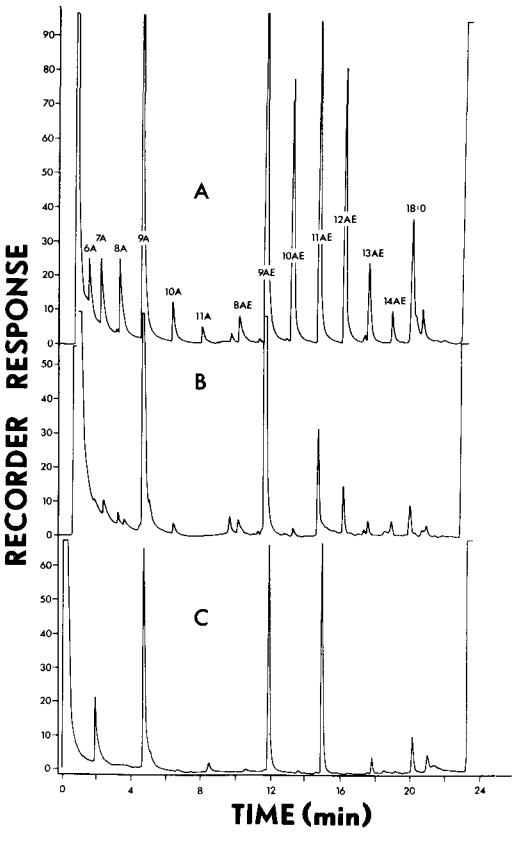

FIG. 1. Typical chromatograms depicting the gas liquid chromatographic analysis of aldehydes (6A-11A) and aldesters (8AE-14AE) resulting from the cleavage of ozonides of methyl octadecenoates derived from: (A) margarine, (B) bovine serum phosphatidylcholine, and (C) hepatoma cell triglycerides. Analyses were performed on a 6 ft 1% OV-17 column. Temperature was programed 50-220 C at 6 C/min. The beginning of the peak at the end of the chromatogram represents the first of three peaks resulting from the triphenylphosphine used to cleave the ozonides.

known structure. A digital integrator (Auto-Lab) was used to quantitate peak areas.

Fatty acid methyl esters and lipid standards were purchased form Nu-Chek-Prep, Elysian, Minn. Spectro grade carbon disulfide was purchased from Matheson, Coleman, and Bell, Norwood, Ohio. All other solvents were glass-distilled and obtained from Burdick and Jackson Lab., Muskegon, Mich. Other chemicals and reagents used were reagent grade or better.

RESULTS

Three typical chromatograms depicting the resolution of aldehydes and aldesters resulting from the cleavage of ozonides prepared from octadecenoate fractions derived from margarine, bovine serum, phosphatidylcholine (PC), and HTC cell triglycerides are shown in Figure 1. The beginning of the last peak shown on the chromatogram (ca. 23 min.) represents the first of 3 large peaks coming from triphenylphosphine which eluted before the elapse of 35 min. Although the technique is capable of detecting and measuring the double bond at practically any position in the molecule (Fig. 1A), most of the samples analyzed contained significant amounts of only two or three isomers (Figs. 1B and 1C). Analyses of mixtures of oleate and vaccenate, the major isomeric monoenes found in these samples, showed that the aldester percentages agreed closely (±5%) with the known wt percentages of the 2 fatty esters without the use of correction factors. Therefore, the percentages given represent values determined by the direct comparison of only the aldester areas. Small peaks eluting in the aldester portion of the chromatograms, as shown in Figure 1B, were not identified or included in the percentages.

The percentages of the major isomeric hexadecenoic acids (16:1) derived from phosphatidylethanolamines (PE), PC, and triglycerides of HTC cells, cultured on 11 different media are given in Table I. Palmitoleic acid, the Δ9 isomer, generally represented 80-90% of the total. The Δ11 isomer was the only other isomer appearing in significant amounts. Except for the somewhat decreased levels of palmitoleic acid in PC and PE when the cells were grown in medium supplemented with 20% bovine serum + 5% fetal calf serum or bovine serum alone, the various media had little or no effect upon isomeric distribution. Generally, the three lipid classes exhibited no preference for either of the isomeric hexadecenoic acids.

Table II contains the percentages of the isomeric octadecenoic acids (18:1) found in PE, PC, triglyceride, and cholesterol esters of HTC cells grown on various media. The percentage distribution of isomeric octadecenoic acids derived from bovine and fetal calf serums also is given in the table. Oleic and vaccenic acids accounted for more than 95% of the octadecenoic acids from all the lipid classes of the cells and serums. Generally, the level of oleic acid decreased, and vaccenic acid increased in all classes as serum and lipid levels in the medium decreased. Except for two or three media, 18:1 isomeric percentages of PC and triglycerides agreed very closely. PE contained slightly higher percentages of oleic acid than the other classes in most cases. Bovine serum contained a high and equal percentage of oleic acid in all lipid classes. In addition to vaccenic acid, bovine serum PC and cholesterol esters

TABLE I

Quantitative Determination of Isomeric Hexadecenoic Acids Derived from Individual Lipid Classes of Minimal Deviation Hepatoma 7288C Cells Cultured on Variety of Media

Medium[c]	Swim's 77 medium modification				Isomeric hexadecenoic acid percentages[a]					
					PE[b]		PC		TG	
	Percent bovine serum	Percent fetal calf serum	Percent lipid-free serum	Other additions or deletions	Δ9	Δ11	Δ9	Δ11	Δ9	Δ11
A	20	5			---	---	72	27	92	8
B	10	5			90	10	83	17	91	9
C	5	5			87	13	83	17	90	10
D		5			88	12	84	16	71	29
E	5				79	21	76	24	ND	
F		2.5			87	13	84	16	88	12
G			5		ND		ND		85	15
H			5	+ Palmitic acid (37.5 μg/ml)	ND		88	12	95	5
I			5	+ Linoleic acid (37.5 μg/ml)	ND		ND		90	10
K	5	5		+ Sterculia foetida oil (50 μg/ml)	ND		ND		89	11
L	5	5		+ Insulin (1 unit/ml)	81	19	82	18	89	11

[a]Differences between the sum of the Δ9 and Δ11 percentages and 100 represent small percentages of other isomers.

[b]PE = phosphatidylethanolamine, PC = phosphatidylcholine, TG = triglyceride, CE = cholesterol ester, and ND = not determined because of small size sample.

[c]Media designation is the same as used in the preceeding article (7).

TABLE II

Quantitative Determination of Isomeric Octadecenoic Acids Derived from Individual Lipid Classes of Minimal Deviation Hepatoma 7288C Cells Cultured on Variety of Media

	Isomeric octadecenoic acid percentages[a]							
	PE[b]		PC		TG		CE	
Medium[c]	Δ9	Δ11	Δ9	Δ11	Δ9	Δ11	Δ9	Δ11
A	67	33	51	47	58	41	52	48
B	70	30	57	43	58	41	49	51
C	57	39	51	45	43	55	33	67
D	60	40	46	52	32	61	28	71
E	58	39	62	36	55	45	51	44
F	51	46	45	49	48	49	ND	
G	47	50	40	55	38	57	ND	
H	66	34	59	41	61	38	ND	
I	54	46	54	46	55	43	ND	
K	70	30	59	37	59	41	ND	
L	55	42	50	46	48	49	ND	
Serum, bovine	ND		83	13[d]	80	20	81	12[d]
Serum, fetal calf	ND		60	40	ND		64	32

[a]Differences between the sum of the Δ9 and Δ11 percentages and 100 represent small percentages of the Δ13 isomer usually present except where noted.

[b]PE = phosphatidylethanolamine, PC = phosphatidylcholine, TG = triglyceride, CE = cholesterol ester, and ND = not determined because of small size sample.

[c]Refer to Table I for complete description of growth media.

[d]Difference between the sum of the percentages of the Δ9 and Δ11 isomers and 100 represents a small percentage of the Δ12 isomer.

also contained a significant level of the Δ12 isomer. Cholesterol esters and PC of fetal calf serum contained a much higher percentage of vaccenic acid than bovine serum classes. Oleic and vaccenic acid percentages in fetal calf serum PC, unlike that of bovine serum, were similar to cellular PC values.

The percentages of the major isomeric eicosenoic acids (20:1) isolated from PE, PC, and triglycerides of HTC cells cultured on various media are given in Table III. The Δ11 and Δ13 isomers predominated, presumably arising from the elongation of oleic and vaccenic acids. The Δ9 isomer was present in most cases; however, caution should be exercised in trying to draw any conclusions from the data where the Δ9 isomer may appear elevated. Linolenic acid, an acid usually not present in more than trace amounts in the lipids of HTC cells, eluted from the preparative chromatograph at the same approximate time as the eicosenoic acids and, therefore, may have contributed partially to the Δ9 isomer percentages. Generally, the Δ13 isomer predominated in all three classes and was even slightly elevated in PC relative to PE and triglycerides. Media appeared to have little effect upon the isomeric compositions.

Table IV shows the isomeric composition of the octadecenoic acids isolated from media sterol esters, the only media lipid class that contained enough of any monoenoic acid for analysis. Oleic and vaccenic acid percentages resembled those of the serum used to supplement the medium; those media containing bovine serum contained higher percentages of oleic acid, whereas those media containing only fetal calf serum contained the highest levels of vaccenic acid. These data indicate that cellular cholesterol esters are not in equilibrium with medium cholesterol esters.

Octadecadienoic acids obtained from triglycerides of cells grown on most of the media, bovine and fetal calf sterol esters, and bovine serum PC consisted of more than 90% linoleic acid. The balance was made up of the Δ8, 11 and Δ11, 14 isomers. Vaccenic acid probably gave rise to the Δ8, 11 isomer, but the probable origin of the Δ11, 14 isomer is not apparent.

DISCUSSION

One of the most important modifications of the Beroza and Bierl ozonization procedure (9) we found necessary was the generation of the ozone in the absence of the sample. Trapping the ozone in the solvent containing the sample resulted in numerous spurious peaks, presumably resulting from secondary reactions. They were present within a matter of secs after ozone generation was initiated, well before all the sample had been ozonized. Privett and Nickell (10,11), who have studied the ozonolysis process in detail, always generated the ozone in

TABLE III

Quantitative Determination of Isomeric Eicosenoic Acids Derived from Individual Lipid Classes of Minimal Deviation Hepatoma 7288C Cells Cultured on Variety of Media

	Isomeric eicosenoic acid percentages[a]								
	PE[b]			PC			TG		
Medium[c]	Δ9	Δ11	Δ13	Δ9	Δ11	Δ13	Δ9	Δ11	Δ13
A		ND			31	69	1	48	50
B	8	48	43		28	72		44	55
C	2	33	65	4	19	77		34	66
D		28	72		15	85		46	54
E	11	45	43	25	32	43		ND	
F	10	32	58	7	21	72	6	37	57
G	11	34	55	4	22	74	3	27	69
H		ND		21	28	51	10	44	46
I		ND			ND			60	39
K		ND			ND		9	51	40
L	12	42	46	7	24	69	7	38	55

[a]Differences between the sum of the Δ9, Δ11, and Δ13 percentages and 100 represent small percentages of other isomers.

[b]PE = phosphatidylethanolamine, PC = phosphatidylcholine, TG = triglyceride, CE = cholesterol ester, and ND = not determined because of small size sample.

[c]Refer to Table I for complete description of growth media.

the absence of the sample to reduce side reaction products. Pyrolysis of standard monoene ozonides, without triphenylphosphine, in the injector of the chromatograph (200-325 C) failed to give quantitative results; spurious peaks were present, and the yield of aldehydes and aldesters was variable and usually lower than when triphenylphosphine was used to cleave the ozonides. We also found that the level of triphenylphosphine could be reduced to a level that would not overload the column or detector without affecting quantitation. It was necessary to make up a new solution of triphenylphosphine periodically because of a breakdown product that gradually built up and eluted in the vicinity of the C-7 aldehyde.

This report appears to be the first to describe, quantitatively, the isomeric composition of hexadecenoic, octadecenoic, and eicosenoic acids derived from individual lipid classes of either neoplastic or normal mammalian tissues. Most of the isomeric composition data reported has been for octadecenoic acids derived from total lipids, and any conclusions one might be tempted to make by comparison with the present data should be considered tenuous. We recently obtained data (R. Wood, J. Falch, and R.D. Wiegand, unpublished data) which shows that the isomeric composition of rat liver octadecenoic acids derived from different classes differ. This is in agreement with earlier data that indicated the level of vaccenic acid in bovine PC and diphosphatidylglycerol differed (12).

Numerous investigators (1-5) have shown

TABLE IV

Distribution of Isomeric Octadecenoic Acids in Sterol Esters of Media after Growth of Minimal Deviation Hepatoma 7288C Cells on Variety of Media[a]

	Isomeric percentages[b]	
Medium[c]	Δ9	Δ11
A	88	12
B	84	15
C	85	15
D	70	30
E	91	9
F	71	29
K	82	18
L	83	17
Serum, bovine	81	12
Serum, fetal calf	64	32

[a]Analyses were performed on sterol esters isolated from media collected from the last half of the growth period.

[b]The difference between the sum of the percentages in any row and 100% represents the sum of minor acids not included in the table.

[c]Refer to Table I for description of growth media.

that the lipids of neoplasms contain elevated levels of oleic acid. In addition, we have shown that both neutral lipid and phospholipid classes of HTC cells contained high percentages of 18:1 fatty acids which increased as the level of serum and lipid in the medium decreased (6,7). The present data show that vaccenic acid represents 30-70% of the 18:1 fraction from the 4 lipid classes examined. The increased

percentage of vaccenic acid in PC, PE, triglycerides, and cholesterol esters as the level of serum and lipid in the medium decreased (Table II) indicates that the increased percentages of the octadecenoate fraction were due to increased quantities of vaccenic acid and not oleic acid. Since the increased level of oleic acid found in most tumor lipids has not been established by double bond positional analysis, it is possible that vaccenic acid, and not oleic, accounts for the increased level of the 18:1 fraction of neoplasms.

Vaccenic acid occurs in normal tissue lipids in varying amounts. Holloway and Wakil (12) demonstrated that the 18:1 fraction derived from rat liver mitochondria total lipids contained ca. 35% vaccenic acid. The octadecenoate fractions from human liver and blood total lipids have been reported to contain 10-30% vaccenic acid (13). Studies with labeled acetate indicate that vaccenic acid also represents 10-15% of the rat adipose tissue 18:1 fraction. These studies show that the occurrence of vaccenic acid in neoplasms is not unique but indicates the very high levels reported here for the HTC may be abnormal. The full significance of these observations will not become apparent until similar data has been obtained for individual lipid classes of normal rat liver. Preliminary results indicate that the 18:1 fraction of all lipid classes does not contain the high level of vaccenic acid reported here for HTC cells. The function of vaccenic acid and the effects of elevated levels upon the mammalian cell are unknown and also will have to await further investigations.

The high levels of vaccenic acid causes one to ponder over their origin. One would predict that vaccenic arises from the elongation of palmitoleic acid. Holloway and Wakil (12) have shown this to be the route of most mitochondrial vaccenic acid in normal rat liver. We presently are looking at HTC cells to determine if vaccenic arises by this pathway. If palmitoleic does give rise to vaccenic acid in the hepatoma cell, it is of interest to note that $\Delta 11$ hexadecenoate isomer (ca. 10%, Table I) did not give rise to significant levels of the $\Delta 13$ octadecenoate (Table II). The isomeric composition of the 20:1 fraction (Table III) indicates that oleic and vaccenic acid probably gave rise to this fraction through the chain elongation system.

Sterculia foetida oil, known to inhibit the desaturation of stearic acid to yield oleic acid (14,15), had no effect upon oleic or vaccenic acid levels (Table II) in the hepatoma cells. The lack of an effect could have resulted from the cells' inability to hydrolyze the triglycerides containing cyclopropene fatty acids or the resistance of the hepatoma cells' monoenoic desaturase system to cyclopropene fatty acids. Additional experiments are necessary to distinguish between these two possible explanations, both of which have important implications. The dramatic reduction of the 18:1 levels that occurred in the neutral lipid and phospholipid classes when cells were cultured in medium containing lipid-free serum plus linoleic acid (6,7) failed to alter the proportions of oleic and vaccenic acids in any of the classes. These results suggest that the biosynthesis of both of these acids was suppressed or replaced equally by linoleic acid.

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Changes in Phospholipid Composition in Hibernating Ground Squirrel, *Citellis lateralis*, and their Relationships to Membrane Function at Reduced Temperatures

ROLAND C. ALOIA and **ERIC T. PENGELLEY**, Department of Biology, University of California, Riverside, California 92502, and **JAMES L. BOLEN** and **GEORGE ROUSER**, City of Hope National Medical Center, Division of Neurosciences, Duarte, California 91010

ABSTRACT

The hearts of hibernating mammals have the capacity to function at reduced temperatures of 1 C in contrast to hearts of nonhibernating mammals which undergo ventricular fibrillation during deep hypothermia. Thus, all vital membrane functions continue in hibernators. This suggests that there may be special features of their lipid and protein composition. Analysis of the hearts of the hibernating ground squirrel, *Citellus lateralis*, in the active (37 C) and hibernating state (1-4 C) reveals differences in the percent composition of the phospholipid classes. There is a decrease in the percentage of diacyl choline, ethanolamine, serine phosphoglycerides, and diphosphatidyl glycerol and an increase in the percentage of lysophosphatidyl choline, lysophosphatidyl ethanolamine, lysodiphosphatidyl glycerol, and phosphatidic acid in the hibernating state. There is also an increase in the molar amount of phosphorus/100 g fresh wt of tissue during hibernation. The most striking difference in phospholipid composition between active and hibernating heart tissue is the 3.5-fold increase in lysoglycerophosphatides. These compounds are implicated as contributing factors controlling membrane fluidity during hibernation and upon which the physiological state of hibernation may be contingent.

INTRODUCTION

The hibernating ground squirrel, like other heterothermic animal species, is able to lower its body temperature to ca. 1 C (1). The low body temperature is maintained throughout the winter season (~ 5 months) with brief (1-2 day) intermittant periods of arousal to 37 C every 8-12 days (2). The heart beat is reduced from ca. 350 beats/min to ca. 2 beats/min at 1 C in the intact animal (3,4), as well as in isolated heart preparations (5-7) from which spontaneous action potentials have been recorded (8). In contrast, reduction of the body temperature of nonhibernating mammals to ca. 15-20 C results in ventricular fibrillation and heart failure (1, 9-11). Spontaneous action potentials cannot be recorded from isolated rabbit heart preparations below ca. 17 C (8).

An invariable feature of adaptation for growth at low temperatures in bacteria (12-14), plants (15-19), and poikliotherms (20-25) appears to be an increase in the degree of unsaturation of membrane lipid fatty acids. The opposite effect, namely an increase in saturated fatty acids at higher temperatures, also is observed in thermophilic organisms, able to grow at high temperatures (26-28), and in red blood cells of hamsters acclimated to elevated ambient temperatures (29). The level of unsaturation of membrane fatty acids has been related to membrane fluidity and the activity of enzymes as a function of temperature. Although at any temperature biological membranes apparently can accommodate both gel-like and liquid-crystalline phases (30,31), numerous membrane-bound enzymes are activated only in an unsaturated, fluid-membrane environment (32-36). Thus, when enzyme activity is plotted as a function of temperature (Arrhenius plot), there is an abrupt change in the slope of the line in the temperature range of the transition of the membrane lipids to the solid state (31,37,38). That the degree of unsaturation or the fluidity of the membrane lipids, and not the enzyme protein, determines the temperature-activity characteristics of these membrane-bound enzymes has been demonstrated conclusively (39,40). Succinic dehydrogenase (a lipid requiring enzyme) from goldfish adapted to either 5 or 25 C was indistinguishable by electrophoresis (41), yet exhibited temperature-activity profiles which were different and related to the degree of unsaturation of the associated phospholipid (40). However, when the pure enzyme protein extracted from the 5 or 25 C animal was reactivated with phospholipid from either source (5 or 25 C animal), the temperature characteristics of the enzyme corresponded precisely with the source of the reactivating lipid (40). Similarly, pure Na^+-K^+-adenosine triphosphatase (ATPase) (another lipid requiring enzyme) isolated from

bovine brain and from frog kidney exhibited closely similar Arrhenius plots when reactivated with lipid from the same source (either from bovine brain or frog kidney), although the plots of the activity of each enzyme activated by endogenous lipid were different and related to the degree of unsaturation of the lipids (39).

