Specific Formation of Arachidonoyl Phosphatidylinositol from 1-Acyl-*sn*-glycero-3-phosphorylinositol in Rat Liver[1]

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ABSTRACT

The conversion of 1-acyl-*sn*-glycero-3-phosphorylinositol-^{3}H into phosphatidylinositol-^{3}H was studied using rat liver microsomal and homogenate preparations. The nature of the molecular species of phosphatidyl inositol so formed in the absence of added acyl moieties was determined after fractionating the radioactive product by means of argentation thin layer chromatography. In other experiments, the possible specificity of the microsomal acyl-CoA:1-acyl-*sn*-glycero-3-phosphorylinositol acyltransferase towards different acyl-CoA derivatives was investigated. Maximum conversion of 1-acyl GPI to the diacyl analogue was dependent on the addition of adenosine triphosphate and CoA when exogenous acyl groups were omitted from the incubation medium. Under these latter conditions, the tetraenoic species comprised 56-74% of the total molecular species of newly-formed phosphatidylinositol. The microsomal acyl-CoA:1-acyl-*sn*-glycero-3-phosphorylinositol acyltransferase showed a marked preference for arachidonoyl-CoA. The present results suggest that the enrichment of rat liver phosphatidyl inositol in arachidonic acid may arise when 1-acyl-*sn*-glycero-3-phosphorylinositol is acylated to form phosphatidylinositol.

INTRODUCTION

Of all the phospholipids present in rat liver, phosphatidylinositol (PI) is unique in that it contains the highest concentration of arachidonic acid (1). The metabolic basis for this biological phenomenon is complicated by the fact that intact phosphatidic acid (PA) apparently enters this phospholipid directly (2) during the de novo biosynthesis of PI; however, arachidonate represents only ca. 7% of the fatty acids present in PA (3,4). Although the CTP:phosphatidic acid cytidylyltransferase (5) could be highly specific for substrates containing arachidonic acid, isotopic data suggest that enzyme specificity alone cannot readily account for the pronounced enrichment of hepatic PI with arachidonate (1,6).

Previous studies in vivo with orthophosphate-^{32}P, glycerol-^{14}C and inositol-^{3}H over extended time intervals (1,7) suggested that monoenoic plus dienoic species of PI possibly might be converted into tetraenoic molecules by way of a deacylation-reacylation cycle involving the entry of arachidonate into PI by reacting with lysophosphatidylinositol (lyso PI). However, the formation of PI from lyso PI has not been demonstrated in liver. The pioneering work of Lands (8) implicated the possible role of the acyl-CoA:phospholipid acyltransferases in the formation of lecithins with varying fatty acid compositions. In addition, the enzymatic acylation of lyso PI in the presence of added acyl-CoA's has been reported in microsomes from pigeon pancreas (9) and rat brain (10).

The purpose of the work described herein is to report on the conversion of 1-acyl-*sn*-glycero-3-phosphorylinositol (1-acyl GPI) to PI in rat liver. The possible physiological importance of this reaction in the formation of hepatic arachidonoyl PI was studied by characterizing the molecular species of PI formed in the absence of added acyl-CoA's or free fatty acids. In addition, the specificity of the acyl CoA:1-acyl-*sn*-glycero-3-phosphorylinositol in rat liver microsomes towards different acyl-CoAs was investigated.

MATERIALS AND METHODS

Chemicals and Substrates

Inositol-^{3}H (2.8 mCi/μmole) was obtained from the New England Nuclear Corp. (Boston, MA). Radioactive inositol was diluted with unlabeled myo-inositol (Calbiochem, La Jolla, CA) to obtain the desired specific activity. Standard lipids were purchased from Serdary Research Laboratories (London, Ontario, Canada). Adenosine triphosphate (ATP) and coenzyme A (CoA) were obtained from Calbiochem. The acyl-CoA esters were purchased from the Grand Island Biological Co. (Grand Island, NY) or synthesized as described previously (11-13). The concentrations of adenine and thiol ester in the acyl-CoA preparations

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used were calculated from the absorbances at 260 and 232 nm, respectively, and the extinction coefficients reported by Seubert (11). The concentration of the thiol ester was confirmed by measuring the amount of free CoA released when aliquots of these preparations were hydrolyzed by pancreatic lipase (14).