The enrichment in membrane lipid unsaturation in organisms acclimated to low temperatures apparently serves to maintain membrane fluidity and, hence, the functional capacity of membrane bound enzymes. However, there is little information concerning the possible influence of different phospholipid classes on membrane function at reduced temperatures. It is known that polar groups of phospholipid molecules can alter the transition temperature at which membrane lipids solidify (42), and changes in phospholipid classes, in response to cold exposure, have been reported in bacteria, yeast and higher plants (43-46), and in cold blooded animals (25). We report here important changes in phospholipid class composition (decrease in diacyl and an increase in monoacyl [lyso-] glycerophosphatides) of the heart lipid classes in hibernating ground squirrels.

MATERIALS AND METHODS

The phospholipid class content of the hearts of 22 active (T_B 37 C) and 20 hibernating (T_B 1.5-4 C) ground squirrels (*Citellus lateralis*) were analyzed. Hibernating squirrels were sacrificed on the fourth-sixth day of an established hibernating cycle (>8 days) to ensure biochemical equilibrium (47) and to avoid possible imminent arousals (2). Within 4 min from the time the hibernating animals were removed from the hibernaculum, their rectal temperature and body wts were measured, they were decollated, and the heart removed and plunged into liquid nitrogen. The hearts were removed from the active animals within 3 min after decollation. Hibernators were sacrificed between November 14 and December 29, 1972, and the hearts pooled into three groups of 3, 8, and 9 organs. Some of the active animals were sacrificed on August 13 and 14, 1973, and hearts were pooled into two groups of 8 and 9 organs. One group of five active animals were sacrificed October 1, 1972, just prior to the onset of their natural hibernating season. All samples were stored at -20 C until extracted.

Lipid Extraction

All procedures were performed under a nitrogen atmosphere, and all solvents were redistilled, and 0.01% butylated hydroxytoluene (BHT) was added as an antioxidant. Frozen, pooled organs were extracted with chloroform/methanol, 2:1, in a Beckman Polytron homogenizer at speed 7 for 90 sec, filtered, and the residue reextracted with chloroform/methanol, 4:1, containing 5% of 28% ammonium hydroxide by homogenization in a precooled metal cup-Waring blender for 3 min (48). The extracts of each homogenization were filtered through a sintered glass funnel (coarse porosity) under a nitrogen atmosphere, pooled, and the solvent removed by reduced pressure-low temperature rotary distillation (49,50). Care was taken to prevent lipid changes by exposure to dry, solid surfaces (51). The sample then was transferred to a Sephadex G-25 column in chloroform/methanol 19:1 saturated with H_2O and the lipid separated from nonlipid contaminants (48,52,53). The first fraction containing all of the phospholipids was concentrated and stored in chloroform/methanol 2:1 containing 0.01% BHT at -20 C until chromatographed (48,50).

Chromatography

Silica Gel H (Merck, Darmstadt, Germany) as a slurry of 25 g in 75 ml H_2O containing 1.875 g magnesium acetate was employed as an adsorbent (52). After spreading (0.25 mm thickness), plates were air-dried, oven-dried (110 C, 30 min), cooled, and stored in a dust-free container. Immediately before use, plates were placed in a plexiglass spotting chamber and allowed to equilibrate in a nitrogen atmosphere of 55% relative humidity (25 C) for at least 45 min prior to spotting. Immediately prior to or during the spotting procedure, an aliquot (usually 25 μliter) equal to the sample load was weighed on a Cahn g electrobalance (48,50). Aliquots (ca. 500 μg each) were spotted on 4 thin layer chromatographic (TLC) plates in a nitrogen atmosphere, and 8 aliquots were taken for total phospholipid determination (52). After spotting, samples were allowed to dry for 5 min. Plates then were chromatographed in chloroform/methanol/28% aqueous ammonia (65:25:5), dried 12 min in a nitrogen atmosphere, and developed in the second dimension with chloroform/acetone/methanol/acetic acid/H_2O (3:4:1:1:0.5) (52). Plates were air-dried, sprayed with sulfuric acid-formaldehyde (97:3), charred at 160 C for 45 min, and photographed (49,50). Charred spots were aspirated into test tubes, digested with 70% perchloric acid (double distilled from Vycor; G. Fredrick Smith Co., Columbus, Ohio) at 180 C for 60 min, cooled, and color reagents added (52,54). Tubes were heated for 5 min at 100 C, centrifuged to sediment silica gel, supernatant solutions decanted into cu-

vettes, and color intensity determined at 797 mu Gilford model 240 spectrophotometer). Two quadruplicate sets of two dimensional chromatograms were analyzed for each pooled heart sample.

RESULTS

Since organs were obtained from squirrels within 3 min after decollation, immediately frozen in liquid nitrogen, and stored at -20 C until extracted, little postmortem change is to be expected. The two dimensional separation of phospholipid classes is shown in Figure 1 (52). Reproducibility was demonstrated by mean values falling within the standard deviation of the previous analysis (Table I). Phospholipid classes were identified from previously chromatographed heart phospholipid samples using the same solvent mixtures (49).

Table I illustrates the molar percentages of phosphorus for each phospholipid class examined. Comparison of the values for active and hibernating animals indicates that the percentages of phosphatidyl ethanolamine (PE), phosphatidyl choline (PC) ($P < 0.01$), phosphatidyl serine (PS), and diphosphatidyl glycerol (DPG) ($P < 0.001$) decrease during the hibernating state, while those of lysophosphatidyl ethanolamine (LPE), lysophosphatidyl choline (LPC), lysodiphosphatidyl glycerol (LDPG) ($P < 0.001$), and phosphatidic acid (PA) ($P < 0.05$) increase. The percentage by which the diacyl phosphatides PE, PC, and DPG decrease seems to be mirrored by a corresponding increase in the lysoglycerophosphatides, LPE, LPC, and LDPG.

Table I also indicates that the amount of total phospholipid (millimoles phosphorus/100 g fresh wt) increases from 3.47 in the active to 3.79 in the hibernating state ($P < 0.01$).

DISCUSSION

The increase in total phospholipids during the hibernating state (Table I) is in agreement with studies of other hibernating mammals (55-57). Individual phospholipid classes of a hibernating mammal have been analyzed in only one previous study by Wells, et al. (55). Accurate comparisons between this study and the present investigation are difficult to make, however, since they examined a different tissue and used column chromatographic procedures as opposed to two dimensional TLC. Recent reports have shown that these column procedures can lead to spurius results (51,58). Wells, et al., (55) found the phospholipid class distributions of brown adipose tissue from active and

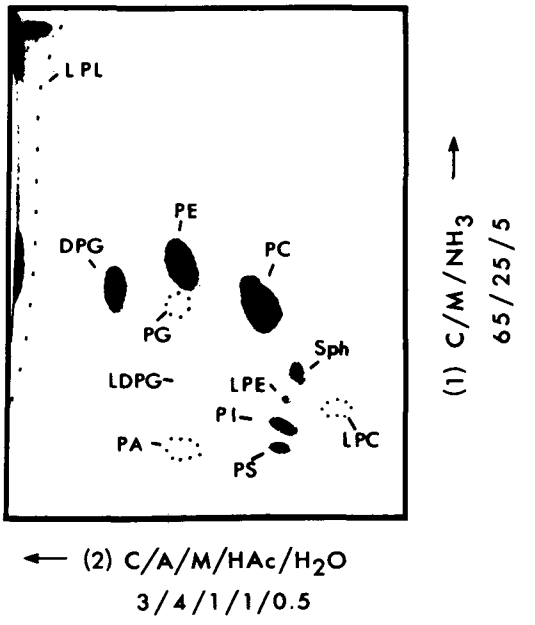

FIG. 1. Two dimensional thin layer chromatogram of ground squirrel heart extract (500 μg applied) after separation of nonlipid contaminants and gangliosides from other lipids by Sephadex column chromatography. See text for details. Solvent systems are as described in "Materials and Methods." Spots difficult to reproduce photographically are outlined. LPL = less polar lipids, DPG = diphosphatidyl glycerol, PE = phosphatidyl ethanolamine, PC = phosphatidyl choline, PG = phosphatidyl glycerol, LDPG = lysodiphosphatidyl glycerol, Sph = sphingomyelin, LPE = lysophosphatidyl ethanolamine, PI = phosphatidyl inositol, LPC = lysophosphatidyl choline, PA = phosphatidic acid, and PS = phosphatidyl serine.

hibernating bats (*Myotis lucifugus*) to be similar, whereas our data for the ground squirrel (*C. lateralis*) show that the proportion of phospholipid in the hearts of active and hibernating animals is different. During hibernation, the molar percentages of PE, PC, PS, and DPG decrease, while those of the lysoglycerophosphatides, LPE, LPC, LDPG, as well as PA, increase.

Since the concentration of lysoglycerophosphatides in active hibernator and other vertebrate hearts is normally low (< 2%, [59,60]), the greatly enhanced content (∿ 8 mole %) of these compounds in the hibernating state may be related to the maintenance of membrane fluidity and the capacity of the hibernating squirrel heart to continue beating at low temperatures. Support for this hypothesis is derived from a consideration of Arrhenius plots of enzyme activity.

Succinate oxidation of mitochondria isolated from hibernating ground squirrels (61) is

TABLE I

Heart Muscle Phospholipids[a]

	Active squirrels						Hibernating squirrels						
	A (n = 9)[b]		B (n = 8)		C (n = 5)		D (n = 9)		E (n = 3)		F (n = 8)		P[c]
Phosphatidyl ethanolamine	33.03 ±0.63	32.22 0.27	33.01 0.33	33.50 0.19	33.77 0.75	33.07 0.66	31.10 0.92	31.50 0.03	31.46 0.82	31.21 0.38	31.83 0.08	31.88 0.29	0.01
Phosphatidyl choline	38.69 0.47	40.25 0.49	39.46 0.20	39.78 0.67	38.18 0.91	38.49 0.81	36.56 1.45	37.22 0.19	38.48 2.15	37.84 0.11	37.37 0.62	37.54 0.30	0.01
Diphosphatidyl glycerol	14.30 0.19	14.04 0.12	14.45 0.06	14.54 0.16	14.04 0.26	14.23 0.87	11.85 0.31	11.79 0.17	12.12 0.57	12.09 0.31	12.57 0.05	12.76 0.13	0.001
Sphingomyelin	3.14 0.02	3.30 0.06	3.28 0.11	3.15 0.07	3.78 0.18	3.79 0.23	4.94[e] 0.14	3.91 0.08	3.14 0.11	3.32 0.22	3.56 0.05	3.38 0.15	NS
Phosphatidyl inositol	4.02 0.07	3.88 0.08	3.83 0.21	3.74 0.05	3.85 0.22	3.80 0.29	3.66 0.17	3.45 0.26	3.62 0.23	3.60 0.09	4.03 0.12	3.76 0.09	NS
Phosphatidyl serine	2.53 0.17	2.55 0.09	2.66 0.26	2.53 0.06	2.64 0.29	2.46 0.36	2.48 0.25	2.21 0.13	2.10 0.09	2.16 0.07	2.27 0.14	2.16 0.04	0.001
Lysodiphosphatidyl glycerol	0.66 0.13	0.44 0.05	0.65 0.09	0.38 0.07	1.09 0.16	0.77 0.19	2.37 0.14	2.26 0.07	2.16 0.10	2.30 0.20	2.09 0.11	1.74 0.05	0.001
Lysophosphatidyl ethanolamine	0.80 0.24	0.52 0.07	0.40 0.08	0.36 0.04	0.98 ---	1.39 ---	2.23 0.20	2.82 0.12	2.70 0.19	2.85 0.08	1.86 0.21	1.74 0.29	0.001
Lysophosphatidyl choline	0.48 0.12	0.62 0.13	0.55 0.04	0.43 0.02	1.03 ---	0.96 ---	2.88 0.15	2.76 0.06	2.79 0.13	2.86 0.06	1.82 0.11	1.75 0.09	0.001
Phosphatidic acid	0.39 0.23	0.40 ---	0.23 0.12	0.29 0.02	--- ---	--- ---	0.42 ---	0.50 0.06	0.33 0.10	0.56 0.03	0.69 0.03	0.62 0.05	0.05
Phosphatidyl glycerol	0.71 0.06	0.77 0.05	0.70 0.08	0.87 0.05	0.32 ---	0.35 ---	0.68 ---	0.66 0.10	0.53 0.10	0.77 0.06	1.13 0.14	0.79 0.08	NS
Miscellaneous	1.27 0.12	1.02 0.07	0.78 0.09	0.43 0.12	0.32 ---	0.69 ---	0.82 ---	0.92 0.12	0.59 0.04	0.46 0.23	0.78 0.10	0.87 0.12	---
TPL[d]	3.54	3.64	3.55	3.60	3.23	3.25	3.67	3.63	3.71	3.74	4.04	3.98	0.01
TPL mean[d]	(3.47 ± 0.17)						(3.79 ± 0.39)						

[a]Expressed as percentage of total lipid phosphorus; mean ± standard deviation; recovery range: 97.7-101.5%.

[b]Repetitions of quadruplicate sets of chromatograms; (n) = number of animals.

[c]P = probability; nonpaired T-test comparing active with hibernating animals; NS = not significant.

[d]TPL = total phospholipid. Millimoles of phosphorus/100 g fresh wt (mean ± standard deviation).

[e]Not included in calculations.

similar to that of poikilothermic animals (62) and chill-resistant plant tissues (63), in that the Arrhenius plot of enzyme activity as a function of temperature is expressed as a straight line with a single, low activation energy. The Arrhenius plot for succinate oxidation by active ground squirrels, homeothermic mammals (rat), and chill-sensitive plant tissues (61-63), on the other hand, exhibits a distinct discontinuity or break and a large increase in activation energy below the transition temperature. The discontinuity is considered to represent a phase transition (64) of the membrane lipids from a liquid-crystalline to a gel-like state as the temperature is lowered below the transition temperature (65) and has been demonstrated by physical techniques in isolated lipids from bacteria (66,67), plant, and animal tissues (68). The membranes of the hibernating animal are apparently sufficiently fluid that a phase transition does not occur and the membranes remain functional at reduced temperatures (69).

Na^+-K^+-ATPase (ATP phosphohydrolase, EC 3.6.1.3, a lipid requiring enzyme) from rabbit kidney exhibits the typical homeothermic Arrhenius profile of a break at a specific temperature (18 C) and a doubling of the energy of activation below the transition temperature (70). However, the discontinuity in the Arrhenius plot can be abolished and a straight line plot with a single activation energy induced by treatment with phospholipase A which produces lysoglycerophosphatides (70).

That lysoglycerophosphatides themselves, however, are responsible for the straight line Arrhenius plot and the single activation energy, is derived from a recent electron spin resonance study (unpublished observations of A.D. Keith, et al.). Arrhenius plots were made of the change in rotational correlation time of a spin label added to pure phospholipids extracted from whole heart and heart and liver mitochondria. As expected, the Arrhenius plot from the hibernating ground squirrel exhibited a straight line with a single activation energy, whereas that from the active ground squirrel showed a line with a single activation energy, whereas that from the active ground squirrel showed a discontinuity at ca. 24 C with an increase in activation energy below the transition temperature. However, by adding 7 mole % of monopalmitoyl PC (similar to the total mole percent of lysoglycerophosphatides found in the hibernating squirrel heart; Table I) to the phospholipids from the active ground squirrels, the discontinuity in the Arrhenius plot was abolished, and a straight line plot was produced. Consequently, it appears that the large increase in molar percent of lysoglycerophosphatides found in hibernating squirrel hearts is, indeed, a major factor in maintenance of membrane "fluidity" at reduced temperatures.

Previous to this, low temperature adaptation and growth in bacteria (12), plants (16), and cold blooded animals (24), membrane permeability (66,71,72), and enzyme activity (32,33) at low temperatures have been related to an increase in lipid unsaturation and consequent membrane fluidity. Additionally, recent freeze-etch electron microscopic evidence has indicated that, at low temperatures, the normal distribution of intramembranous particles is observed only when the lipids are unsaturated (73,74). However, it appears that, in the tissues of hibernating mammals, the degree of unsaturation may not be the only factor operative in controlling membrane fluidity at reduced temperatures. Although it has been demonstrated recently that ground squirrel heart lipids do, indeed, become more unsaturated during hibernation (75), the present investigation reveals that the molar percentage of the lysoglycerophosphatides also is elevated dramatically. Additionally, our electron spin resonance data clearly demonstrate that lysoglycerophosphatides can increase the disorder of the membrane phospholipids and thus sustain a fluid environment at reduced temperatures.

The phospholipase and transacylase enzymes responsible for de- and reacylation of glycerophosphatides (76,77) have been demonstrated in many membrane types (71-81), and it is entirely possible, therefore, that enzymatically controlled, physiological concentrations of lysoglycerophosphatides could be produced and influence the physiochemical state of the membrane core (82). Mitochondrial phospholipase A has been shown to be activated by free fatty acids and $CaCl_2$, inducing the formation of lysoglycerophosphatides and permeability changes resulting in maximal mitochondrial swelling (83). The fact that free fatty acid levels in some hibernating species (hedgehog [84]) and the heart-muscle Ca^{++} content of other species (hamster [85]) are almost doubled during hibernation indicates a possible mechansim for the high content of lysocompounds found in the heart of the hibernating ground squirrel. Conversely, if the transacylase enzymes were simply more cold-inhibited than the phospholipases, this too would account for the accumulation of lysoglycerophosphatides, as well as the decrease in PE, PC, DPG, and PS, seen in Table I. Additionally, if the enzymes involved in the conversion of PA to other phospholipids (phosphatide phosphatase and cytidyl transferase, EC 3.1.3.4 and EC 2.7.7.41, respectively), were cold sensitive, PA would

tend to accumulate, while the synthesis of PE, PC, DPG, and PS would tend to be retarded.

Examination of the data in Table I indicates that except for DPG, other phospholipid values fall within the normal range of other vertebrate heart data from studies employing comparable quantitative techniques (61,62). DPG appears to be slightly higher in the squirrel heart, possibly reflecting a higher content of mitochondria/unit volume compared to other vertebrate hearts. Comte, et al., (86) and Fleischer, et al., (87) have reported DPG values as high as 18.1 and 20.5% for isolated pig and beef heart mitochondria, respectively.

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Effect of Light Intensity upon Lipid Composition of *Nitzschia closterium* (*Cylindrotheca fusiformis*)[1]

DAVID M. ORCUTT[2], and **GLENN W. PATTERSON**, Department of Botany, University of Maryland, College Park, Maryland 20742

ABSTRACT

Total fatty acid, total sterol, fatty acids of specific lipid classes, and unsaturated fatty acids produced in *Nitzschia closterium* were compared qualitatively and quantitatively as a function of changes in light intensity. Increased levels of total fatty acids were observed in cells grown at high light intensity when compared to cells grown at low light intensity. However, the percentage of unsaturated fatty acid decreased under high light conditions. Fatty acid analysis of triglyceride and 1,3 diglyceride fractions indicated an increase in levels of fatty acid at high light intensity when compared to low light intensity, while levels of polar lipid fatty acids increased at low light intensity. These analyses can be taken to indicate an increase in triglyceride and diglyceride at high light and a decrease in polar lipid at high light. Levels of free fatty acids did not differ significantly with light intensity. The levels of total sterol also were unaffected by changes in light intensity. However, levels of sterol isolated as free sterol and sterol associated in a yet unknown manner in the polar lipid fraction varied with changes in light intensity. Levels of polar lipid sterol increased at high light intensity compared to low light intensity, while the opposite was true for free sterol. The greatest percentage of total sterol was found in the polar lipid class regardless of light intensity.

INTRODUCTION

Little information is available concerning the effect of light intensity upon total lipid composition and composition of specific lipid fractions in algae. What information is available has been in relation to lipid changes in the green alga *Chlorella* and the phytoflagellate *Euglena* when grown heterotropically, photoheterotrophically, or photoauxotropically (1-3). However, in *Euglena gracilis* (3) decreases in chlorophyll and total lipid were observed with increased light intensity when cells were grown photoauxotropically. Increased percentages of some polyunsaturated fatty acids occurred with increased light intensity.

Changes in total lipid and sterol found in natural populations of red and brown algae have been correlated with seasonal changes in sunlight and depth distribution at which the organisms were collected (4,5). Generally, more lipids and sterols were present when algae were collected in the summer months and in shallow water. Increases in sterol concentration also have been observed for laboratory cultures of *Chlorella* when grown at increased light intensity (6). Brandt, et al., (7) found an unidentified bound form of sterol in *E. gracilis* Z and noted that the bound sterol decreased and free sterol increased in light grown cultures, while the reverse was true of dark grown cultures.

Apparently, no studies have been conducted with diatoms in relation to the effect of light intensity upon lipids and specific lipid fraction composition. In view of the role of lipid components in functioning of the photosynthetic apparatus in plants and utilization of data on sterol (8) and fatty acid (9) composition in taxonomic and phytogenetic studies, it is essential to understand how environmental factors affect lipid composition both qualitatively and quantitatively.

EXPERIMENTAL PROCEDURES

Axenic cultures of the diatom *Nitzschia closterium* (*Cylindrotheca fusiformis*; Indiana Culture Collection no. 640) were grown in synthetic seawater medium as described by Lewin (10). Cells were cultured in 500 ml Pyrex carrot tubes at 17 C for 7 days. Both low and high light (LL and HL) intensity cultures were grown simultaneously utilizing fluorescent illumination of 200 and 2000 foot-candles. The lower intensity was achieved by blocking part of the illumination with fine meshed wire screen. Cultures were inoculated with cells adapted for 4 days to the 2000 foot-candle intensity and bubbled with 1% CO_2 in air. Cells were harvested by continuous centrifugation, freeze-dried, and dry wts determined.

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[2]Present address: Department of Plant Pathology and Physiology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061.

Total lipids were obtained by Soxhlet extraction using chloroform-methanol (2:1, v/v) for 24 hr. The extract was dried, redissolved in chloroform, and filtered through Whatman no. 1 filter paper. The filtrate was evaporated to dryness and the dried residue weighed as the total lipid. Lipid classes were separated utilizing a thin layer chromatographic (TLC) technique outlined by Ginger and Fairbairn (11). Lipid classes corresponding to authentic free sterol, free fatty acid (FFA), 1,3 diglyceride (DG), triglyceride, (TRIG), and polar lipid (PL) (origin) were eluted from TLC plates with diethyl ether. DG plus free sterol (these do not separate), TRIG, and PL bands were refluxed with 20% KOH in methanol (1 mg KOH/mg lipid) for 45 min. The samples were acidified with 6N HCl and partioned with n-hexane, evaporated, and subjected to TLC, as previously described. Fatty acid methyl esters and sterols were eluted from TLC plates and analyzed qualitatively and quantitatively by gas liquid chromatography (GLC) utilizing a 15% Hi-EFF 1BP and a 3% SE-30 column, respectively.

Total fatty acid and sterol analysis was performed as in the above procedure beginning with saponification of an aliquot of the total lipid sample.

RESULTS AND DISCUSSION

Relative quantities of various lipid fractions were compared in *N. closterium* when grown at HL and LL intensities (Table I).

Under HL intensity, the percentage of fatty acid was nearly twice that at LL, although the total lipid at HL intensity increased only 2% above the lipid concentration at LL intensity. The concentration of total sterol remained the same at HL and LL intensities. Lipid fraction analysis of PL, DG, TRIG, and FFA (Table I) indicated the most obvious changes occurred in TRIG fatty acids. At HL intensity, more than 5 times as much fatty acid was observed than at LL intensity. However, in the PL fatty acid more than twice as much fatty acid was observed at LL intensity than at HL intensity. Low concentrations of fatty acids were found in the form of DG and FFA. No significant differences were observed in FFA concentration as a function of light intensity. Differences observed for DG were small considering the total fatty acid concentrations.

Table II illustrates the concentration of

TABLE I

Effects of Light Intensity upon Lipid Composition in *Nitzschia closterium*[a]

Lipid fraction	HL[b]	LL[c]
Polar lipid fatty acid	2.6	5.4
Free fatty acid	0.3	0.4
Diglyceride fatty acid	0.5	0.1
Triglyceride fatty acid	9.2	1.7
Polar lipid sterol	0.4	0.3
Free sterol	0.1	0.2
Total unsaturated fatty acid	8.3	5.8
Total saturated fatty acid	4.3	1.8
Total fatty acid	12.6	7.6
Total sterol	0.5	0.5
Total lipid	22.8	20.8

[a]Average of two experiments; data expressed as percent of dry wt.

[b]HL = high light.

[c]LL = low light.

TABLE II

Effect of Light Intensity upon Lipid Fraction Fatty Acid Concentrations in *Nitzschia closterium*[a]

	Lipid fraction[b]									
	PL		DG		FFA		TRIG		Total concentration	
Fatty acid	HL[c]	LL[d]	HL	LL	HL	LL	HL	LL	HL	LL
14:0	2.6	6.5	0.7	<0.1	0.2	0.2	3.8	0.9	7.3	7.6
15:0	---	---	<0.1	<0.1	<0.1	<0.1	---	0.2	Trace	0.2
16:0	7.1	10.0	1.1	0.3	0.5	0.5	27.3	3.9	36.0	14.7
18:0	---	---	<0.1	<0.1	<0.1	0.1	0.3	---	0.3	0.1
16:1	12.3	31.9	1.0	0.2	0.7	0.8	54.0	6.5	68.0	39.4
16:2	0.7	2.9	---	<0.1	<0.1	0.1	1.2	0.3	1.9	3.3
16:3 + 18:1	1.5	10.4	0.7	0.1	0.2	0.4	2.7	2.6	5.4	13.5
18:2	1.1	2.1	0.6	<0.1	<0.1	<0.1	---	0.4	1.7	2.5
18:3	---	---	0.3	<0.1	---	<0.1	---	---	0.3	Trace
20:5	3.9	7.9	2.0	0.1	1.0	1.5	---	2.4	6.9	11.9

[a]Average of two experiments; data expressed as mg of fatty acid/g dry wt.

[b]PL = polar lipid fatty acid, DG = 1,3 diglyceride fatty acid, FFA = free fatty acid, and TRIG = triglyceride fatty acid.

[c]HL = high light.

[d]LL = low light.

TABLE III

Effect of Light Intensity upon Relative Percentages of Fatty Acids in *Nitzschia closterium*[a]

	Lipid fraction[b]							
	PL		DG		FFA		TRIG	
Fatty acid	HL[c]	LL[d]	HL	LL	HL	LL	HL	LL
14:0	8.7	8.5	11.7	5.6	8.4	6.0	3.8	5.3
15:0	---	---	0.7	2.0	1.1	1.3	---	1.2
16:0	25.3	14.7	20.6	30.1	20.4	13.3	30.9	22.4
18:0	---	---	0.6	5.6	2.2	2.3	0.2	---
16:1	43.3	45.2	21.0	25.3	23.9	26.4	61.1	36.4
16:2	1.4	3.7	---	1.0	1.0	3.0	1.3	1.9
16:3 + 18:1	5.1	15.3	9.6	14.8	4.4	11.3	2.7	14.9
18:2	3.7	2.8	8.1	3.5	3.4	1.3	---	2.4
18:3	---	---	2.0	1.8	---	0.2	---	---
20:5	12.5	9.8	25.6	10.3	35.2	34.9	---	15.5

[a]Average of two experiments; data expressed as percent of total fatty acid in each fraction.

[b]PL = polar lipid, DG = 1,3 diglyceride, FFA = free fatty acid, and TRIG = triglyceride.

[c]HL = high light.

[d]LL = low light.

individual fatty acids in *N. closterium* in relation to light intensity. The highest concentrations of fatty acid were found in the TRIG and PL fractions. Increases in concentration of all fatty acids in the PL class were observed for cells grown at LL intensity as compared to HL intensity. The opposite was true for most fatty acids in the TRIG class, except for 15:0 and 18:2, where slight increases were observed at LL intensity. Considerably more 20:5 was produced at LL intensity in this class. Most fatty acids in the DG class increased in concentration at HL intensity when compared to LL intensity. Little difference in concentration of any fatty acid was observed at either light intensity in the FFA class. The most drastic changes in concentration of individual fatty acids in the TRIG class was with respect to increases of 16:1 and 16:0 at HL intensity. More than 8 and 7 times, respectively, of these fatty acids were produced at HL than at LL intensity. However, the same two fatty acids increased in concentration at LL intensity in the PL class, especially 16:1. It is also interesting to note that most 20:5 fatty acid is associated with the PL class and increases in concentration at LL intensity in the PL and TRIG classes. In view of the obvious increased production of fatty acid under HL conditions and the relatively small change in total lipid as a function of light intensity, it is thought that this disparity might be accounted for by significant increases observed visually in the amount of pigments produced at LL intensity.

Table III compares relative percentages of fatty acid in each lipid class as a percentage of total fatty acid in each respective class. The most significant changes in relative fatty acid composition were with respect to 16:0, 16:1, 16:3 + 18:1, and 20:5. The percentage of 16:0 was greater at HL intensity in all lipid classes except DG. The percentage of 16:1 remained unchanged in all lipid classes except TRIG, where higher percentages were observed under HL intensity. Percentages of 16:3 + 18:1 and 20:5 were highest at HL intensity for all lipid classes except for TRIG where 20:5 was higher at LL intensity.

The sterol composition of *N. closterium* was determined in a previous study (12). The predominant sterol found was the 24S isomer of brassicasterol, (24S)-24-methylcholesta-5,22 E-dien-3β-ol. In the present study, sterol was found in *N. closterium* as free sterol, sterol ester, and sterol associated with the PL class in a yet undetermined form. The highest concentrations of sterol were found in the PL class under both HL and LL intensities with the next highest concentration occurring as free sterol. Changes in the concentration of sterol in the form of PL sterol and free sterol appear to be a function of light intensity. At HL intensity, more sterol was observed in the polar lipid class than at LL intensity. The opposite was true for free sterol under the same conditions. A duplicate experiment verified these differences.

In *E. gracilis*, the concentration of total lipid and chlorphyll has been shown to decrease with increased light intensity over a range of 120-610 foot-candles (3). Sharp increases in the percentages of linolenic and 4, 7, 10, 13-hexadecatetraenoic acids were observed with increased light intensity. Correlations between increases in polyunsaturated fatty acids and Hill reaction activity with increased light intensity were suggested. Kates and Volcani (13) in their

analysis of diatom fatty acids have indicated that diatoms are apparently Hill reaction organisms and yet contain only small amounts of linolenic acid. This suggests to them that the degree of unsaturation of a fatty acid may be of importance in the Hill reaction and not a specific fatty acid.

Changes in specific fatty acids reflect, in part, changes in specific lipid fractions. It is of interest from the standpoint of the present study that the only fatty acids which increased significantly in total concentration at HL intensity were 16:0 and 16:1. All other fatty acids decreased in concentration at HL intensity. Also the percentage of unsaturated fatty acid increased at LL intensity, while total lipid increased at HL intensity. Although direct measurements were not made, cellular pigments comprised a significant portion of the total lipid and appeared to decrease with increased light intensity.

It has long been known that "oil" serves as an energy storage compound in the diatoms (14). The differences observed in the TRIG composition of *N. closterium* apparently reflect the accumulation of these energy storage compounds in response to more optimal environmental conditions.

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New Series of Fatty Acids in Northern Pike (*Esox lucius*)[1]

R.L. GLASS, THOMAS P. KRICK, and **ALLEN E. ECKHARDT,** Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55101

ABSTRACT

The presence in Northern Pike (*Esox lucius*) liver and testes lipids of a group of eight homologous fatty acids of as yet unknown structure is reported. They occur esterified to cholesterol and to glycerol as triglycerides but are absent from the phospholipids. They contain three oxygens and are characterized further by being more resistant to hydrogenation than normal unsaturated fatty acids and by an inability to form urea inclusion compounds. They also have been found to be major constituents of the liver fatty acids of four additional species of fish.

INTRODUCTION

An unfamiliar fatty acid was observed by us as a major component in the liver lipids of a male Northern Pike (*Esox lucius*). Its methyl ester migrated, during gas liquid chromatography (GLC) on a diethylene glycol succinate (DEGS) column, similarly to that of a 22:4 ω6 fatty acid. Its mol wt was determined by mass spectrometry to be 364 which corresponds to a 23:2 fatty acid methyl ester. Hydrogenation under 8 psig hydrogen with PtO_2 catalyst for 20 min converted the common unsaturated acids present to the normal saturated compounds but did not affect this compound. Hydrogenation also revealed the presence of seven other compounds similarly unaffected by hydrogenation but which did not correspond to any known saturated or unsaturated fatty acid.

Kluytmans and Zandee (1) have reported the presence in Northern Pike testes of a compound which they tentatively identified as either a 22:3 or a 23:1 fatty acid. We concluded that their acid was identical to the one we had observed in livers. Northern pike testes then were found to contain the entire group of 8 compounds in amounts which ranged up to 60% of the total fatty acids present.

We have since found these compounds in the liver lipids of a number of other species of male freshwater fish. They are seldom found in females. Structural work presently underway has thus far failed to reveal their exact nature. The present communication describes their preparation and distribution in Northern Pike.

[1]Scientific Journal Series 8943 Agricultural Experiment Station, University of Minnesota, St. Paul, Minn. 55101.

EXPERIMENTAL PROCEDURES

Materials

Live fish were obtained by trapping or by angling from lakes in the St. Paul, Minn., area. The testes and livers were removed and extracted in a blender with chloroform-methanol according to the aqueous wash procedure of Folch, et al. (2). The lipids so obtained were dissolved in Skellysolve F to make a 10% solution and then were stored under nitrogen at -10 C.

Methods

GLC was done on an F&M dual column hydrogen flame instrument model 5750. The columns were 1.5 m x 6 mm (outside diameter) copper tubing packed with diethylene glycol succinate (C6DEGS, Analabs, North Haven, Conn.) 10% on 80-100 mesh Chromosorb W (Applied Science Laboratories, State College, Pa.). Temperature programing was from 150-240 C at 6°/min.

Low resolution mass spectrometry was done on an LKB-9000 combined gas liquid chromatograph-mass spectrometer (GLC-MS). The column was glass tubing, (1.3 m x 2 mm inside diameter) packed with the same material described for conventional GLC. The ionization potential was 70 eV, and the ion source temperature was 290 C.

Lipids were separated into classes by thin layer chromatography (TLC) using 1.5 mm layers of Silica Gel H on 20 x 20 cm glass plates. Three ml of a 10% solution of total lipid (TL) in Skellysolve F was applied as a streak to the plates which then were developed in paper lined glass chambers using the solvent system Skellysolve F-diethyl ether (85:15 v/v). The clearly visible zones of cholesteryl esters (CE), triglycerides (TG), and phospholipids (PL) were scraped off, and the lipid eluted from the gel with diethyl ether.

Methyl esters were prepared for GLC by methanolysis using methanolic sodium methoxide and methanol-benzene (1:1, v/v) as a solvent (3). Acid catalyzed esterification of fatty acids produced by saponification or by pancreatic lipase hydrolysis produced identical products as determined by GC-MS.

Preparation of PMFA[2] Methyl Esters

These were prepared in good yields from a

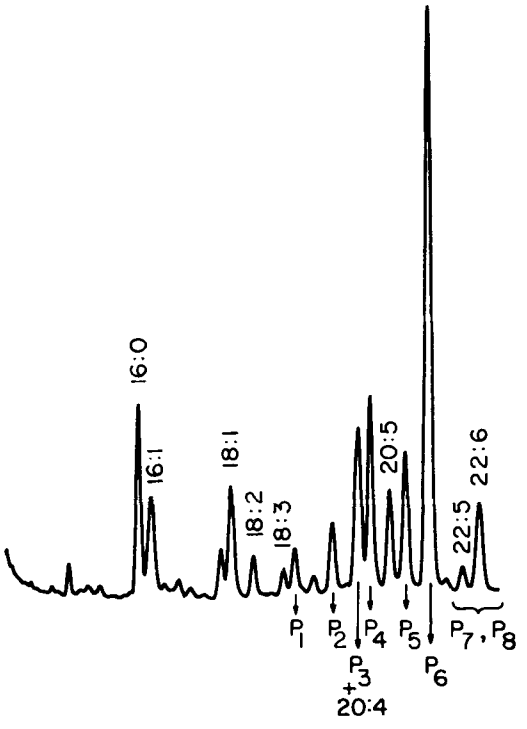

FIG. 1. Chromatogram of fatty acid methyl esters from Northern Pike testes.

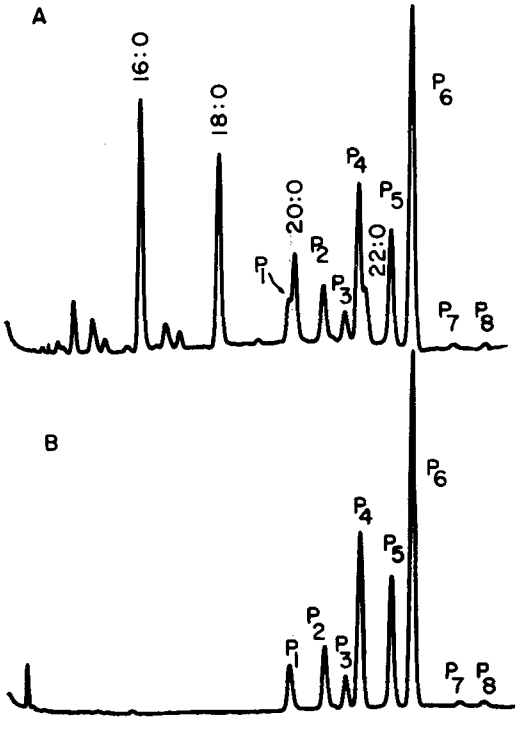

FIG. 2. A. Chromatogram of hydrogenated fatty acid methyl esters from Northern Pike testes. B. Hydrogenated fatty acid methyl esters after urea precipitation of normal methyl esters.

mixture of fatty acid methyl esters by taking advantage of the facts that: (A) they are not hydrogenated readily and (B) they do not form urea inclusion compounds. Selective hydrogenation of the methyl ester mixture was carried out under 8 psig hydrogen, PtO_2 catalyst and methanol-benzene or chloroform solvent for 20 min in a Parr hydrogenator. The PMFA methyl esters were not altered by this treatment.

The solution was decanted from the catalyst and the solvent removed under reduced pressure. The methyl esters were dissolved in warm methanol-urea solution (1 g methyl esters/30 ml methanol containing 5 g urea [4]). After crystallization for 2-4 hr at 4 C, the mixture was centrifuged, and the supernatant, which contained essentially only PMFA methyl esters, was collected. These were removed from the methanol-urea solution by extraction with Skellysolve F. The PMFA methyl esters obtained by this procedure may be used for preparative GLC.

[2]PMFA = pike male fatty acids and includes only the new compounds described.

RESULTS

Figure 1 shows a chromatogram of the fatty acid methyl esters obtained from the lipids of Northern Pike testes. The PMFA esters are labeled P_1-P_8 in the order of increasing retention time. Other fatty acid esters are indicated in the conventional manner. P_7 and P_8 are not visible at this stage of purification. Similarly P_3, which migrates with 20:4, only can be detected following hydrogenation. In this sample, the PMFA esters constituted 55.7% of the total. P_6 is invariably the major member of the series, usually constituting from 50-60% of the total PMFA. P_4 usually comprises ca. 20%. The remaining members of the series are quite variable in amount and one or another may even be absent.

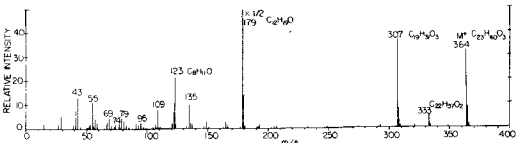

FIG. 3. Low resolution mass spectrum of methyl P_6.

Figure 2A is a chromatogram of hydrogenated Northern Pike testes fatty acid methyl esters. As indicated, all eight members of the

TABLE I

Summary of Mass Spectral Data from Pike Male Fatty Acid Methyl Esters

Methyl ester	Molecular ion (M^+) m/e	Base peak m/e	Relative intensities[a]			Elemental composition[b]
			M^+	$M^+ -29$	$M^+ -57$	
P_1	308	151	18.6	24.7	0.0	$C_{19}H_{32}O_3$
P_2	322	165	18.9	0.4	8.1	$C_{20}H_{34}O_3$
P_3	336	179	29.0	0.3	39.9	$C_{21}H_{36}O_3$
P_4	336	151	29.0	26.5	0.0	$C_{21}H_{36}O_3$
P_5	350	165	22.2	0.2	7.8	$C_{22}H_{38}O_3$
P_6	364	179	41.4	0.4	36.9	$C_{23}H_{40}O_3$
P_7	378	165	38.6	0.5	7.1	$C_{24}H_{42}O_3$
P_8	392	179	37.0	0.2	34.0	$C_{25}H_{44}O_3$

[a]Relative intensity for each fragment as compared to its base peak as 100.0.