1-acyl GPI-^{3}H was prepared from PI-^{3}H which was obtained by incubating rat liver microsomes with 0.33 mM inositol-^{3}H in the presence of 2mM $MnCl_2$ and 50 mM Tris-HCl buffer (pH 7.4) as described previously (15). The radioactive PI was extracted and isolated from liver microsomes (15) and diluted with unlabeled PI from the same source. The PI-^{3}H so obtained was then subjected to enzymatic hydrolysis (9) with phospholipase A_2 (*Crotalus adamanteus* venon). Thin layer chromatography (TLC) revealed that > 91% of the PI-^{3}H was converted to the corresponding monoacyl derivative. After removing the small amount of undigested PI-^{3}H (<10% of the total radioactivity) by partitioning the reaction mixture (9,16), the 1-acyl GPI-^{3}H was further purified by thin layer (17) or column chromatography (1) to remove all traces of free fatty acids. The concentration of the 1-acyl GPI was determined by gas liquid chromatography (15). Its fatty acid composition was similar to that reported previously (18) and consisted mainly of saturated fatty acids with stearate as the major component.

Animals and Incubation Procedures

Male Wistar rats weighing 150-170 g were purchased from Woodlyn Farms (Guelph, Ontario, Canada). They were maintained as described (15) until being sacrificed. Liver microsomes were prepared (15) after homogenizing fresh livers in 0.25 M sucrose containing 1 mM EDTA (pH 7.4). Microsomal acyl-CoA:1-acyl-*sn*-glycero-3-phosphorylinositol acyltransferase was measured under optimal conditions such that the rate of PI formation was linear with respect to time and protein concentration. Acyltransferase activity was measured in a medium containing 50 mM phosphate buffer (pH 7.4), 37 μM 1-acyl GPI-^{3}H, 29 μM acyl-CoA, and 150 μg of microsomal protein in a total volume of 0.6 ml. The reaction was stopped after 1.5 min and the formation of PI-^{3}H was determined by the method of Keenan and Hokin (9) following the preparation of total lipid extracts. At least 98% of the radioactivity was found in the upper phase of such extracts prepared from zero-time control incubations or from incubations with no added acyl-CoA.

The entry of 1-acyl GPI-^{3}H into PI was also studied using rat liver microsomal or homogenate preparations and suitable cofactors necessary for the formation of acyl-CoAs from endogenous acyl moieties. For such studies with liver microsomes, the standard assay mixture consisted of 50 mM phosphate buffer (pH 7.4), 16.7 mM ATP, 0.4 mM CoA, 13.3 mM $MgCl_2$, 10-67 μM 1-acyl GPI-^{3}H containing 2-6(x 10^3) cpm per incubation, and 0.4 mg of protein in a final volume of 0.3 ml. After incubating at 37 C for 5 min, the reaction was terminated and the newly formed PI-^{3}H was isolated from lipid extracts.

For the experiments with homogenate preparations, livers were rapidly excised, rinsed in cold 0.25 M sucrose, and weighed. The livers were then homogenized in 4 vol of 0.25 M sucrose solution containing 1 mM EDTA using a Potter-Elvehjem homogenizer with a Teflon pestle. The crude homogenate was centrifuged at 800 x g for 10 min to remove cellular debris. The assay medium for monitoring PI formation contained 50 mM phosphate buffer (pH 7.4), 5.0 mM ATP, 0.13 mM CoA, 4 mM $MgCl_2$, 68.7 mM NaF, 11.5 μM 1-acyl GPI-^{3}H and 200 μl of the purified homogenate (5.1 mg protein) in a final volume of 1.0 ml. The incubation was conducted for 15-45 min at 37 C. PI synthesis was assessed as outlined above.

Thin Layer Chromatography

The radioactive PI was purified by TLC as described previously (15). The molecular species of PI were separated by argentation TLC into monoenoic plus dienoic, trienoic, tetranoic, and polyenoic (>tetraenoic) subfractions (7). The various classes were visualized under untraviolet light after spraying with 2',7'-dichlorofluorescein, eluted from the gel scrapings, and their associated radioactivity was measured by scintillation counting (15). As judged by fatty acid analyses, all chemical classes were essentially free of contamination from all other molecular species with the exception of the trienes which contained ca. 10% of the total monoenoic plus dienoic species. Control experiments with radioactive PI revealed that 76-88% of the phospholipid applied to plain or silver nitrate plates could be recovered following development and elution from the gel.