[b]Only the P_6 methyl ester composition is based upon elemental analysis. Others were calculated from the molecular ion with the assumption that each has three oxygens.

series are clearly visible, although P_1 and P_4 are resolved poorly from 20:0 and 22:0, respectively. P_7 and P_8 never are present in amounts greater than shown in Figure 2A. Figure 2B shows the same sample following urea treatment. The removal of the normal saturated fatty acid methyl esters virtually is complete. P_6 has been obtained chromatographically pure by subsequent preparative GLC. Elemental analysis of methyl P_6 (Galbraith Laboratories, Knoxville, Tenn.) gave carbon = 75.68%, hydrogen = 10.96%, and oxygen = 13.36%. This corresponds to $C_{23}H_{40}O_3$. High resolution mass spectrometry (Shrader Analytical Laboratories, Detroit, Mich.) showed the molecular ion (M^+) to have m/e = 364.2958 which also corresponds to $C_{23}H_{40}O_3$. It is concluded that the parent fatty acid is $C_{22}H_{38}O_3$.

Figure 3 shows the mass spectrum of methyl P_6. The ion formulae shown were obtained by high resolution MS. The spectrum is not typical of fatty acid methyl esters, being much simpler and possessing few high intensity peaks. Only four fragments have relative intensities greater than 20%, whereas methyl linoleate has over 20. The seven other members of the PMFA group give entirely analagous spectra, with differences as noted in Table I, which indicate that the members of the group are related structurally. As shown in Table I, all give strong molecular ion (M^+) peaks, and these progress by a single methylene unit from m/e 308-392, with P_3 and P_4 being isomers. In the group, there are only 3 base peaks, at m/e 151, 165, and 179 which, again, differ by single methylene units. All but P_1 and P_4 show prominent peaks at m/e $M^+ - 57$ ($-C_4H_9$) and these instead have a peak at m/e $M^+ - 29$ ($-C_5H_5$). All showed the $M^+ - 31$ peak characteristic of methyl esters. The base peak ion fragment given by methyl P_6 contains the unidentified oxygen. It appears to be located centrally, since the fragment at m/e 307 presumably resulted from the loss of C_4H_9 from the tail end of the molecule, while the base peak arose from cleavage at the ester end.

We have been unable as yet to characterize methyl P_6 further by either physical or chemical means. Work presently underway, which presumably will permit us to give a detailed description of these compounds, will be described later.

Distribution of PMFA in Northern Pike Tissue

Although we now find small amounts of PMFA in all tissues examined (muscle, heart, and blood), only in the testes and liver do they occur as major components. Table II shows the fatty acid composition and distribution in the testes and liver of a single male pike caught in August 1973. In the testes lipids the PMFA account for 29.6% of the total fatty acids. These are concentrated in the TG and absent from the PL which contain, instead, the greater proportion of the polyunsaturated acids. In the liver lipids, where the PMFA constitute 26.4% of the total fatty acids, they are seen to be concentrated in the CE and, to a lesser degree, in the TG. This distribution may be considered typical, but there are marked variations, both seasonal and individual. From January-March, the PMFA content of liver is seldom above 5% of the total fatty acids. It rises thereafter to values shown in Table II. Even at this time, however, individuals are found with low PMFA in the liver and considerably higher amounts in the testes which are always a good source of these compounds.

TABLE II

Fatty Acid Composition of Northern Pike[a] Liver and Testes Lipids

Methyl ester[b]	Liver			Testes			
	Total lipids %	Triglycerides %	Phospholipids %	Total lipids %	Cholesteryl esters %	Triglycerides %	Phospholipids %
16:0	12.8	8.0	21.2	11.1	2.3	10.7	23.7
16:1	7.6	7.2	9.3	7.4	2.6	9.2	2.5
18:0	4.0	0.8	4.9	3.3	0.5	3.7	5.0
18:1	7.6	6.4	11.4	13.8	2.6	16.3	7.1
18:2ω6	2.1	2.8	2.6	6.7	T[c]	8.5	2.8
18:3ω3	2.0	2.4	2.6	3.2	T	4.1	1.4
20:4ω6	14.7	1.6	17.0	6.5	---	5.5	12.3
20:5ω3	7.3	1.2	14.5	4.4	---	4.6	6.4
22:5ω6	1.8	T	1.6	1.5	---	1.5	2.8
22:5ω3	2.4	T	2.1	3.1	---	3.3	2.1
22:6ω3	7.8	2.0	12.9	12.6	---	9.2	31.6
P_1	1.5	3.2	---	1.2	---	1.5	---
P_2	3.0	5.6	---	2.2	---	3.0	---
P_3	ND[c]	1.6	---	ND	2.6	ND	---
P_4	5.5	15.1	---	6.5	18.2	6.5	---
P_5	4.9	12.0	---	1.7	2.6	1.3	---
P_6	14.7	30.3	---	14.8	68.6	11.1	---
P_1-P_6	29.6	67.8	---	26.4	92.0	23.4	---
Percent fat	ND			11.8			

[a]Male, 450 g, caught August 1973.

[b]Identification in all cases made by comparison with authentic samples and gas-liquid chromatograph-mass spectrometer.

[c]T = trace, less than 0.1%, and ND = not determined.

We now have found individuals of other species of fish with large (20-50% of the total fatty acids) amounts of PMFA in their livers. These are the Bigmouth Buffalo (*Ictiobus cyprinellus*), the Northern Black Bullhead (*Ictalurus melas*), the Bluegill (*Lepomis machrochirus*), and the Northern Rock Bass (*Ambloplites rupestris*). Again, the PMFA are predominantly a male characteristic but, in these fish, occur only in trace amounts in the testes. As with the Northern Pike, P_6 is invariably the major member of the series.

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Sphingophospholipids of Species of *Aedes* and *Culex* Mosquito Cells Cultivated in Suspension Culture from Logarithmic and Stationary Phases of Growth

T.K. YANG, E. MC MEANS, L.E. ANDERSON, and **H.M. JENKIN,** The Hormel Institute, University of Minnesota, Austin, Minnesota 55912

ABSTRACT

Two sphingophospholipids, sphingomyelin (ceramide phosphorylcholine), and ceramide phosphorylethanolamine, were isolated and purified from cells of four mosquito species obtained from the logarithmic and stationary phases of growth. Quantitation of the two lipids in both phases of growth from cells of each species was made. The fatty acid composition of the two lipids was compared between the cell types and the two phases of growth. There was a tendency for an increase of ceramide phosphorylcholine and a decrease of ceramide phosphorylethanolamine in the stationary phase. Longer chain fatty acids of ceramide phosphorylcholine were observed in the stationary phase than in the logarithmic phase of growth of the mosquito cells. The four species had distinctly different fatty acid patterns in the two complex lipids which might be useful for taxonomic purposes and cell identification.

INTRODUCTION

Mosquito cells, in many ways, are satisfactory models for lipid metabolic and biochemical studies, since they contain high amounts of lipid, are cultivated easily, and are vectors of virus diseases (1). These cells are used as host cells in this laboratory to cultivate arboviruses for the study of viral membrane lipids. Sphingomyelin (ceramide phosphorylcholine [CPC]) has been studied in humans by a number of investigators and has been suggested to be a structural component of cell membranes influenced by a complex interplay of physiological and biochemical factors (2-5). Ceramide phosphorylethanolamine (CPE), an analogue of sphingomyelin, has been isolated from marine invertebrates (6), dipterous larvae and adults (7-11), and cultured mosquito cells (12). However, few studies have been performed associating the chemical nature and metabolism of these two sphingophospholipids in insects. Such information could aid in determining lipid differences between mosquito species, the time to study lipid metabolism of arbovirus infected cells and associate virus susceptibility to lipid composition of the cells. In this report, we have compared the two sphingophospholipids of cells of four mosquito species from the logarithmic and stationary phases of growth.

MATERIALS AND METHODS

Cells: Aedes albopictus cells, Singh's line (13) were obtained from F. Paul, Naval Medical Research Institute, Bethesda, Md. *Aedes aegypti* cells, Singh's line (13), *Culex tritaeniorhynchus* (14), and *Culex quinquefasciatus* (15) were received from S.H. Hsu, Naval Medical Research Unit No. 2, Taipei, Taiwan. The cells were grown at 28 C in 250 ml spinner units containing 150 ml Leafhopper medium (16) supplemented with 20% newborn calf serum, 100 units penicillin, and 100 μg streptomycin/ml medium. The cells were harvested in the logarithmic (2-3 days) and stationary (10 days) phases of the growth (E. McMeans, T.K. Yang, L.E. Anderson, and H.M. Jenkin, unpublished data). The cells were sedimented at 500 x g for 10 min, the supernatant fluid removed, and the pellet washed 3 times in Hanks's balanced salt solution without calcium and magnesium. The wet packed cells were pooled and extracted immediately or held no more than 1 week at -20 C under nitrogen.

Extraction and Analysis of Lipids

The general analytical procedures employed in this study have been described elsewhere (17,18). The lipid was extracted from the wet cells by the procedure of Bligh and Dyer (19). The total lipids (50-100 mg) were fractionated into neutral lipids, glycolipids, and phospholipids by the method of Rouser (20) using silicic acid column chromatography. Phospholipids were separated on thin layer chromatographic (TLC) plates coated with Silica Gel H (E. Merck AG, Darmstadt, Germany) using chloroform-methanol-acetic acid-water, 25:15:4:2. The band containing phosphatidylserine, phosphatidylinositol, and CPE was rechromatographed on TLC plates coated with Silica Gel H containing magnesium acetate (1.5 g magnesium acetate and 20 g Silica Gel H suspended in 55 ml water). The solvent system for the separation was chloroform-methanol-

TABLE I

Relative Percentage of Sphingophospholipids in Phospholipid Fraction of Mosquito Cells Cultivated in vitro

Mosquito cell species	Ceramide phosphorylcholine		Ceramide phosphorylethanolamine	
	Logarithmic phase	Stationary phase	Logarithmic phase	Stationary phase
Aedes aegypti	1.2 ± 0.0[a]	1.7 ± 0.2	6.0 ± 0.8	4.7 ± 0.1
Aedes albopictus	1.9 ± 0.0	2.6 ± 0.1	7.0 ± 0.4	5.7 ± 0.1
Culex quinquefasciatus	1.9 ± 0.1	6.1 ± 0.3	3.8 ± 0.5	4.6 ± 0.2
Culex tritaeniorhynchus	3.2 ± 0.2	3.1 ± 0.1	3.8 ± 0.3	3.6 ± 0.2

[a]Mean ± standard error of duplicate samples from two independent experiments.

ammonia, 65:25:5.

For purification of the sphingophospholipids, a sample of the phospholipid fraction (10-15 mg) was subjected to mild alkaline methanolysis to destroy the ester-linked glycerophospholipids according to Sweeley (21). The remaining amide-linked sphingolipids subsequently were separated by TLC using chloroform-methanol-acetic acid-water, 25:15:4:2. Tentative identification of lipids was made by two dimensional TLC using a solvent system of chloroform-methanol-7N ammonia, 65:30:4, in the first direction and chloroform-methanol-acetic acid-water, 170:25:25:6, in the second direction and sprayed with ninhydrin and molybdenum reagents. Further identification was made by IR spectrophotometry (model 21, Perkin-Elmer, Norwalk, Conn.). Methyl esters of the fatty acids were prepared by transesterification of the lipids with 5% HCl-methanol at 100 C for 4 hr. The methyl esters were analyzed by gas liquid chromatography (GLC), as described previously (18). The quantity of the lipids present in the sample was calculated from the amount of methyl ester analyzed by GLC using an internal standard (21:0 fatty acid, 22).

RESULTS

Lipid Content of Mosquito Cells

The results are based upon duplicate analyses of two independent cell samples prepared under identical conditions. The total lipid content of the cells (percent of dry wt) was 18.2-23.7% for the two species of *Aedes* and 30.9-40.7% for the two species of *Culex*. The phospholipids constituted 48.9-69.5% of the total lipid in the *Aedes* cells and 40.5-52.8% in the *Culex* cells. The major phospholipid components were phosphatidylethanolamine and phosphatidylcholine. The minor phospholipids were phosphatidylinositol, phosphatidylserine, lysophosphatidylcholine, CPE, and CPC. Two dimensional TLC showed that two components had remained after the mild alkaline methanolysis; using reference sphingophospholipid standards, one of them was shown to be sphingomyelin; the other was proven to be CPE by means of two dimension TLC, IR spectra, and ninhydrin and molybdenum sprays.

Table I illustrates the amounts of the two sphingophospholipids found in the cells of four species of mosquito from the logarithmic and stationary phases of growth. CPC constituted 1.2-3.2% and 1.7-6.1% of the total phospholipids in the logarithmic phase and stationary phase, respectively. The CPC content was found to be slightly higher in the stationary phase then in the logarithmic phase of cells from three species, whereas *C. tritaeniorhynchus* had ca. the same amount of CPC in both phases of growth. The CPE content of cells decreased in the stationary phase of all species except *C. quinquefasciatus*.

Fatty Acid Composition of Sphingophospholipids

The fatty acid profiles of CPC in the four species of cells are shown in Table II. CPC in all species contained at least 90% saturated fatty acids. The major fatty acids were 16:0, 20:0, and 22:0 (64-88%). Longer chain fatty acids of the CPC were observed in the stationary phase of all cell types, as noted by an increase of the 20:0 and 22:0 fatty acids, whereas 16:0 and 18:0 fatty acids decreased compared to cells analyzed from the logarithmic phase.

Table III shows the fatty acid profile of CPE in cells of the four species. The fatty acid patterns of the *Aedes* cells and the *Culex* cells differed from each other. Of the fatty acids, 84-92% were 20:0 and 22:0 from both stages of growth of *Aedes* cells. The 20:0 fatty acid alone represented 71-80% of the total fatty acid. In *Culex* cells, ca. equal amounts of 20:0 and 22:0 were found in both phases of growth. No significant differences were found in the fatty acid composition of CPE between the logarithmic and stationary phases of growth of all species.

TABLE II

Fatty Acid Profiles of Ceramide Phosphorylcholine in Mosquito Cells

Fatty acid	*Aedes aegypti*		*Aedes albopictus*		*Culex quinquefasciatus*		*Culex tritaeniorhynchus*	
	Log phase[a]	Sta. phase[b]	Log phase	Sta. phase	Log phase	Sta. phase	Log phase	Sta. phase
14:0[c]	0.6 ± 0.1[d]	0.2 ± 0.1	0.4 ± 0.2	0.3 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
15:0	0.6 ± 0.1	0.5 ± 0.2	1.2 ± 0.1	0.5 ± 0.0	0.3 ± 0.1	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0
16:0	8.1 ± 0.2	3.7 ± 0.6	36.1 ± 0.1	9.4 ± 0.3	14.6 ± 0.6	9.2 ± 0.2	20.5 ± 0.9	10.1 ± 0.5
17:0					0.3 ± 0.1	0.1 ± 0.0	0.7 ± 0.2	0.2 ± 0.0
18:0	4.7 ± 0.2	4.5 ± 0.2	9.1 ± 0.3	4.6 ± 0.1	7.3 ± 0.1	4.3 ± 0.7	10.0 ± 2.0	4.9 ± 0.3
18:1	0.9 ± 0.0	0.8 ± 0.6	0.7 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.3 ± 0.0
19:0						0.3 ± 0.1	0.7 ± 0.3	0.2 ± 0.0
20:0	65.5 ± 0.1	72.5 ± 2.2	16.4 ± 0.6	59.5 ± 0.2	35.3 ± 0.2	41.7 ± 0.1	22.6 ± 2.8	29.4 ± 1.1
22:0	14.1 ± 0.6	12.0 ± 0.2	11.7 ± 0.3	15.4 ± 0.4	32.4 ± 2.1	36.0 ± 1.0	26.1 ± 0.3	40.8 ± 0.8
23:0	1.1 ± 0.3	1.9 ± 0.1	3.5 ± 0.2	3.2 ± 0.5	1.9 ± 0.1	1.6 ± 0.1	3.0 ± 0.3	2.1 ± 0.2
24:0	1.9 ± 0.0	2.0 ± 0.1	8.5 ± 0.5	2.9 ± 0.1	4.3 ± 0.2	3.6 ± 0.2	7.8 ± 0.1	7.6 ± 0.6
24:1	2.6 ± 0.4	1.3 ± 0.3	12.0 ± 0.8	2.7 ± 0.1	3.1 ± 1.4	2.8 ± 0.1	7.9 ± 0.3	4.2 ± 0.3

[a]Logarithmic phase of growth

[b]Stationary phase of growth.

[c]Number of carbon atoms in fatty acids : number of double bonds.

[d]Relative percent of the total fatty acids, mean ± standard error of duplicate samples from two independent experiments.

TABLE III

Fatty Acid Profiles of Ceramide Phosphorylethanolamine in Mosquito Cells

Fatty acid	*Aedes aegypti*		*Aedes albopictus*		*Culex quinquefasciatus*		*Culex tritaeniorhynchus*	
	Log phase[a]	Sta. phase[b]	Log phase	Sta. phase	Log phase	Sta. phase	Log phase	Sta. phase
16:0[c]	0.7 ± 0.1[d]	1.1 ± 0.2	0.8 ± 0.2	0.6 ± 0.0	0.9 ± 0.2	1.1 ± 0.0	0.8 ± 0.1	0.8 ± 0.2
18:0	4.5 ± 0.3	4.7 ± 0.5	4.5 ± 0.5	3.7 ± 0.0	4.9 ± 0.3	3.3 ± 0.8	8.2 ± 1.5	5.1 ± 0.6
18:1	0.2 ± 0.0	0.8 ± 0.6	0.4 ± 0.3	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.7 ± 0.4
18:2					0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1
19:0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.3 ± 0.1
20:0	80.4 ± 2.4	79.6 ± 0.3	71.1 ± 0.4	73.7 ± 0.7	49.9 ± 0.5	48.2 ± 2.5	45.9 ± 2.7	41.4 ± 0.6
22:0	12.6 ± 3.0	11.9 ± 2.0	19.9 ± 0.2	18.8 ± 0.6	40.5 ± 1.2	43.3 ± 2.9	38.7 ± 0.9	45.0 ± 1.9
23:0	0.8 ± 0.4		1.8 ± 1.4	1.9 ± 0.1	0.8 ± 0.2	0.7 ± 0.0	0.9 ± 0.0	0.9 ± 0.2
24:0	0.7 ± 0.1	0.7 ± 0.1	1.1 ± 0.2	1.0 ± 0.1	2.2 ± 0.0	2.4 ± 0.4	4.4 ± 0.4	5.5 ± 0.2

[a]Logarithmic phase of growth.
[b]Stationary phase of growth.
[c]Number of carbon atoms in fatty acids: number of double bonds.
[d]Relative percent of the total fatty acids, mean ± standard error of duplicate samples from two independent experiments.

DISCUSSION

Rouser and Solomon (4) found the amount of CPC in human aorta increased with increase in age, whereas most other phospholipids decreased. Chin (5) reported that the CPC content of human serum tended to rise and chain elongation of the CPC fatty acids occurred with increasing age, suggesting aging is one of the significant factors in the regulation of this lipid and its fatty acid composition. Ställberg-Stenhagen and Svennerholm (2) reported that in normal human frontal lobe, the stearic acid decreased and the C_{22}-C_{26} acids increased with age. Svennerholm and Ställberg-Stenhagen (3) later suggested there was a relative lack of enzymes associated with fatty acid elongation in the infant brain and a rapid increase in the activity of such enzymes with age. Lipsitz, et al., (23) found a distinct age difference in the fatty acid composition of neutral lipids of the house cricket *Acheta domesticus*; the most marked developmental change was a reduction in the myristic acid content of the monoglyceride. High concentration of short chain fatty acids was found in at least three neutral lipid components, and considerable alterations in the concentration of their fatty acids occurred during development. Stephen and Gilbert (24) demonstrated that chain elongation of fatty acids was a function of the developmental stage in *Hyalaphora cecropia*.

In our study, it was found that the amount of CPC was increased in the stationary phase of both species of *Aedes* and *Culex quinquefasciatus*. Longer chain fatty acids ($\geq$20:0) of CPC were observed in the stationary phase of the four species studied. These results suggest that there are close similarities in the regulation of the sphingomyelin and its fatty acid composition between human and insect systems.

The four species studied have distinctly different fatty acid patterns which might be useful for taxonomic purposes and cell identification, particularly to distinguish tissue culture cells when a question of mislabeling or contamination may have occurred. The four species could be distinguished from each other by the fatty acid patterns of CPC, whereas the fatty acid patterns of CPE can only be used to differentiate *Aedes* species from *Culex* species.

Differences in fatty acid compositions of CPC and CPE were found within the same cell species. Small amounts of 14:0 and 15:0 were present in CPC but absent in CPE. The CPC had higher amounts of 16:0 than that found in CPE. There was a tendency for an increase of CPC and a decrease of CPE in the stationary phase. These observations suggest that the

metabolism of CPC and CPE might be different and deserves further study.

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Metabolism of 2,4-^{3}H-14α-Methyl-5α-Ergost-8-Enol and 2,4-^{3}H-5α-Ergosta-8,14-Dienol in *Chlorella Ellipsoidea*[1]

L.B. TSAI and **G.W. PATTERSON**, Botany Department, University of Maryland, College Park, Maryland 20742, and **C.F. COHEN**, Insect Physiology Laboratory, Plant Protection Institute[2], Beltsville, Maryland 20705 and **P.D. KLEIN**, Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois 60439

ABSTRACT

5α-Ergosta-8,14-dienol and 14α-methyl-5α-ergost-8-enol were synthesized chemically from ergosterol and labeled with tritium at the C-2 and C-4 positions. Both labeled sterols, when incubated with growing cultures of *Chlorella ellipsoidea*, were converted to ergost-5-enol but not to C-27 or C-29 sterols. *Chlorella ellipsoidea*, thus, has the capability of removing the C-14 methyl group and converting the 8(9) and 8,14 double bond systems to the 5(6) position. Brassicasterol produced by this organism is not labeled in these experiments, indicating that it is not derived from a saturated side chain precursor.

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[2]ARS, USDA.

INTRODUCTION

In the last few years, an increasing amount of work has been concerned with the identification of compounds involved in the sterol biosynthetic pathway immediately prior to and after the removal of the 14α-methyl group. The conversion of 14α-methyl-9β,19-cyclo-5α-cholestan-3β-ol (pollinastanol) into cholesterol has been reported to occur in tobacco leaves (1). Cholesta-8,14-dien-3β-ol has been demonstrated to be converted into cholesterol using enzymatic preparations from animal systems (2-7). The accumulation of $\Delta^{8,14}$-sterols and 14α-methyl Δ^8-sterols has been observed as a result of AY-9944 treatment and triparanol treatment, respectively, in *Chlorella ellipsoidea* (8,9). These sterols have been suggested to be precursors in sterol biosynthesis in this green

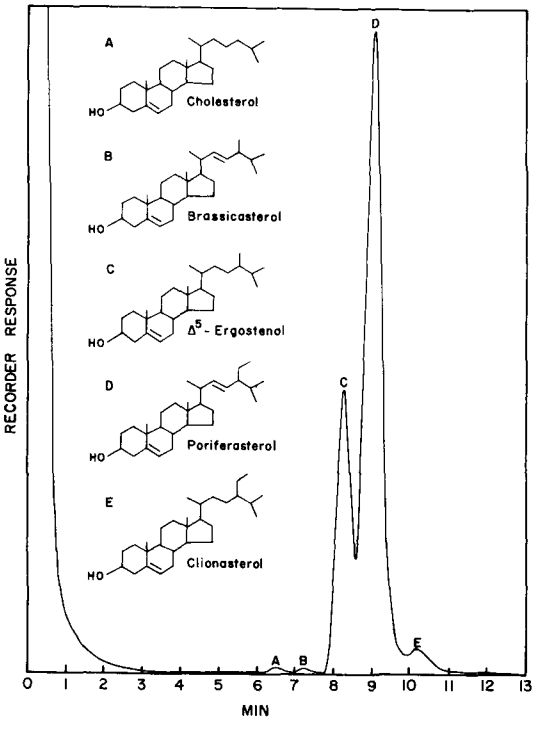

FIG. 1. Gas chromatograms and structures of sterols from an autotrophic culture of *Chlorella ellipsoidea*.

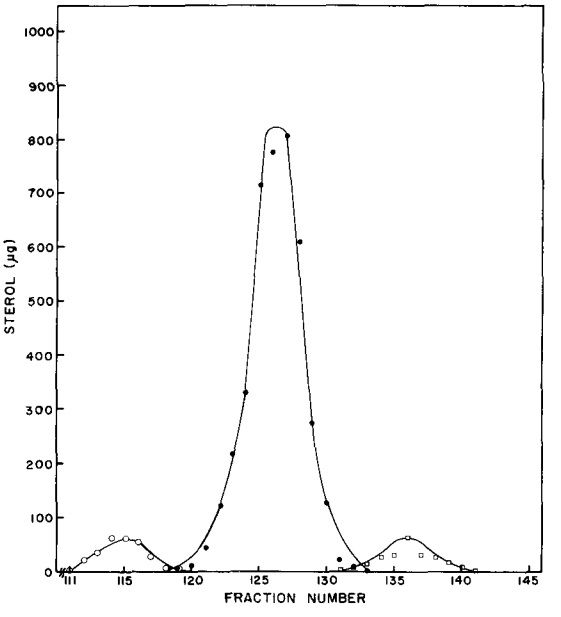

FIG. 2. Separation of cholesterol, Δ^5-ergostenol, and clionasterol on a lipophilic Sephadex column —●— = Δ^5-Ergostenol, —□— = clionasterol, and —○— cholesterol.

alga (9). With the exception of the pollinastanol work (1), potential sterol precursors, such as these, have not been shown to be converted to normally occurring sterols of plants. In this paper, we demonstrate the incorporation of [2,4-^{3}H]-14α-methyl-5α-ergost-8-en-3β-ol and [2,4] ^{3}H]-5α-ergosta-8,14,-dien-3β-ol into Δ^5-ergostenol in growing *C. ellipsoidea* cultures.

EXPERIMENTAL PROCEDURES

$\Delta^{8,14}$-Ergostadienol (5α-ergosta-8,14-dien-3β-ol) and 14α-methyl Δ^8-ergostenol (14α-methyl-5α-ergost-8-en-3β-ol) were synthesized chemically from ergosterol (10,11) and labeled with tritium at the C-2 and C-4 positions, as described by Thompson, et al. (12). Purity of the labeled sterols was established by gas liquid chromatography (GLC), thin layer chromatography, and IR.

The desired amount of labeled sterol was dissolved in 0.1-0.2 ml 85% ethanol and added to a growing culture of *C. ellipsoidea*.

C. ellipsoidea (Gerneck, Ind. culture collection no. 247) was grown autotrophically at 27 C in sterile inorganic medium in Pyrex "carrot" tubes. The cultures were bubbled with a 1% CO_2-in-air mixture and illuminated with white light of 900 foot candles.

Sterol Extraction and Isolation

Algal cells were grown for 6-8 days in the presence of labeled sterol and harvested by centrifugation. Sterols were extracted from freeze-dried cells with chloroform-methanol (2:1, v/v) and partially purified by digitonin precipitation (13).

In the $\Delta^{8,14}$-ergostadienol labeling experiments, the total free sterol isolated was acetylated, and unconverted $\Delta^{8,14}$-ergostadienol acetate was separated from the naturally occurring sterol acetates (Fig. 1) of *C. ellipsoidea* (cholesterol, 1.2%; brassicasterol, 0.8%; Δ^5-ergostenol, 31%; poriferasterol, 61%; and clionasterol, 6%) by column chromatography on 12% AgNO-impregnated Anasil B. Elution was attained with increasing percentages of diethyl ether in hexane (14). Rechromatography of the faster eluting *Chlorella* sterol acetates was sufficient to remove all traces of $\Delta^{8,14}$-ergostadienol acetate.

The sterol diene acetates of *Chlorella* were separated from the sterol monoene acetates by column chromatography on Anasil B (15). Elution was attained with 7% diethyl ether in hexane. Final isolation and purification of all five *C. ellipsoidea* sterol acetates were obtained by chromatography on a lipophilic Sephadex column (Figs. 2 and 3) which was prepared

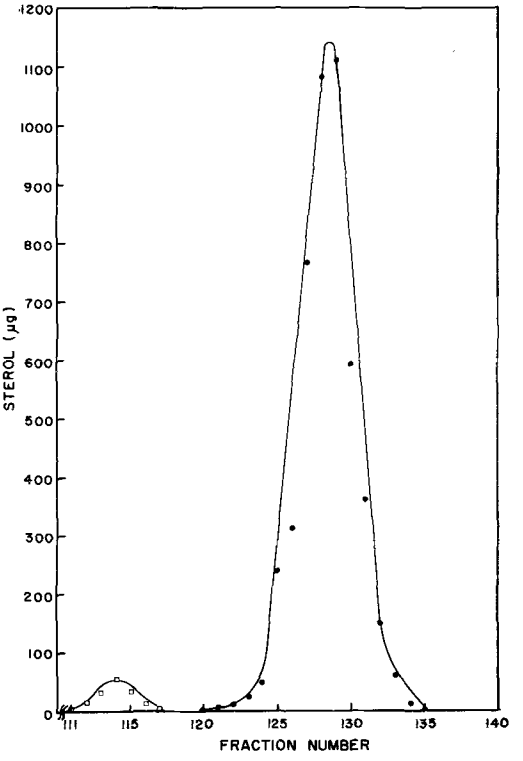

FIG. 3. Separation of brassicasterol and poriferasterol on a lipophilic Sephadex column —●— = Brassicasterol and —□— = poriferasterol.

from Sephadex LH-20 by the method of Ellingboe, et al. (16). Sterol acetates were eluted in 20 ml fractions by gravity flow (20 ml/hr) of 5% hexane in methanol.

The total sterol fraction from the culture incubated with 14α-methyl-Δ^8-ergostenol was chromatographed on an Anasil B. Column. Free sterols were separated into monoenes (including 14α-methyl-Δ^8-ergostenol) and dienes by elution with 20% diethyl ether in hexane. The diene fraction was acetylated and rechromatographed on Anasil B (elution with 7% ether in hexane) to remove all traces of 14α-methyl-Δ^8-ergostenol. The sterol diene acetates were separated on Sephadex, as previously described. The monoene fraction also was acetylated and chromatographed on Sephadex. The elution of 14α-methyl-Δ^8-ergostenol acetate coincided with that of cholesterol acetate and slightly contaminated the Δ^5-ergostenol acetate fractions. Clionasterol acetate was obtained in pure form. Rechromatography of the contaminated Δ^5-ergostenol acetate fractions produced pure Δ^5-ergostenol acetate. No further attempt was made to isolate cholesterol.

Quantitation and identification of sterols in

TABLE I

Incubation of *Cholorella ellipsoidea* with [2,4-^{3}H]-14α-Methyl Ergost-8-Enol

Sterol	Sterol wt. (μg)	Radiation (dpm)	Specific activity (dpm/μg)
Total ^{3}H-sterol added	1450 (3400)	1 x 10^8 (2 x 10^8)	64000 (64000)
Total sterol extracted[a]	53400 (33600)	9 x 10^6 (2 x 10^7)	170 (600)
Brassicasterol[b]	20 (–)	150 (–)	7 (–)
Ergost-5-enol[b]	1900 (1000)	84400 (62500)	45 (63)
Clionasterol[b]	90 (90)	393 (764)	5 (9)
Poriferasterol[b]	470 (660)	177 (95)	0 (0)

[a]Total cell dry wt: experiment I = 18.4 g. Experiment II = 11.6 g. Values in parenthesis are from the second experiment.

[b]Most highly purified fractions.

TABLE II

Incubation of *Chlorella ellipsoidea* with [2,4-^{3}H]-Ergosta-8,14-Dienol

Sterol	Sterol wt (μg)	Radiation (dpm)	Specific activity (dpm/μg)
Total ^{3}H-sterol added	581 (796)	6.2 x 10^7 (8.8 x 10^7)	108000 (108000)
Total sterol extracted[a]	18400 (20100)	1.6 x 10^6 (4.2 x 10^6)	87 (209)
Cholesterol[b]	21 (30)	67 (102)	3 (3)
Brassicasterol[b]	16 (35)	73 (82)	4 (5)
Ergost-5-enol[b]	490 (996)	24100 (91600)	49 (92)
Poriferasterol[b]	783 (1560)	444 (217)	1 (0)
Clionasterol[b]	66 (69)	187 (291)	3 (4)

[a]Total cell dry wt: experiment I = 6.0 g. Experiment II = 5.0 g. Percent of incorporation into 4-desmethyl sterols: experiment I = 0.35%. Experiment II = 0.72%. Values in parenthesis are from the second experiment.

[b]Most highly purified fractions.

all experiments were made by GLC on a 3% SE-30 column, and radioactivity of isolated sterols was determined with a scintillation counter to determine specific activities.

RESULTS AND DISCUSSION

Incorporation of [2,4-^{3}H]-14α-Methyl-Ergost-8-Enol

[2,4-^{3}H]-14α-Methyl-Δ^8-ergostenol was converted only into Δ^5-ergostenol by *C. ellipsoidea*. The results are shown in Table I. This indicates that *C. ellipsoidea* contains an enzyme system which can remove the 14α-methyl group of the exogenous sterol precursor. It also shows that removal of the 14α-methyl group is not dependent upon the presence of a 4α-methyl group on the sterol nucleus. This demonstrates that 14α-methyl-Δ^8-sterols can serve as intermediates in sterol biosynthesis in this alga. Such sterols probably are intermediates in other *Chlorella* species, such as *Chlorella emersonii* and *Chlorella sorokiniana*, since 14α-methyl Δ^8-sterols also have been isolated from drug treated cultures of these organisms (13,17).

Incorporation of [2,4-^{3}H]-$\Delta^{8,14}$-Ergostadienol

The conversion of $\Delta^{8,14}$-ergostadienol into

Δ^5-ergostenol of this alga (Table II) provided strong evidence that the alga contains Δ^{14}-reductase to reduce the C-14(15) double bond and other enzymes to convert the Δ^8 bond to the Δ^5 position. None of the other *Chlorella* sterols contained significant radioactivity.

Neither 14α-methyl Δ^8-ergostenol nor $\Delta^{8,14}$-ergostadienol has been found to be converted into brassicasterol (Table I and Table II). This indicates the addition of the Δ^{22} bond of brassicasterol must arise from a pathway other than direct introduction into a saturated side chain. However, in yeast, direct introduction of the Δ^{22} bond has been demonstrated (18). The low incorporation and conversion of labeled sterols to *Chlorella* sterols occurs with known intermediates (unpublished) of sterol biosynthesis and is probably due, in part, to the very tough cell wall of *Chlorella*, which may be viewed as a significant barrier to sterol absorption by the cell.

Neither C-29 sterols nor cholesterol of *C. ellipsoidea* was found to be labeled in these studies. This was expected, since the second alkylation reaction in the side chain requires a 24-methylene group as substrate (19) and dealkylation at C-24 has not been demonstrated in plants (20).

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Studies of Serum Lecithin-Cholesterol Acyl Transferase Activity in Rat: Effect of Vitamin E Deficiency, Oxidized Dietary Fat, or Intravenous Administration of Ozonides or Hydroperoxides

TAKEHIKO TAKATORI and **O.S. PRIVETT**, The Hormel Institute, University of Minnesota, Austin, Minnesota 55912

ABSTRACT

Serum lecithin-cholesterol acyl transferase activity in the rat was studied in animals raised on diets devoid of vitamin E or containing oxidized fat and in the serum of normal animals after intravenous administration of hydroperoxides or ozonides. Lecithin-cholesterol acyl transferase activity was suppressed by a vitamin E deficiency but was elevated in the serum of the animals fed diets containing oxidized fat. The intravenous injection of hydroperoxides or ozonides of linoleate into the tail vein of rats caused an immediate depression of serum lecithin-cholesterol acyl transferase activity. The effect of hydroperoxides was more severe than the ozonides, but, with sublethal doses of these compounds, the activity of the enzyme became normal within 24 hr. Hydroperoxides and ozonides also suppressed the activity of lecithin-cholesterol acyl transferase of rat serum of normal animals in vitro. The suppression of lecithin-cholesterol acyl transferase activity by hydroperoxides of ozonides was only partially restored by the addition to the serum of 2-mercaptoethanol.

INTRODUCTION

Hydrogen peroxide and oxidized lecithin have been shown to inhibit the activity of lecithin-cholesterol acyl transferase (LCAT) in vitro (1,2). Serum LCAT activity also was suppressed in human subjects exposed to high oxygen tensions (3). In the course of these studies, it was demonstrated that inhibition of LCAT activity involved reaction with sulfhydryl (SH) groups, inasmuch as SH blocking agents, such as p-chloromercuribenzoate, inhibited the activity of the enzyme (4) and also protected it against hydrogen peroxide (2). Accordingly, it also was demonstrated that the SH groups could be unblocked and the activity restored by SH-containing compounds, such as 2-mercaptoethanol (2). The oxidation of SH groups in proteins in vivo and in vitro during lipid peroxidation has been demonstrated by several investigators (5-7). Clark, et al., (8) demonstrated similar reactions between hydrogen peroxide and lipoproteins. Therefore, it appeared that LCAT might be affected by the toxicity of hydroperoxides and ozonides injected intravenously (9) and by the ingestion of oxidized fat (10). It also appeared that the activity of LCAT might be affected by a vitamin E deficiency because of the relationship of this vitamin to in vivo oxidation (11-13).

Reported here are studies of effects upon serum LCAT activity of rats fed diets depleted of vitamin E or containing oxidized fat. Effects of hydroperoxides or ozonides injected intravenously into the tail vein of rats, as well as in in vitro experiments on serum LCAT activity, also were studied.

MATERIALS AND METHODS

Highly purified methyl linoleate (ML) was obtained from the Lipids Preparation Laboratory, The Hormel Institute, Austin, Minn.; 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) from Aldrich Chemical Co., Rochester, N.Y.; and bovine serum albumin (fraction V) (BSA) and dithiothreitol (DTT) from Sigma Chemical Co., St. Louis, Mo. Scintillation chemicals, 2,5-diphenyl oxazole (PPO) and 1,4-bis-2-(4-methyl-5-phenyl oxazolyl)-benzene (dimethyl POPOP), were obtained from Packard Instrument Co., Downers Grove, Ill. Cholesterol-4-^{14}C (specific activity, 55.6 mCi/mmole) was obtained from Amersham Searle Corp., Arlington Heights, Ill., and purified by thin layer chromatography (TLC) using Silica Gel H-coated plates with a solvent system consisting of petroleum ether-diethyl ether-acetic acid, 85:15:1.

Methyl linoleate hydroperoxide (MLH) and methyl linoleate ozonide (MLO) were prepared in highly purified form from ML, as previously described (9,14,15).

Experiments on the toxic effects of MLH and MLO were carried out with mature male rats of the Sprague-Dawley strain, weighing 250-350 g, purchased from Dan Rolfsmeyer Co., Madison, Wisc., and fed a basic sucrose-casein diet containing all of the required vita-

mins and minerals and 10% safflower oil, as previously described (16). MLO, MLH, or ML was injected into the tail vein of these animals in the form of an emulsion with 0.2 ml fresh rat serum at a dosage of 20 mg/100 g body wt. Blood samples were withdrawn from the eye (retroocular plexus) of animals under a light ether anesthesia after fasting them overnight. The blood was held at 0 C for 1 hr to clot and the serum separated by centrifugation.