RESULTS

Table I gives the effect of various cofactors on the conversion of 1-acyl GPI into PI in rat liver microsomes in the absence of added acyl moieties. When the higher concentration of 1-acyl GPI was employed (67 μM), maximum

conversion was obtained in the presence of $MgCl_2$, ATP, and CoA. The requirement for CoA was nearly absolute since only 5% of optimal activity was observed in its absence. Omission of $MgCl_2$ andATP resulted in conversions which were 81% and 36%, respectively, of that observed in the complete system. At the lower concentration of 1-acyl GPI (10 μM), the requirement upon added ATP for maximum acylation was observed again, but was less pronounced than at the higher concentration of the acyl acceptor. Furthermore, no dependency on added $MgCl_2$ was observed using the lower concentration of 1-acyl GPI, which may reflect the presence of minor amounts of this metal in these microsomal preparations.

The pattern of molecular species of PI produced when 1-acyl GPI was converted into PI using rat liver preparations is given in Table II. When 21.5 μM 1-acyl GPI was added, 70% of the newly-formed PI in liver microsomes were tetraenoic species while the monoenes plus dienes, trienes, and polyenes represented 22%, 4%, and 4%, respectively. A somewhat similar pattern of molecular species of microsomal PI was produced when 10 μM rather than 21.5 μM 1-acyl GPI was employed. In this latter case, however, an even greater proportion of tetraenoic (74%) relative to monoenoic plus dienoic (15%) species was formed. When exogenously added ATP, CoA, and $MgCl_2$ were replaced by oleoyl-CoA in the microsomal acylating system, 91% of the newly synthesized PI were monoenoic plus dienoic species. With rat liver homogenates, the major species of PI formed were tetraenes (56%), while the monoenes plus dienes, trienes, and polyenes contributed 18%, 13%, and 13%, respectively.

TABLE I

Effect of Various Components on the Conversion of 1-Acyl-*sn*-glycero-3-phosphorylinositol-^{3}H (1-acyl GPI-^{3}H) into Microsomal Phosphatidylinositol-^{3}H

Omission	1-Acyl GPI-^{3}H incorporated[a] (%)	
	10 μM 1-acyl GPI	67 μM 1-acyl GPI
None[b]	78.4 (73.7)	38.1
$MgCl_2$	81.3 (76.1)	31.0
ATP	56.3 (46.5)	13.9
CoA	9.5 (3.7)	1.8
ATP, CoA		1.0
ATP, $MgCl_2$		5.8

[a]Values are given as % of added 1-acyl GPI-^{3}H that was incorporated into PI-^{3}H. Incubation procedures were as described in Materials and Methods. The data in parentheses are from experiments with a separate microsomal preparation.

[b]Refers to complete system which contained $MgCl_2$, adenosine triphosphate (ATP), and coenzyme A (CoA).

The specificity of the acyl-CoA:1-acyl-*sn*-glycero-3-phosphorylinositol acyltransferase in liver microsomes towards different acyl-CoA derivatives is given in Table III. Higher rates of reaction were observed with unsaturated as compared to saturated acyl-CoAs. The acyltransferase rates with arachidonoyl-CoA, however, were 3-fold greater than those obtained with either oleoyl- or linoleoyl-CoA.

TABLE II

Incorporation of 1-Acyl-*sn*-glycero-3-phosphorylinositol-^{3}H (1-acyl GPI-^{3}H) Molecular Species of Phosphatidylinositol using Endogenous Acyl Donors[a]

Chemical classes	Distribution of radioactivity (%)			
	Microsomal preparations			Homogenates
	Experiment 1	Experiment 2	plus 18:1-CoA	
Monoenes + Dienes	15.0 ± 0.7	21.6 ± 1.5	90.8 ± 0.7	18.0 ± 2.1
Trienes	2.3 ± 0.4	4.1 ± 0.8	2.0 ± 0.6	12.6 ± 2.7
Tetraenes	73.6 ± 1.4	70.2 ± 2.8	66.2 ± 1.1	56.1 ± 5.9
Polyenes[b]	9.1 ± 1.3	4.1 ± 1.3	0.9 ± 0.2	13.3 ± 1.3
Concentration of 1-acyl GPI	10 μM	21.5 μM	10 μM	11.5 μM
Incorporation (%)	83.5 ± 1.2	78.6 ± 1.1	15.4 - 32.0	95.9 ± 1.5
Number of experiments	4	3	3	3

[a]Values are given as % distribution of radioactivity among the various molecular species of phosphatidyl inositol (PI) (means ± S.E.). Incubation conditions with microsomes (Expt. 1 and 2) and homogenates were as described in Materials and Methods with no added acyl donors. Incubations with added 18:1-CoA (28 μM) were conducted for periods of 2-5 min in the presence of 50 mM phosphate buffer (pH 7.4) and 0.2 mg of microsomal protein.