Determination of LCAT activity was carried out on freshly prepared serum, generally as described by Stokke and Norum (17). For this analysis, an albumin-cholesterol-4-^{14}C solution was prepared similar to the method of Porte and Havel (18). A benzene solution of cholesterol-4-^{14}C containing ca. 2.5 μCi was added to a volumetric flask (5 ml); the solvent was evaporated under a stream of nitrogen, and the residue dissolved in 0.1 ml absolute alcohol. Five ml 5% BSA in 0.15 M NaCl was injected rapidly into the solution. The volume was reduced by evaporation under a stream of nitrogen to remove the alcohol and the solution made to 5 ml by the addition of distilled water. DTNB, 2-mercaptoethanol, and DTT were dissolved in 0.1 M potassium phosphate buffer (pH 7.1) to give a final concentration of 1.5 mM, 0.1 M, and 0.1 M of each, respectively (17). The standard incubation mixture consisted of 0.1 ml fresh rat serum and 0.1 ml albumin-cholesterol-4-^{14}C solution. If 2-mercaptoethanol, DTNB, MLH, or DTT were used alone or in combination, they were added in appropriate amounts to the incubation mixture with or without preincubation at 0 C for 10 min. The incubations were carried out in glass test tubes for 30 min at 37 C with shaking in a water bath. Under these conditions, the rate of the reaction was constant for ca. 50 min. The reaction was stopped by the addition of 20 volumes of chloroform-methanol, 2:1 v/v. The reaction mixture was allowed to stand overnight; it then was filtered and the precipitate washed twice with fresh chloroform-methanol. The filtrates were combined and evaporated in vacuo at room temperature. The residue was dissolved in a small amount of chloroform and dried further by evaporation of the solvent in vacuo. The final residue then was fractionated by TLC on plates coated with Silica Gel H with petroleum ether-diethyl ether-acetic acid, 85:15:1. The spots were visualized by spraying the plate with 1% iodine in methanol. The spots of cholesterol and cholesteryl ester were marked, and, after evaporation of the iodine, they were scraped into scintillation vials containing 5 g PPO and 0.3 g dimethyl POPOP/liter toluene for determination of their radioactivity

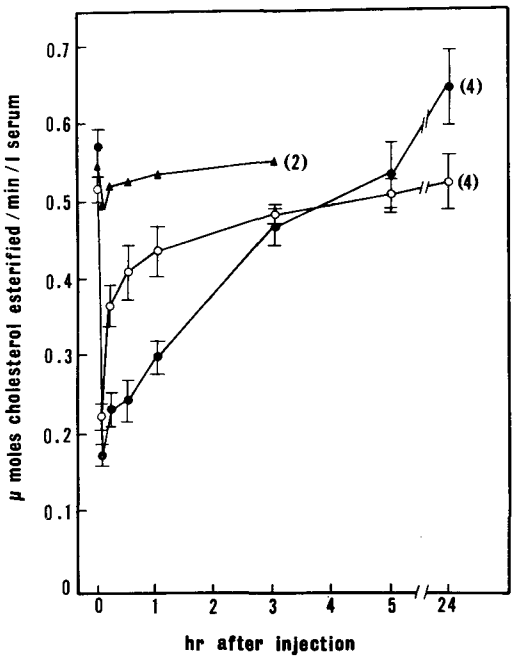

FIG. 1. Time course effect of methyl linoleate hydroperoxide (MLH), methyl linoleate ozonide (MLO), and methyl linoleate (ML) injected intravenously on lecithin-cholesterol acyl transferase activity. Number of animals in parenthesis. The vertical bar at each point represents the standard deviation. For ML injection, each point represents the average value from two animals. See experimental details in the text. ▲—▲ = ML, ●—● = MLH, and ○—○ = MLO.

in a Packard Tricarb model 3002 liquid scintillation spectrometer. The concentration of cholesterol or cholesteryl ester in the original serum was determined in separate experiments by quantitative TLC using pure cholesterol as a standard by the charring photodensitometric technique (19,20). LCAT activity was expressed as nmoles cholesterol esterified/min/liter of serum at 37 C by multiplying the percentage of labeled cholesterol esterified by the free cholesterol of the original sample.

For determination of the effect of a vitamin E deficiency upon LCAT activity, male Sprague-Dawley rats were fed from weaning for 9 months on a vitamin E-free diet prepared as previously described (10). Evidence of a vitamin E deficiency was determined by the susceptibility of the erythrocytes to dialuric acid-induced hemolysis (10,21).

The effect of ingestion of oxidized fat upon LCAT activity was determined with animals fed diets containing oxidized safflower oil or menhaden oil (containing 2% safflower oil) from weaning to 10 months of age. The nutritional effects of these diets have been defined in previous studies (10). Two animals were se-

TABLE I

Lecithin-Cholesterol Acyl Transferase (LCAT) Activity as a Function of 2-Mercaptoethanol in Rat Serum 5 and 15 Min after Injection of MLH[a] or MLO[b]

Serum	Number of animals	MLH				MLO			
		no treatment		2-mercaptoethanol		no treatment		2-mercaptoethanol	
		LCAT[c]	P[d]	LCAT	P	LCAT	P	LCAT	P
Control (before injection)	4	574 ± 25[e]	---	717 ± 28	---	517 ± 17	---	671 ± 34	---
5 Min after injection	4	186 ± 6	≪0.001	526 ± 27	≪0.001	223 ± 27	≪0.001	533 ± 30	<0.005
15 Min after injection	4	237 ± 16	≪0.001	598 ± 11	<0.001	367 ± 17	≪0.001	650 ± 35	NS

[a]MLH = methyl linoleate hydroperoxide.
[b]MLO = methyl linoleate ozonide.
[c]Nmoles cholesterol esterified/min/liter serum.
[d]Student's t-test vs control; NS = non significance.
[e]Values represent the mean ± standard deviation, N = 4.

lected from the group fed the oxidized safflower oil and the control groups fed fresh safflower or menhaden oil; three animals were used from the group fed the oxidized menhaden oil. Each of the groups contained 8-10 animals. LCAT activity was determined on the serum of each animal after incubation periods of 10, 20, 40, and 60 min.

RESULTS

LCAT activity was suppressed immediately in the serum of animals injected with MLH or MLO as shown in Figure 1 by assays at intervals from 5 min-24 hr after the injection of these compounds. The recovery of LCAT activity was much faster in the animals injected with MLO than in those injected with MLH and approached normal 5 hr after the injection of either of these compounds. ML, which was used as a control for MLO and MLH, did not affect LCAT activity (Fig. 1).

The effect of 2-mercaptoethanol upon LCAT activity of the serum of animals 5 and 15 min after injection of MLO or MLH is shown in Table I. This reagent counteracts the inhibitory action of SH-blocking agents, such as DTNB (17) or p-chloromercuribenzoate (4), on the enzyme in normal serum. The addition of 2-mercaptoethanol to the serum of animals 5 min after the injection of hydroperoxides or ozonides produced an increase in LCAT activity but did not restore it to normal, as it does when the activity of the enzyme is inhibited by DTNB. The LCAT activity was not restored to normal by 2-mercaptoethanol until after at least 15 min after the injection of hydroperoxides. The ozonides were less effective than the hydroperoxides in the inhibition of LCAT activity, as shown in Figure 1 and as indicated by the fact that the activity of the enzyme was restored essentially to normal by 2-mercaptoethanol 15 min after the injection of the ozonides (Table I).

The addition of MLH to the serum of normal animals also gave a marked inhibition of LCAT activity, as shown in Table II. The effect of the addition of DTNB or DTT to normal serum upon the activity of the enzyme also is shown in this table. The inhibitory action of DTNB was reversed completely by the subsequent addition of 2-mercaptoethanol. Prior treatment of the serum with DTNB did not protect the enzyme against the effect of the hydroperoxide. Likewise, 2-mercaptoethanol only exhibited a partial effect in reversing the activity of the enzyme in serum preincubated with DTNB prior to treatment with the hydroperoxide. In all experiments, the 2-mercapto-

TABLE II

Serum Lecithin-Cholesterol Acyl Transferase (LCAT) Activity in Presence of Different Reagents

Number	Sample[a]	LCAT activity[b]	Percent
1	Control (normal serum)	779 ± 7[c]	100.0
2	Control + DTNB – preincubation	85 ± 5	10.9
3	Control + DTT – preincubation	51 ± 8	6.5
4	Control + 2-mercaptoethanol	857 ± 2	110.0
5	Control + DTNB – preincubation + 2-mercaptoethanol	870 ± 21	111.7
6	Control + MLH – preincubation	181 ± 17	23.2
7	No. 6 + 2-mercaptoethanol	418 ± 18	53.7
8	No. 6 + DTNB – preincubation + 2-mercaptoethanol	416 ± 25	53.4
9	Control + DTNB – preincubation, + MLH – preincubation, + 2-mercaptoethanol	472 ± 16	60.6

[a]Each assay was carried out in quadruplicate on fresh serum pooled from two animals. Twenty μliter 1.5 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), 0.1 M dithiothreitol (DTT), and 0.1 M 2-mercaptoethanol were added into the standard incubation mixture, except the addition of 40 μliter 0.1 M 2-mercaptoethanol into samples 7-9. Fifty μliter methyl linoleate hydroperoxide (MLH) emulsion (0.1 mg) containing 5% bovine albumin in 0.15 M NaCl were added into samples 6-9. Preincubation was carried out for 10 min at 0 C.

[b]Nmoles cholesterol esterified/min/liter.

[c]Mean ± standard deviation.

TABLE III

Effect of Vitamin E upon Lecithin-Cholesterol Acyl Transferase (LCAT) Activity and Lipid Classes in Rat Serum

Hemolysis, LCAT, and lipid analyses	Vitamin E (+) (5 animals)	Vitamin E (–) (6 animals)	P[a]
Hemolysis[b] (%)	3.1 ± 1.2	89.0 ± 5.9[c]	<0.001
LCAT activity[d]	778 ± 128	531 ± 117	<0.02
Free cholesterol (mg/dl)	16.0 ± 4.0	16.5 ± 2.3	NS
Cholesterol ester (mg/dl)	80.8 ± 7.7	93.6 ± 6.7	<0.05
Free fatty acid (mg/dl)	22.5 ± 7.6	22.1 ± 5.6	NS
Triglyceride (mg/dl)	51.4 ± 4.1	60.2 ± 13.0	NS

[a]P (Student's t-test for significance) of vitamin E (+) vs vitamin E (–) groups.

[b]Value induced by dialuric acid.

[c]Mean ± standard deviation.

[d]Nmoles cholesterol esterified/min/liter.

ethanol was added in large excess.

LCAT activity also was decreased by a vitamin E deficiency as illustrated in Table III. Evidence of a vitamin E deficiency in the animals fed the vitamin E-free diet was the susceptibility of the erythrocytes to dialuric acid-induced hemolysis. There was no apparent effect of the vitamin E deficiency upon fatty acid composition (not shown) cholesterol, free fatty acid, or triglyceride content of the serum. The level of cholesteryl esters was elevated significantly in the serum, however.

Serum LCAT activity was increased by feeding diets containing oxidized menhaden or safflower seed oil, as shown in Table IV. In these experiments, LCAT activity was determined in the serum of each animal at incubation periods of 10, 20, 40, and 60 min. Thus, 8 analyses were conducted on the 2 animals in each of the control groups and the group receiving the oxidized safflower oil, and 12 analyses were conducted on the 3 animals of the oxidized menhaden oil groups. The relative rates of the reaction, as well as absolute values, are taken into account by this procedure. The difference between LCAT activity of the combined control groups (four animals) and the combined oxidized groups (five animals) was significant, as indicated in Table IV. The levels of both cholesterol and cholesteryl esters were also higher in the serum of animals receiving the oxidized fat than in those fed the corresponding fresh oils.

DISCUSSION

In the course of lipid peroxidation, interactions occur that cause damage to proteins and

TABLE IV

Effect of Oxidized Fat in Diet of Rats upon Serum Lecithin-Cholesterol Acyl Transferase (LCAT) Activity[a]

Dietary fat	Number of animals[b]	Cholesterol (mg/dl)		Cholesteryl esters (mg/dl)		LCAT activity[c] (nmoles cholesterol esterified/min/liter serum)	
Safflower oil (control)	2	14.2	(13.5-15.0)[d]	74.7	(73.5-76.0)	785	(645-1030)[d]
Oxidized safflower oil	2	29.5	(28.5-30.5)	86.7	(86.5-87.0)	1150	(900-1750)
Menhaden oil (control)	2	6.5	(5.5- 7.5)	48.5	(39.5-57.5)	651	(580-800)
Oxidized menhaden oil	3	18.5	(16.0-23.0)	99.5	(87.0-121.0)	1500	(1050-2180)

[a]Student's t-test for significance between combined control groups (4 animals) and combined oxidized fat groups (5 animals) (7° of freedom ts = 4686) had a P value of <.005 (718 ± 103 vs 1359 ± 223 obtained on the basis of grouping the high and low values for the 4 animals of the control groups and the high, low, and medium values for the 5 animals of the oxidized group to provide mean ± standard deviation for the calculation).

[b]Selected from 8-10 animals or original groups.

[c]The analyses were made at reaction time of 10, 20, 40 and 60 min on serum of each animal.

[d]Average value; values in parentheses show the range.

enzymes (5-7). Clark, et al., (8) has shown that the physical properties of lipoproteins are altered by hydrogen peroxide in vitro. Hence, it is predictable that LCAT activity should be suppressed by the intravenous injection of hydroperoxides or ozonides, especially because the activity of the enzyme involves sulfhydryl groups which are oxidized readily. However, LCAT activity also is related to a specific high density lipoprotein cofactor (22,23) and the ratio of cholesterol to lecithin (24-26). Hydroperoxides react readily with erythrocytes and appear to be cleared from the blood quickly, in that over 90% of the radioactivity of IV injected, C^{14}-labeled linoleate ozonide accumulated in the lung in less than 1 hr (T. Takatori and H. Shimasaki, Hormel Institute, unpublished observations).

The inhibitory action of hydroperoxides or ozonides on LCAT cannot be explained by a simple blocking of SH groups of the enzyme, as suggested for the action of DTNB, because only ca. half of the activity was restored by the action of 2-mercaptoethanol. It appears that the same reaction occurs in vivo with injected hydroperoxides as in vitro when hydroperoxides are added to normal serum, inasmuch as 2-mercaptoethanol was only partially effective in the restoration of the activity of the enzyme in serum of animals up to 15 min after the injection of these compounds. The recovery of LCAT activity in vivo apparently occurs by replacement or resynthesis of active constituents. If the enzyme itself is destroyed partially, it could be replaced readily, because it is synthesized in the liver. Cofactors, particularly HDL, that are required for the activity of the enzyme likewise would be resynthesized, and, hence, recovery of LCAT activity from sublethal doses of hydroperoxides or ozonides can be explained readily, inasmuch as the toxic substances are detoxified in vivo.

Although the toxic reaction of the hydroperoxides appears to be the same in vitro as in vivo, it cannot be explained at present. Mercaptoethanol does not appear to function in the same way as it does against DTNB, inasmuch as ca. only one-half of the activity is restored. Apparently, some of the reactions of the hydroperoxides are reversible by 2-mercaptoethanol which results in the restoration of part of the activity of the enzyme. LCAT has not been isolated, and its cofactor requirement is not entirely clear. Likewise, hydroperoxides or ozonides might react with the substrates, in part, particularly lecithin, which is not homogenous. Although the inhibitory reaction of the hydroperoxides is not clear, the use of these compounds may provide a means to examine the mechanism of the action of this enzyme in greater detail.

Reduction of serum LCAT activity in a vitamin E deficiency could be due to a number of factors, including the alteration of proteins by in vivo oxidation, as in the production of aging pigments (27). There also, apparently, is some alteration in the phospholipids in a vitamin E deficiency (27,28); hence, LCAT activity may be influenced as a result. The increase in serum cholesteryl esters seems para-

doxical, but LCAT activity does not appear to be related directly to the level of serum cholesteryl esters. On the other hand, there is a high correlation between LCAT activity and the level of serum cholesterol (29). Hence, the increase in LCAT activity of the animals fed oxidized fat might be expected as a consequence of the increase in serum cholesterol. There also is an increase in serum cholesteryl esters in these animals. These observations may be related to effects of oxidized fat upon lipid transport or lipoprotein synthesis. Choiesteryl esters may well contain fatty acids derived from oxidized fat that, in turn, may effect their metabolism. Although oxidized fat consumed in the diet is toxic (30-32), the secretion of cholesterol into serum could well remain unaffected, inasmuch as it generally is found to be lower in the liver of animals fed these fats (30).

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Effect of Dietary Bile Acids, Cholesterol, and β-Sitosterol upon Formation of Coprostanol and 7-Dehydroxylation of Bile Acids by Rat

B.I. COHEN, R.F. RAICHT, and **E.H. MOSBACH,** Department of Lipid Research of the Public Health Research Institute of the City of New York, Inc., New York, New York 10016 and the Manhattan Veterans Administration Hospital, New York, New York 10010

ABSTRACT

During studies of sterol metabolism in the rat, the fecal neutral sterol fraction was analyzed by a combination of thin layer chromatography and gas liquid chromatography. On a stock diet of rat chow supplemented with 5% corn oil, the rats excreted 14.5 mg/day of total neutral sterols. Coprostanol comprised 35% (5 mg/day) of this fraction. When the diet was supplemented with 0.5% sodium taurochenodeoxycholate, the amount of coprostanol in the feces remained the same as in the controls (3.2 mg/day, 32%). The addition of 0.5% sodium taurocholate to the diet resulted in a fivefold reduction of coprostanol formation (0.6 mg/day, 8%). When 1.2% cholesterol was added to the stock diet, the amount of coprostanol present in the feces decreased to an average of 11% compared to controls, but the absolute amount formed was greater (35 mg/day). On a diet enriched with 0.8% β-sitosterol, the rats, on the average, converted 23% of the cholesterol to coprostanol. Feeding diets enriched with sodium taurochenodeoxycholate and sodium taurocholate reduced the 7-dehydroxylation of primary bile acids in the feces by 28% and 42%, respectively. The conversion of primary bile acids to secondary bile acids in the feces of control, cholesterol, and β-sitosterol fed rats was the same (64%).

INTRODUCTION

The microbial reduction of the $\Delta^{5,6}$ double bond of cholesterol to form coprostanol occurs in the large intestine of many species (1). The exact mechanism of this transformation has been the topic of several studies (2-4). Thus, it has been suggested that coprostanone is an intermediate in the formation of coprostanol (5), since coprostanone was isolated from the feces of man; the ketone was not found in rat feces (6). Plant sterols have been reported to be converted to saturated metabolites in a manner similar to the neutral sterols, since β-sitosterol was converted to coprositostanol (7). Different diets supplemented with milk (8), lactose (9), Tween 80, and sodium taurocholate (10) all markedly inhibit coprostanol formation. The conversion of cholesterol to coprostanol can be abolished by feeding antibiotics (11), by incomplete digestion of carbohydrates (12) and by removal of the caecum (13). An increase in the transformation of cholesterol to coprostanol was reported in rats fed a diet rich in linoleic acid (6). The physiological significance of the microbial transformation of cholesterol is not known.

In connection with our sterol metabolism studies in the rat, we have investigated the effect of feeding bile acids (sodium taurochenodeoxycholate and sodium taurocholate) and sterols (cholesterol, and β-sitosterol) upon the formation of coprostanol. We have examined the corresponding transformation of β-sitosterol to coprositostanol. The ratio of the primary to secondary bile acids in the feces of the rats also was studied. The data suggest that differences in coprostanol formation are accompanied by similar differences in the degradation of the plant sterols and are associated with differences in the conversion of primary to secondary bile acids.

EXPERIMENTAL PROCEDURES

Animals and diet: Male, Sprague-Dawley derived rats weighing 225-250 g were purchased from Charles River Breeding Laboratories, Wilmington, Mass. The animals were weighed and placed into individual metabolic cages. The cages allow for quantitative recovery of feces, as well as determination of food intake. The animals had access to food and water ad lib. The animals were fed a stock diet consisting of ground raw chow supplemented with 5% corn oil. The cholesterol and β-sitosterol concentrations of this diet were 0.3 mg/g and 0.6 mg/g, respectively. To the stock diet was added either 1.2% cholesterol, 0.8% β-sitosterol, 0.5% sodium taurocholate or 0.5% sodium taurochenodeoxycholate. The animals were fed the experimental diet for 10 days. Feces were collected in three 2 day pools on days 6, 8, and 10 of this period.

TABLE I

Wt, Food Intake, and Fecal Output of Control, Cholesterol, β-Sitosterol, and Bile Acid Fed Rats

Diet fed[a]	Initial wt[b] (g)	Wt at sacrifice[c] (g)	Daily food intake[d] (g/day)	Daily fecal output[e] (g/day)
Stock				
Average	258	332	25.9	7.1
Range	232-276	282-359	24.6-29.0	5.7-7.6
Stock + 1.2% cholesterol				
Average	259	341	25.0	7.5
Range	241-275	333-351	24.6-26.5	6.7-8.2
Stock + 0.8% β-sitosterol				
Average	271	337	24.6	7.5
Range	266-281	330-348	23.1-25.6	6.7-7.9
Stock + 0.5% sodium taurochenodeoxycholate				
Average	239	296[f]	23.0	5.9
Range	231-249	280-318	19.1-25.7	4.9-7.1
Stock + 0.5% sodium taurocholate				
Average	237	300[f]	24.5	6.8
Range	220-243	290-330	21.0-28.8	5.8-7.8

[a]Animals used were male Sprague-Dawley derived rats from Charles River Breeding Laboratories of ca. the same wt.

[b]Wt of rats prior to being placed into metabolic cages for the experimental period where they had access to food and water ad lib.

[c]Wt of the animals after the experimental feeding period.

[d]Average daily intake of rat chow.

[e]Average daily wt of dried feces of the rats over the 6 day collection period.

[f]Wt not significantly different from controls ($p<0.01$).

Reference compounds: Cholesterol U.S.P. (Nutritional Biochemical Corporation) was used after crystallization from ethanol.

β-Sitosterol was shown to be 92% pure by gas liquid chromatography (GLC) on SE-30. Campesterol (7%) and stigmasterol (1%) were the major impurities.

5α-Cholestane (Applied Science Laboratories, State College, Pa.) was used as an internal standard for GLC.

Sodium taurocholate and sodium taurochenodeoxycholate were synthesized by methods previously described (14,15).

Thin layer chromatography (TLC): The TLC separation of the neutral steroids was carried out on 0.5 mm plates of Florisil TLC (Floridin Corp., Berkeley Heights, W. Va.). The plates were activated at 120 C for 1 hr and stored over Drierite prior to use.

GLC: Separations of the neutral sterols and bile acids were carried out on a Hewlett Packard gas chromatograph model 7610A equipped with flame ionization detectors and a dual pen recorder. U shaped glass columns (4 mm inside diameter, 180 mm length) were silanized and packed with 3% SE-30 on 100-120 mesh Supelcoport (Supelco, Bellefonte, Pa.). Prepurified nitrogen was used as a carrier gas at a flow rate of 20-30 ml/min and an inlet pressure of 40 psi. Measurements of peak areas were accomplished with a Hewlett Packard automatic integrator 3370B adjusted to record retention time in min (with automatic baseline correction). Operating conditions for the column, inlet, and detector were 250, 280, and 280 C, respectively.

Methods for isolation and quantitation of neutral and acidic steroids from feces: The techniques used have been described in detail (16-18). Dried feces were extracted with ethanol in a Soxhlet extractor for 48 hr to remove the neutral and acidic steroids. The neutral sterols were purified by TLC on Florisil plates. The bands corresponding to the parent sterols and their 5β-reduction products were scraped into a funnel containing a sintered glass disc and were eluted with five 3 ml portions of ethyl ether. GLC analysis of each band was carried out on a column packed with 3% SE-30 after preparing the trimethylsilyl (TMS) derivatives. 5α-Cholestane was added to each sample as an internal standard for GLC.

The fecal acidic steroids were purified by TLC on silica gel. They were analyzed as the TMS ether derivatives of their methyl esters by GLC on 3% SE-30, as previously described

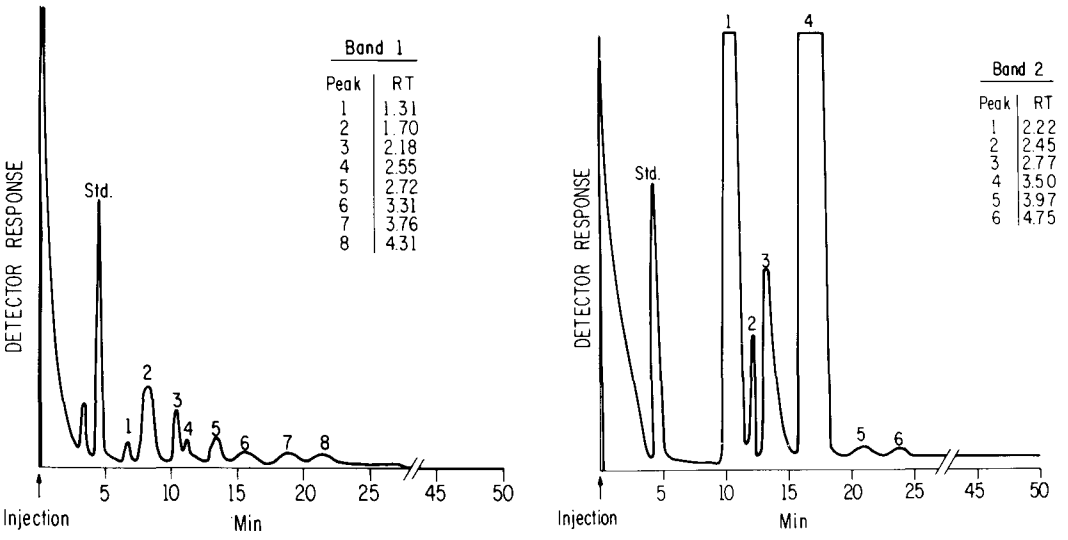

FIG. 1. Gas liquid chromatographic (GLC) analysis of fecal sterols. Representative GLC profile of the components of the fecal neutral sterols after Florisil TLC (thin layer chromatography) for the rats fed diets of either stock, stock + 1.2% cholesterol, stock + 0.8% β-sitosterol, or stock + 0.5% sodium taurochenodeoxycholate. Bands 1 and 2 represent the material obtained after the TLC in which the 5β-reduction products (band 1) were separated from the parent sterols (band 2). GLC analysis was performed by adding 5α-cholestane to each sample and preparing the trimethylsilyl ether derivatives. Retention times are reported relative to the internal standard (actual retention time = 4.93 min). Conditions of the GLC analysis are given in the text. The components in each band used for the calculation of the sterol data are: band 1-peak 2, coprostanol; peak 5, coprositostanol; band 2-peak 1, cholesterol; peak 5, β-sitosterol. Std. = internal standard. RT = relative retention time.

(16,18).

RESULTS AND DISCUSSION

Previous studies have demonstrated that dietary modifications affect the transformation of cholesterol to coprostanol in the intestine (7-14). Most of the early studies had to rely upon techniques which made analysis of the total fecal neutral sterols imprecise. In addition, the presence of plant sterols in the diet may have caused problems in obtaining correct quantitative measurements. In connection with sterol metabolism studies in rats, we have examined the composition of the neutral and plant sterols in feces using a combination of TLC-GLC procedures (11, 17-19).

The animals in our studies were treated under similar conditions during the 10 day experimental period. Table I shows that, during this period, the rats in each group gained similar amounts in wt (60-80 g), ate about the same amount of chow (25 g/day), and had similar fecal outputs (ca. 6.5 g/day dry wt). The chow fed to the rats contained large amounts of sterols and bile acids so that their effect upon the bacterial transformation of the neutral sterols, plant sterols, and bile acids could be determined.

TLC on Florisil permitted us to separate the neutral and plant sterols (band II) from their 5β-reduction products (band I) (18). No band corresponding to keto metabolites could be detected. Quantitative analysis of the TMS ether derivatives of the sterols in each band was carried out by GLC. This procedure allowed for accurate determination of the neutral sterols, plant sterols, and their corresponding 5β-reduction products. Figure 1 shows a typical separation of the TMS ethers of the fecal sterols of animals fed either stock, 1.2% cholesterol, 0.8% β-sitosterol, or 0.5% sodium taurochenodeoxycholate. Feeding 0.5% sodium taurocholate drastically reduces the amount of bacterial degradation of the neutral and plant sterols; Figure 2 shows a typical separation of the sterols in the taurocholate-fed rats. Most of the fecal sterols isolated from these rats were present as the parent sterols in band II. Input-output measurements demonstrated quantitative recovery of β-sitosterol. Therefore, no correction for losses of sterols during intestinal transit was necessary (16).

The effects of stock diet and diets containing 1.2% cholesterol and 0.8% β-sitosterol on the 5β-reduction of the neutral and plant sterols are summarized in Table II. In rats fed stock chow, 34% of the neutral sterols and 35%

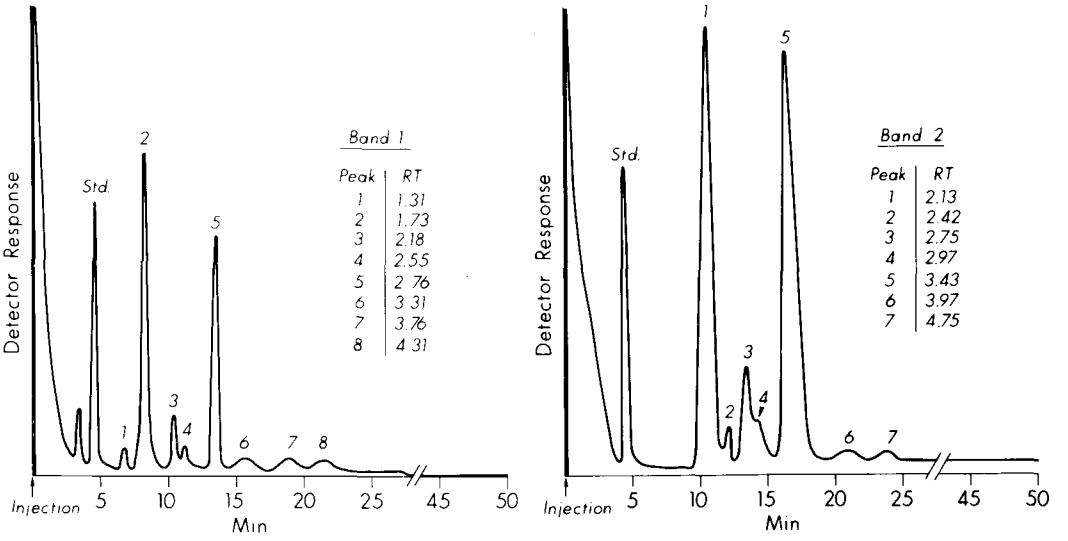

FIG. 2. Gas liquid chromatographic (GLC) analysis of fecal sterols on a sodium taurocholate diet. Representative GLC profile of the sterol components after Florisil TLC (thin layer chromatography) for the rats fed a 0.5% sodium taurocholate diet. The identity of the peaks used for the calculations are: band 1-peak 2, coprostanol; peak 5, coprositostanol; band 2-peak 1, cholesterol; peak 4, β-sitosterol. Std. = internal standard and RT = relative retention time.

of the plant sterols had been transformed to their corresponding bacterial metabolites, coprostanol and coprositostanol.

When the rats were fed the stock diet supplemented with 1.2% cholesterol, the percent transformation of the neutral and plant sterols was similar (12 and 13%, respectively), although the absolute amount of coprostanol formed was 6 times greater than in the controls. In rats fed the stock diet supplemented with 0.8% β-sitosterol, 11% of this sterol was transformed to its bacterial metabolite coprositostanol, whereas 23% of the cholesterol was transformed to coprostanol. It is not clear at this time why there was greater percent transformation of β-sitosterol to coprositostanol than of cholesterol to coprostanol in the β-sitosterol fed animals. This discrepancy was not observed in any other experimental group. The increased excretion of cholesterol in the feces of the β-sitosterol fed rats was caused by the plant sterol which interrupted the normal enterolymphatic circulation of cholesterol, thus stimulating hepatic cholesterol synthesis (19). Thus, these feeding experiments indicate that, in most instances, the intestinal bacteria transform both the neutral and plant sterols in a chemically analogous manner but not to a similar extent when large amounts of these sterols are present in the diet. Earlier studies have shown that anerobes were responsible for the conversion of cholesterol to coprostanol (20). We suggest that, in the presence of large amounts of sterols in the diet, the capacity of the intestinal anaerobic bacteria is no longer sufficient to transform the sterols in the same percent as in the usual manner.

The formation of secondary bile acids appeared to be correlated with the cholesterol/coprostanol ratio in the feces. These data are summarized in Table III. The percent of deoxycholic acid formed from cholic acid in the intestine of the rats fed either stock, 1.2% cholesterol, or 0.8% β-sitosterol enriched diets was identical (64%). This was the case even though the cholesterol-fed rats are known to excrete larger amounts of bile acids than the other groups.

The effects of supplementing the diet with 0.5% sodium taurochenodeoxycholate or sodium taurocholate are shown in Table II. The animals did not develop diarrhea from this amount of bile acid added to the diet during the entire 10 day experimental period. Feeding these bile acids in amounts greater than normally present physiologically revealed a selective inhibitory effect of sodium taurocholate. Taurocholate feeding reduced the conversion of cholesterol and β-sitosterol to their bacterial metabolites (cholesterol, 8% conversion; β-sitosterol, 10% conversion). On the other hand, the bacterial transformation of these sterols in the taurochenodeoxycholate-fed rats was ca. the same as in the control rats (cholesterol, 32% conversion; β-sitosterol, 33% conversion). These data suggest that the two bile acids may have a

TABLE II

Fecal Output of Sterols and Their Bacterial Metabolites[a]

Number of rats and diet[b]	A Cholesterol (mg/day)	B Coprostanol (mg/day)	B/A+B (%)	C β-Sitosterol (mg/day)	D Coprositostanol (mg/day)	D/C+D (%)
Stock[c]						
9						
Average	9.4 ± 1.1	5.1 ± 0.7	34	11.8 ± 0.8	6.2 ± 0.8	35
Range	6.5 - 16.7	1.7 - 9.3	22-47	8.8 - 15.4	2.7 - 9.2	19-45
Stock + 1.2% cholesterol[d]						
6						
Average	217 ± 5.8	28.8 ± 3.0	12[e]	14.0 ± 0.4	2.0 ± 0.2	13[e]
Range	192 - 232	6.6- 40.6	3-16	13.2 -15.8	0.4 - 2.6	3-17
Stock + 0.8% β-sitosterol[f]						
5						
Average	20.9 ± 1.4	5.9 ± 0.5	23[e]	196 ± 7.1	24.5 ± 3.0	11[e]
Range	17.5 -26.5	3.8- 7.4	17-34	172 - 205	12.9 -40.3	7-17
Stock + 0.5% sodium taurochenodeoxycholate[g]						
4						
Average	7.0 ± 0.6	3.2 ± 0.4	32	7.8 ± 0.7	3.8 ± 0.5	33
Range	4.7 - 9.4	1.5 - 5.3	14-37	5.8 -10.6	1.6 - 6.3	14-49
Stock + 0.5% sodium taurocholate[g]						
4						
Average	6.5 ± 0.4	0.6 ± 0.0	8[e,h]	10.9 ± 0.4	1.1 ± 0.1	10[e,h]
Range	5.2 - 8.2	0.3 - 0.8	4-12	9.5 -12.9	0.6 - 1.7	5-15

[a]Separation of cholesterol and β-sitosterol from their respective bacterial metabolites, i.e. coprostanol and coprositostanol, was performed using a combination of thin layer chromatography and gas liquid chromatography, as described in the text.

[b]Represents the number of animals used in each feeding experiment.

[c]The results reported represent the average ± standard error of 27 fecal pools.

[d]The results reported represent the average ± standard error of 18 fecal pools.

[e]Differs significantly from the control rats fed a stock diet ($p<0.01$).

[f]The results reported represent the average ± standard error of 15 fecal pools.

[g]The results reported represent the average ± standard error of 12 fecal pools.

[h]Differs significantly from the taurochenodeoxycholate fed rats ($p<0.01$).

different effect upon the bacterial flora which reduces the $\Delta^{5,6}$ double bond of the sterols. Further studies will be needed to determine which bacteria are involved. As in the sterol-fed animals, on the average, both the neutral and plant sterols were transformed into their bacterial metabolites to a similar extent.

Significant reductions in the 7-dehydroxylation of the primary bile acids occurred in the intestine of the rats receiving bile acid enriched diets. Rats receiving sodium taurochenodeoxycholate converted only 38% of this bile acid to its corresponding 7-dehydroxylated metabolite lithocholic acid. Since the amount of cholic acid in the feces of the taurochenodexoycholate fed rats was small (less than 10% of the total fecal bile acids), the extent of its 7-dehydroxylation could not be determined with accuracy. The rats fed taurocholate containing diets showed more drastic alterations in the bacterial degradation of the primary bile acids. 7-Dehydroxylation of cholic acid in these animals was only 22%. The data suggest that sodium taurocholate is more effective than sodium taurochenodeoxycholate in reducing both the formation of coprostanol and its own dehydroxylation. The significance of this observation must be studied further in light of the proposed large scale administration of chenodeoxycholic acid to man in an effort to study gallstone dissolution (21-23).

The data obtained in these studies in rats show that large amounts of dietary sterols and bile acids exert different effects upon the bacterial degradation of cholesterol, plant sterols, and bile acids in the intestine. These differences may have importance since the interaction of both bile acids and sterols with the intestinal wall had been implicated as possible etiological agents in the pathogenesis

TABLE III

Effects of Diet upon 7-Dehydroxylation of Fecal Bile Acids

Diet[a]	Number of rats	Secondary fecal bile acids[b]
Stock	9	
Average		64 ± 10[c]
Range		56 - 81
Stock + 1.2% cholesterol	6	
Average		64 ± 10[c]
Range		48 - 74
Stock + 0.8% β-sitosterol	5	
Average		64 ± 7[c]
Range		56- 71
Stock + 0.5% sodium taurochenodeoxycholate	4	
Average		38 ± 18[d,e]
Range		24 - 56
Stock + 0.5% sodium taurocholate	4	
Average		22 ± 12[c,e,f]
Range		13 - 39

[a]Rats fed a diet of stock chow supplemented with sterols and bile acids for the 10 day experimental period.

[b]Numbers represent the percent of the secondary bile acids in the feces on day 8 of the experimental period.

[c]Numbers represent the ratio cholic and deoxycholic acid in feces.

[d]Numbers represent the ratio of chenodeoxycholic acid and lithocholic acid in feces.

[e]Differs significantly from rats fed a stock diet, ($p<0.01$).

[f]Differs significantly from taurochenodeoxycholate fed rats, ($p<0.01$).

of colon cancer (24,25).

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SHORT COMMUNICATIONS

Essential Fatty Acids in Maternal Diet and in Rat Milk Phospholipids

ABSTRACT

The administrations of semisynthetic diets supplemented with oils and fats containing different levels of linoleic acids to lactating rats result in corresponding changes in the polyunsaturated fatty acids of triglycerides in the collected milk. Milk phospholipids show a quite different trend, polyunsaturated acids of the linoleic acid family being highest with low dietary linoleic acid supply, and vice versa, suggesting a control in the secretion of polyunsaturated fatty acids in milk.

INTRODUCTION

Maternal intake of essential fatty acids (EFA) regulates the levels of polyunsaturated fatty acids in milk lipids (1-4). In previous studies of the effects of dietary lipids upon milk fatty acid profile in the rat, the stomach contents of the sucklings have been analyzed (1-3). The considerable degree of lipolysis occurring in the stomach, however, may lead to modifications of the fatty acid composition of individual lipid classes (5). Furthermore, little attention has been paid to the effects of the diets upon the milk phospholipid fraction, containing higher levels of long chain highly unsaturated fatty acids in respect of triglycerides.

The effects of diets containing different levels and ratios of EFA upon the fatty acid profile of triglycerides and phospholipids of rat milk, collected by microaspiration at the

TABLE I

Fatty Acid Composition (Wt Percentage) of Dietary Lipids[a]

Fatty acid methyl esters	N	OO	SO	T	SF
10:0	--	--	--	1.1	--
12:0	0.1	--	--	0.6	--
14:0	1.5	0.2	0.3	5.7	3.3
15:0	0.2	--	0.1	0.7	0.9
15:1	--	--	--	0.3	0.2
16:0	17.9	12.2	7.9	32.4	31.6
16:1	2.5	1.2	0.3	5.0	0.8
17:0	0.4	--	0.1	1.4	2.5
17:1	0.4	0.2	--	0.9	0.2
18:0	5.6	2.9	5.3	2.2	56.5
18:1 (n-9)	28.0	74.6	22.2	44.0	1.1
18:2 (n-6)	32.6	6.3	59.7	2.5	0.3
20:0	0.3	0.4	0.4	0.2	0.6
18:3 (n-3)	4.1	1.1	0.6	0.8	--
20:2	0.5	0.3	0.3	0.5	0.2
20:3 (n-9)	--	--	--	--	0.2
20:3 (n-6)	0.2	--	0.9	--	--
20:4 (n-6)	0.4	--	--	--	--
20:5 (n-3)	1.8	--	--	--	--
22:3 (n-9)	--	--	--	--	0.3
22:3 (n-6)	--	--	--	0.3	--
22:4 (n-6)	0.3	--	--	0.3	--
22:5 (n-3)	0.2	--	--	0.7	--
22:6 (n-3)	0.2	--	--	--	--

[a]N = normal diet, OO = olive oil, SO = sunflower seed oil, T = tallow, and SF = saturated fraction obtained from tallow.

twelfth day of lactation, have been investigated in the present study.