[b]Refers to molecular species with more than 4 double bonds per molecule and consists of pentaenoic plus hexaenoic species.

TABLE III

Relative Rates of 1-Acyl-*sn*-glycero-3-phosphorylinositol Acylation in Rat Liver Microsomes with Different Acyl-CoA Esters[a]

Acyl-CoA	Acylation rate (nmoles/min/mg protein)
16:0-CoA	3.7 ± 0.7
18:0-CoA	8.1 ± 1.6
18:1-CoA	10.6 ± 1.5
18:2-CoA	9.6 ± 0.8
20:4-CoA	31.0 ± 2.9

[a]All values are given as means ± S.E. Assay conditions were as described in Materials and Methods. The incubation medium contained 37 μM 1-acyl GPI-^{3}H, 29 μM acyl-CoA, and 150 μg of microsomal protein in a total volume of 0.6 ml of 50 mM phosphate buffer (pH 7.4).

DISCUSSION

Previous isotopic studies in vivo (1,7) and in vitro (6) suggested that the de novo biosynthesis of PI via phosphatidic acid and cytidine diphosphate-diglyceride gives rise mainly to a mixture of monoenoic plus dienoic and tetraenoic molecules, whereas PI may become further enriched in arachidonic acid by way of a deacylation-reacylation cycle. To date, however, the conversion of lysophosphatidylinositol to PI in liver has not been reported. The present results provide direct evidence for the reacylation of 1-acyl GPI to form PI in rat liver as suggested from previous experiments in vivo (1,7) with radioactive inositol, glycerol, and orthophosphate over extended time intervals. Maximum conversion of monoacyl GPI to diacyl GPI using microsomal preparations, in the absence of added acyl moieties, was dependent on ATP and CoA, which implicates their importance for the activation of endogenous fatty acids. The reacylation of other lyso compounds to form diacyl phosphoglycerides has been shown to have similar cofactor requirements (19,20).

The possible physiological importance of 1-acyl GPI conversion to PI in the production of specific molecular species of this latter phospholipid was investigated using hepatic microsomal and homogenate preparations to which no exogenous fatty acids were added. The nature of the molecular species of PI formed under such conditions would be expected to provide the best estimate of the products of 1-acyl GPI acylation in vivo. The present results indicate that in vitro acylation of 1-acyl GPI gives rise to predominantly arachidonoyl species of PI (56-74% of the total) in rat liver. These latter patterns are in good agreement with the occurrence of a high concentration of arachidonate in PI isolated from rat liver (1). Tetraenoic species represent ca. 67% and 80% of the total PI isolated from microsomes and whole liver, respectively (7,15); they contain almost exclusively arachidonate as the only unsaturated fatty acid. Subsequent isotopic studies in vivo (1,7) and in vitro (6) suggested that arachidonate might enter liver PI by acylation of lysophosphatidylinositol.

The preferential formation of arachidonoyl PI using the generating system could be due to a differential availability and activation of endogenous fatty acids in the tissue preparations as well as the specificity of the acyl-CoA: 1-acyl-*sn*-glycero-3-phosphorylinositol acyltransferase. Studies on this latter enzyme, furthermore, indicate a striking preference for arachidonoyl-CoA relative to other acyl-CoA derivatives. Thus, the fatty acid specificity of the acyltransferase may well be an important factor in regulating the preferential formation of arachidonoyl PI from monoacyl GPI. Keenan and Hokin (9) observed that microsomes from pigeon pancreas preferred oleoyl- to palmitoyl-CoA for the acylation of 1-acyl GPI while Baker and Thompson (10) showed that microsomes from rat brain had a selectivity for arachidonoyl-CoA. These latter findings (10) clearly substantiated earlier double label experiments in brain which indicated that the entry of arachidonate into PI could occur without the synthesis of the corresponding phosphatidic acid (21). The possible physiological role of the acyl-CoA:1-acyl-*sn*-glycero-3-phosphorylcholine acyltransferase in the formation of hepatic lecithin containing arachidonic acid has been supported by enzymatic studies with microsomal preparations (22,23). Furthermore, isotopic studies in vivo (24) and with rat liver slices (25) have revealed that 1-acyl-*sn*-glycero-3-phosphorylcholine is selectively incorporated into tetraenoic species of phosphatidylcholine. In conclusion, the present results from experiments with both endogenous and exogenous acyl donors suggest that the high concentration of arachidonate in liver PI may arise at the step whereby 1-acyl GPI is acylated to form PI.