EXPERIMENTAL PROCEDURES

Five groups of 4 month old fertile female rats (300-400 g) were fed either a standard diet, containing 5% lipid, or semisynthetic diets containing 10% of either olive oil (00), sunflower seed oil (SO), tallow (T), or a saturated fraction obtained from tallow (SF). The semisynthetic diets contained 18% protein, 65% carbohydrate, 10% fat, 4% α cellulose, 1% Wesson salt mix (supplemented with 4 ppm zinc sulfate), and 2% vitamin mix (containing 27 mg/100 g diet vitamin E). The diets were fed starting 10 days before mating up to the twelfth day of lactation. The average caloric intakes were practically the same for all dietary groups. Milk was collected by microaspiration according to the technique of Feller and Boretos (6). After lyophilzation of milk, lipids were extracted; triglycerides and total phospholipids were isolated by thin layer chromatography (TLC), and methyl esters were prepared (7), purified, and analyzed by combined direct gas chromatography (GLC) (8) and GLC after separation by $AgNO_3$ impregnated TLC.

RESULTS AND DISCUSSION

The fatty acid composition of the lipid fraction in the various diets is shown in Table I. Lipids extracted from the standard diet and the SO diet have high polyenoic acid contents. In contrast, diets 00 and T contain low polyenoic acid levels, whereas the polyenoic acid content in diet SF is minimal, linolenic acid being virtually absent.

Table II shows the wt percentage levels of polyunsaturated fatty acids of triglycerides and phospholipids in milk collected from lactating rats fed the diets with various lipid supplements. The levels of linoleic acid in milk triglycerides appear to be influenced greatly by maternal dietary intake of this fatty acid, as observed by other investigators (1,2,4). The concentrations of arachidonic acid in the triglyceride fraction is also dependent upon the dietary levels of linoleic acid, but the range of values is narrower. The milk phospholipid fraction in the various dietary groups shows changes which are different from those detected in the triglyceride fraction, especially in the levels of polyunsaturated fatty acids. In fact, the levels of arachidonic acid (20:4) and of docosatetraenoic acid (22:4) are higher in milk phospholipids from rats fed diets with low linoleic acid levels (SF and T groups), and they

TABLE II

Wt Percentage of Polyunsaturated Fatty Acids in Rat Milk Triglycerides (TG) and Phospholipids (PL) at 12 Days of Lactation under Various Dietary Conditions[a]

Fatty acids	N		OO		SO		T		SF	
	TG	PL	TG	PL	TG	PL	TG	PL	TG	PL
18:2 (n-6)	14.0 ± 1.22	11.3 ± 1.12	3.0 ± 0.43	6.1 ± 0.74	28.7 ± 2.9	22.1 ± 2.3	1.9 ± 0.35	5.3 ± 0.62	0.8 ± 0.10	3.0 ± 0.41
18:3 (n-3)	1.1 ± 0.21	0.1	0.2 ± 0.01	--	0.1	0.1	0.08	--	0.08	--
20:3 (n-9)	--		0.2 ± 0.01	0.5 ± 0.03	0.4 ± 0.04	--	0.4 ± 0.01	1.0 ± 0.12	0.3 ± 0.02	2.8 ± 0.31
20:3 (n-6)	0.5 ± 0.06	2.0 ± 0.34	0.2 ± 0.02	2.8 ± 0.22	1.0 ± 0.22	3.3 ± 0.42	0.2 ± 0.02	2.6 ± 0.23	--	0.7 ± 0.06
20:4 (n-6)	1.2 ± 0.21	5.7 ± 0.72	0.7 ± 0.06	3.4 ± 0.52	2.4 ± 0.34	3.7 ± 0.43	0.5 ± 0.04	6.5 ± 0.77	0.2 ± 0.02	7.1 ± 0.82
22:4 (n-6)	--	3.7 ± 0.41	--	3.6 ± 0.32	0.8 ± 0.09	3.4 ± 0.41	--	4.9 ± 0.55	--	5.0 ± 0.44
22:5 (n-6)	--	0.2 ± 0.01	--	0.5 ± 0.06	--	0.8 ± 0.19	--	0.2 ± 0.01	--	0.5 ± 0.02
22:5 (n-3)	--	0.4 ± 0.02	--	0.2 ± 0.01	--	--	--	0.2 ± 0.02	--	--
22:6 (n-3)	--	0.8 ± 0.09	--	0.5 ± 0.04	--	--	--	0.5 ± 0.05	--	0.5 ± 0.06

[a]N = normal diet, OO = diet containing 10% olive oil, SO = diet containing 10% sunflower seed oil, T = diet containing 10% tallow, and SF = diet containing 10% saturated fat. Values are the average ± standard error of determination performed on six samples of milk in each dietary group.

are lower in milk phospholipids of the SO (high linoleic in the diet) group in respect of the control values. It, thus, appears that, although, in milk phospholipids, the level of linoleic acid in the SO group is 7 times higher than in the SF group, the level of tetraenes (20:4 and 22:4 n-6) is almost half in the former group with respect to the second. The data suggest increased conversion of linoleic acid to polyunsaturated derivatives with low dietary levels of linoleate and inhibition in this conversion in the presence of high levels of linoleate in the diet. The observations of an increased formation of long chain polyunsaturated fatty acids of the (n-6) series with low dietary levels of the precursor linoleic acid, and vice versa, suggest a control of the supply of polyunsaturated fatty acids to the sucklings in conditions of reduced maternal intake of EFA.

C. GALLI
C. SPAGNUOLO
Institute of Pharmacology
and Pharmacognosy
University of Milan
20129 Milan, Italy

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Myocardial Alterations Resulting from Feeding Partially Hydrogenated Marine Oils and Peanut Oil to Rats

ABSTRACT

Myocardial alterations were observed in 5 groups of rats fed diets containing 20% fat for 16 weeks. The incidence was comparable to that from other studies and uniform at 6/20 in hearts from rats fed: partially hydrogenated herring oil to give dietary levels of either 16.7% or 4.6% 22:1; partially hydrogenated redfish/flatfish oil to give 4.5% 22:1 in the dietary fat; and peanut oil (of unknown origin) containing 0.1% 22:1. The incidence was 9/20 in the hearts of rats fed an unrefined and unprocessed redfish oil at a dietary level of 16.0% 22:1 in fatty acids.

INTRODUCTION

Several of the reports of myocardial alteration in experimental animals fed marine oils in partially hydrogenated form have originated in one Canadian laboratory (1-5). The results from one earlier study are difficult to ascribe to marine oils due to the mixture of marine oil and oil from plants of the genus *Brassica* which were involved (6) and a more recent experiment involved only a study of short term lipidosis (7).

EXPERIMENTAL PROCEDURES

To broaden the data base on this subject, the Fisheries and Marine Service, Environment Canada, contracted for the execution of long term studies comparable to those carried out elsewhere, with three marine oil samples as part of 20% (w/w) fat in diets. The levels of docosenoic acids (shorthand notation 22:1), which are thought to be one of the causative agents of myocardial alterations (8,9), were adjusted by dilution with lard-corn oil mixture so that two different partially hydrogenated oils would provide 22:1≈5% of dietary fat. This level was recommended to industry by Health and Welfare Canada as a voluntary restriction pending further studies (10). The other two oils

are lower in milk phospholipids of the SO (high linoleic in the diet) group in respect of the control values. It, thus, appears that, although, in milk phospholipids, the level of linoleic acid in the SO group is 7 times higher than in the SF group, the level of tetraenes (20:4 and 22:4 n-6) is almost half in the former group with respect to the second. The data suggest increased conversion of linoleic acid to polyunsaturated derivatives with low dietary levels of linoleate and inhibition in this conversion in the presence of high levels of linoleate in the diet. The observations of an increased formation of long chain polyunsaturated fatty acids of the (n-6) series with low dietary levels of the precursor linoleic acid, and vice versa, suggest a control of the supply of polyunsaturated fatty acids to the sucklings in conditions of reduced maternal intake of EFA.

C. GALLI
C. SPAGNUOLO
Institute of Pharmacology
and Pharmacognosy
University of Milan
20129 Milan, Italy

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were the same partially hydrogenated herring oil blended with corn oil (3:1) and an unrefined and unprocessed redfish oil also blended with corn oil (3:1). These oils were selected so that, after corn oil supplementation, the percentage of 22:1 was ca. the same percentage (ca. 16%) in both dietary fats primarily (see below) to compare the effects of the refining and partial hydrogenation. In addition to the control lard-corn oil mixture (3:1), a vegetable oil not hitherto tested in Canada (peanut oil, origin unknown) was included in the experiment. Pertinent details of fatty acid composition obtained in this laboratory are given in Table I. All marine oils fed were supplemented with 0.05% of commercial antioxidant mixture, and α-tocopherol was blended into the diet at 50 mg/100 g diet. Peroxide values at the beginning and end of the experiment were respectively: lard, 0.6 and 0.8; corn oil, 3.3 and 1.8; partially hydrogenated herring oil, 0.9 and 0.8; partially hydrogenated redfish/flatfish oil, 1.3 and 2.8; unprocessed redfish oil plus corn oil, 2.1 and 8.2; and peanut oil, 1.4 and 4.8.

The Sprague-Dawley male rats were Caesarian originated, barrier-sustained, from the Bio-Breeding Laboratories, Ottawa, Canada. At weaning, 20 rats were started on each diet contained 20% fat. The balance of the semisynthetic diet comprised 20% casein, 20% sucrose, 30% cornstarch, 1% vitamin mix (TD 71048, General Biochemicals, Shagrin Falls, Ohio), 4% Bernhart-Tomarelli salt mixture, and 5% Alphacel. After 16 weeks the rats were executed. Organs recovered for wt studies included the adrenals, hearts, and livers (Table II). The hearts were fixed in buffered 10% formalin, embedded in paraffin, and a single frontal section prepared and stained with hematoxylin and eosin. The sections were examined on a "blind" basis by a certified pathologist and scored for incidence and severity of myocardial alterations. Staining of ventricular sections with Sudan IV did not show any fatty deposits associated with the experiment.

RESULTS

The histopathological results, Table III, show no experiment induced myocardial alterations in the animals on the control fat of lard-corn oil mixture. The three diets containing partially hydrogenated fish oils showed a moderate incidence of lesions of low severity. The unrefined fish oil showed a higher incidence with a slightly higher severity score. The adrenals for the rats fed this oil showed slight enlargement. The hearts of the rats on the two oils high in 22:1 showed slight enlargement, respectively 0.307 and 0.292% of body wt for the partially hydrogenated herring oil and unrefined redfish oil, vs 0.276% for controls. Liver wt was increased for all four marine oil diets and depressed for peanut oil compared to the control rats. Basically, our results support the findings of Beare-Rogers, et al., (3) and can be interpolated numerically into their data. With 5% 22:1 from partially hydrogenated herring oils included in diets with 20% (w/w) fat, the balance being lard-corn oil, for 16 weeks, their histopathological score for rat hearts was 2/10. At 15% 22:1 the score was 6/10.

The slightly higher score for the unrefined redfish oil in our experiment may reflect the concentration of effectively all 22:1 into three particular isomers (normally 22:1ω11>22:1ω13>22:1ω9) in lieu of the greater isomer spread found in partially hydrogenated oils (4,11). Alternatively, the animals may have been more highly stressed metabolically because of the inclusion of volatile or thermolabile oxidation products (12) normally removed during hydrogenation and on refining and thus were less able to adapt to the inclusion of 22:1 in the diet.

Astorg and Rocquelin (7) tested peanut oil, partially hydrogenated herring oil, and partially hydrogenated herring oil plus corn oil in short term rat experiments. They found no difference in heart wt, i.e. no short term lipidosis, for the marine oils, but their fatty acid analyses showed more 22:1 deposited from both herring oil diets than from peanut oil containing 0.9% of this acid. Our analysis of the control oil supplied as pure peanut oil (Table I) shows virtually no erucic acid. It is always possible, but improbable, that some low erucic acid Brassica oil could have been mixed into any particular lot of peanut oil. In our study, the amount of such admixture would have been limited, as proportions of other acids, especially 18:3ω3, were not unusual. In an earlier experiment with peanut oil containing no erucic acid, 2.5% behenic acid (22:0), and probably some lignoceric acid (24:0), Rocquelin and Cluzan (13) reported "doubtful myocarditis" in 3 out of 20 rats. Other studies from this group present innocuous results for peanut oil (14,15). Partially hydrogenated marine oils contain 22:0 at 2-5% of fatty acids and 24:0 at 0.5% or less. In these oils, the digestibility of 22:0 and 24:0 is comparable to 22:1 (16,17), but no physiological effect has been attributed to the saturated acids from either animal or vegetable sources.

TABLE I

Details of Important Fatty Acids in Lard and Corn Oil Used for Control Diet in 3:1 Mixture of Dietary Fat Mixtures Used in Rat Feeding Experiments and of Peanut Oil[a]

Fatty acid	Lard	Corn oil	Partially hydrogenated herring oil + Corn oil	Partially hydrogenated herring oil + Lard-corn oil (3:1)	Partially hydrogenated redfish/flatfish oil + Lard-corn oil (3:1)	Unprocessed redfish oil + Corn oil	Peanut oil
14:0	1.7	---	5.2	1.7	3.7	4.1	---
16:0	25.6	10.8	12.8	20.4	18.5	10.2	13.6
18:0	16.4	2.2	2.7	10.1	7.1	1.0	2.8
20:0	0.3	0.4	0.6	0.1	0.9	0.1	1.0
22:0	---	---	1.0	0.2	0.7	---	2.7
24:0	---	---	---	---	---	---	0.4
16:1	2.8	0.1	10.2	4.3	6.8	11.7	0.1
18:1	42.1	24.9	17.1	33.2	27.3	17.2	41.7
20:1	0.8	0.2	12.9	3.9	7.2	14.0	0.7
22:1	---	0.4	16.7	4.6	4.5	16.0	0.1
24:1	---	---	0.6	0.1	0.1	0.4	0.01
18:2	9.1	60.2	18.8	20.8	20.5	16.3	36.5
18:3	0.6	0.8	---	---	---	---	0.1
20:4	---	---	---	---	---	0.2	---
20:5	---	---	---	---	---	5.0	---
22:6	---	---	---	---	---	2.3	---

[a]Further details on all samples, except peanut oil, available from author.

TABLE II

Relative Organ Wt (Percent of Body Wt) for 4 Organs from Rats on 6 Different Dietary Fat Mixtures at 20% (w/w) of Diet for 16 Weeks

	Lard-corn oil (3:1) control	Dietary Oil: Partially hydrogenated herring oil + Corn oil	Partially hydrogenated herring oil + Lard-corn oil (3:1)	Partially hydrogenated redfish/flatfish + Lard-corn oil (3:1)	Unprocessed redfish oil + Corn oil	Peanut oil
Percent 22:1	0.1	16.7	4.6	4.5	16.0	0.1
Adrenal R	0.005	0.005	0.005	0.005	0.006[a]	0.005
Adrenal L	0.005	0.005	0.005	0.005	0.006[a]	0.005
Heart	0.276	0.307[a]	0.274	0.284	0.292[a]	0.284
Liver	2.091	2.497[a]	2.251[a]	2.402[a]	2.463[a]	1.871[a]

[a]Statistically different from control.

TABLE III

Histopathological Scoring[a] of Hearts from Rats Kept on 6 Different Dietary Fat Mixtures at 20% (w/w) for 16 Weeks

Oil or mixture	Percent 22:1 in dietary fat	Histopathological observations of myocardial alterations	
		Severity	Incidence
Lard and corn oil (3:1 mixture)	0.1	0	0/20
Partially hydrogenated herring oil and corn oil (3:1 mixture)	16.7	0.30	6/20
Partially hydrogenated herring oil (same batch) in corn oil and lard	4.6	0.30	6/20
Partially hydrogenated redfish-flatfish oil in corn oil and lard	4.5	0.30	6/20
Unprocessed redfish oil and corn oil (3:1 mixture)	16.0	0.60	9/20
Peanut oil	0.1	0.35	6/20

[a]The following is the grading system that was employed in the assessment of the heart lesions: (A) ± this heart lesion was represented by occasion focal scars or groups of swollen intersitial fibroblasts representing a lesion or an effect of a low grade focal myocardial injury *not related* to experimental treatment and occurring in all groups in occasional animals; (B) + this lesion was regarded as treatment induced and consists of multiple small foci of collapse or replacement fibrosis composed of capillaries, fibroblasts, mononuclear cells, and Anitschkow's myocytes; (C) ++ this lesion represented multiple foci of confluent areas of cellular proliferation or scars surrounding and replacing the degenerating cardiac muscle fibres; and (D) +++ this lesion signified massive myocardial alteration in which remnants of necrotic or degenerating cardiac muscle cells were surrounded by reactive mononuclear cell infiltrates, hemosiderin-laden macrophages, or proliferating fibroblasts. It is emphasized that the histopathological examinations were carried out by the pathologist on a "blind" basis without prior knowledge of the various experimental treatments.

R.G. ACKMAN
Canada Environment Fisheries and Marine Service
Halifax Laboratory
Halifax, Nova Scotia B3J 2R3

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LETTER TO THE EDITOR

Concerning Annelid Triglycerides and Active Lipases

Sir: We read with great interest the "Letter to the Editor" in *Lipids* 9:363 (1974) by R.P. Hansen and Zofia Czochanska reporting on the presence of triglycerides in earthworms. The authors' suggestion that earlier studies describing an absence of di- and triglycerides in earthworms may have been misleading, and their conclusion that such analyses must be performed on fresh tissues fit very nicely with our own findings on a polychaetous annelid.

In 1971, we published a paper on the lipids in a marine annelid (*Nereis virens*) that included a detailed section on "Artifacts: Products of Hydrolysis" (D.M.-E. Pocock, J. Marsden, and J.G. Hamilton, *Comp. Biochem. Physiol.* 39A:683 [1971]). To describe briefly, in 1971, we corrected the report in our earlier paper (D.M.-E. Pocock, J. Marsden, and J.G. Hamilton, *Comp. Biochem. Physiol.* 30:133 [1969]) that included results obtained in 1968 from stored, frozen tissues and indicated relatively larger proportions of both diglycerides and free fatty acids than of triglycerides. Later (1969), when we lyophilized fresh tissues immediately after dissection from live, healthy, feeding worms, there were abundant triglycerides and only traces of free fatty acids and diglycerides. The 1971 publication dealt with a comparison of healthy, feeding, immature worms and nonfeeding, virtually starving mature worms which were ready to spawn. Tissues from the virtually starved, mature worms contained only sterols and polar lipids—no depot lipids—whereas tissues from the well nourished, immature worms contained triglycerides, neutral plasmalogens (alk-1-enyl glyceryl ether diesters), and glyceryl ethers (alkyl glyceryl ether diesters) as depot lipids. Starvation experiments (7-21 weeks) further confirmed these three classes of depot lipids (D.M.-E. Pocock, et al., *Comp. Biochem. Physiol.* 39A:683 [1971]).

In conclusion, we wish to note we have known for some time that the annelid *Nereis virens* contains extremely active lipases. Hansen and Czochanska have stated: "The foregoing results suggest that, in earthworms, there is present an active enzyme which affects lipolysis of the triglycerides of dead worms to free fatty acids and glycerol." We are pleased with this statement, because we think that, in poikilotherms, such as annelids and capelin fish (R.G. Ackman, P.J. Ke, W.A. Maccalluum, and D.R. Adams, *J. Fish Res. Bd. Can.* 26:2037 [1969]), active lipases may be an efficient means of rapidly furnishing bioenergetic requirements from depot lipids.

JOAN R. MARSDEN
Department of Biology
McGill University
Montreal, P.Q., Canada
DOROTHY M.-E. POCOCK
University Medical Clinic
Montreal General Hospital
Montreal, P.Q., Canada

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