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Effect of Cold Stress on Rapeseed Oil Fed Rats

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ABSTRACT

No mortality was observed in 6 week old male Sprague-Dawley rats subjected to cold at 4 C for 3 weeks and fed either a control diet (Chow) or a semisynthetic diet containing 20% by wt rapeseed oil high in erucic acid (23.6%). All rats fed the Chow diet and 17 of 20 rats fed the rapeseed oil-containing diet survived 4 weeks in the same environment. Three rats on the latter diet died of self-mutilation. Marked myocardial lipidosis as well as a large accumulation of 20:1 and 22:1 was observed in the hearts of rats fed the rapeseed oil-containing diet. Five of 20 rats on the Chow diet and 2 of 20 rats on the rapeseed oil-containing diet had focal necrotic areas in the myocardium.

INTRODUCTION

The myocardial lipidosis and necrosis produced in male rats by feeding diets containing high levels of rapeseed oil has been documented by many laboratories (1-5). Beare, et al., (6) were the first to report that rats fed rapeseed oil succumbed to cold stress more readily than those fed corn oil. More recently, Beare-Rogers and Nera (7) reported mortalities of up to 80% in 6-week old Sprague-Dawley rats fed, at 4 C, a diet containing 20% by wt rapeseed oil (23.1% 22:1) for 3 weeks, but only 40% mortality in rats fed canbra oil (low erucic acid rapeseed oil) during the same period. Because of the potential importance of these findings, we decided to reinvestigate the effect of cold stress on rats fed rapeseed oil containing a high level of erucic acid.

METHODS

Six-week old male Sprague-Dawley rats (Bio-Breeding Laboratories, Ottawa, Canada) were housed, one per cage, in a randomized block design in a walk-in cold room maintained at 4 C and 75% relative humidity. Individual rat wts were recorded at the beginning and end of each experiment. The rats were fed ad libitum Chow (Ralston-Purina, Woodstock, Ontario) diet from weaning (3 weeks) to 6 weeks of age, at which time they were placed in the cold and half the number were kept on the Chow diet and the other half were fed a semisynthetic diet consisting of 20% casein, 20% sucrose, 30% cornstarch, 4% USP XVII salt mixture (Nutritional Biochemicals Corp., Cleveland, OH), 1% vitamin mixture (containing per Kg diet: 100 mg p-aminobenzoic acid, 0.2 mg biotin, 30 mg calcium pantothenate, 2 mg folic acid, 500 mg inositol, 5 mg menadione, 50 mg niacin, 10 mg pyridoxine hydrochloride, 10 mg riboflavin, 10 mg thiamin hydrochloride, 91 mg DL-α-tocopherol, 1.5 mg retinyl palmitate, 0.02 mg vitamin B-12, 0.025 mg vitamin D-2, and 2 g choline bitartrate), 5% pure wood cellulose (Alfa Floc BW40 Brown Co., Berlin, NH), and 20% by wt rapeseed oil (RSO) containing 23.6% erucic acid.

It was reported (7) that 60% of the rats died in the first week of feeding in the cold; therefore, a preliminary trial (experiment I) using 4 rats on each diet was conducted for 1 week. In experiment II, 25 rats were placed on each diet; 5 from each diet were killed after 1 week and the remainder after 4 weeks.

Cardiac fatty acids were determined at each time period as previously described (3). The hearts were examined histologically for lipid accumulation at both time periods and for necrosis at 4 weeks as previously described (8).

RESULTS AND DISCUSSION

As indicated in Table I, none of the rats in the study reported here died during the first 3 weeks of feeding either the Chow diet or the RSO-containing diet (23.6% 22:1). This observation contradicts the results of Beare-Rogers and Nera (7) who reported mortality of 50-60% after 1 week and cumulative mortality of up to 80% after 3 weeks of feeding the same strain and age of rat under similar conditions of diet, level of erucic acid, and temperature.

In experiments I and II, the initial mean body wt of rats fed the Chow diet was 182 ± 6.9 g and 177 ± 0.8 g, respectively, and for those fed the RSO-containing diet, 173 ± 6.8 g and 175 ±0.9 g, respectively. As indicated in Table II, rats fed the RSO-containing diet gained considerably less wt than did the Chow-fed rats. This finding is in agreement with the well documented growth retarding effects of RSO-containing diets (1-4,9).

The level of 20:1 and 22:1 in the hearts of the rats fed the RSO diet for 1 week was high

TABLE I

Mortality of Male Rats Placed in Cold (4 C) at 6 Weeks of Age and Fed Test Diets

Experiment	Diet	Mortality (week) 1	2	3	4
I	Chow[a]	0/4			
	RSO[b]	0/4			
II	Chow	0/25	0/20[c]	0/20	0/20
	RSO	0/25	0/20	0/20	3/20[d]

[a]Chow—Purina Laboratory Rat Chow, Woodstock, Ontario.

[b]RSO—Rapeseed oil containing 23.6% 22:1 and 12.3% 20:1.

[c]Five rats were killed at the end of the first week for oil red O staining and fatty acid analyses of the heart.

[d]Three rats died as a result of self-mutilation.

TABLE II

Lipidosis[a], Necrosis,[b] and Fatty Acid[c] Composition of Hearts and Mean Body Wt of Male Rats Fed Control and Experimental Diet for 1 and 4 Weeks at 4 C

	1 Week		4 Weeks	
	Chow[d]	RSO[e]	Chow	RSO
Lipidosis	0	++++	0	+ to +++
Necrosis	-	-	5/20	1/17[f]
20:1	0.6 ± 0.04	8.0 ± 0.08	0.5 ± 0.02	5.8 ± 0.3
22:1	0.8 ± 0.02	17.2 ± 2.6	0.2 ± 0.03	8.0 ± 1.1
Mean body wt (g)	200 ± 4.5	192 ± 6.3	295 ± 6.6	229 ± 4.8

[a]Results represent degree of lipid accumulation by oil red O staining: 0 = No fat present; + = very slight; ++ = slight; +++ = moderate; ++++ = marked.

[b]Numbers indicate the number out of 20 Chow-fed and 17 RSO-fed rats showing necrotic lesions.

[c]Fatty acid composition is expressed as mole % of total fatty acids. Each value represents the mean of 5 hearts ± pooled standard error of the mean (SEM). Fatty acids are designated by number of carbon atoms: number of double bonds.

[d]Chow—Purina Laboratory Rat Chow, Woodstock, Ontario.

[e]RSO—Rapeseed oil containing 23.6% 22:1 and 12.3% 20:1.

[f]One of 3 rats that died during the fourth week as a result of self-mutilation had a necrotic lesion.

and decreased considerably by the fourth week of feeding, which is in agreement with the results of others (1-5). Oil red O staining of myocardium at this time period failed to reveal the presence of fat in the hearts of the Chow-fed rats, while marked lipid accumulation was found in the hearts of the RSO-fed rats. The evaluation of oil red O stained sections of myocardium as described previously (8) was 4+ in all hearts from RSO-fed rats at the end of 1 week. At 4 weeks, the grading of the RSO-fed rat hearts ranged from 1 to 3+, whereas, the hearts of the Chow-fed rats remained negative (Table II).

Beare-Rogers and Nera (7) emphasized the fact that a high frequency of death coincided with the period of pronounced cardiac lipidosis. Although in the study reported here pronounced myocardial lipidosis existed at 1 week in rats fed the RSO-containing diet, none of the animals died. However, during the fourth week, 3 of the RSO-fed rats died as a result of self-mutilation. Seventeen of 20 rats survived the RSO regimen for 4 weeks at 4 C compared to only 1 of 19 rats to survive a similar period, diet, and environment as previously reported (7).

In the current study, 15 of the rats fed the Chow diet and all 20 rats fed the RSO diet for 4 weeks developed necrotic areas on the tips of the ears and tail. The affected areas became swollen, hemorrhagic, and necrotic, and, in many instances, sloughing of the ears and tail tip occurred. This effect was more severe in the

RSO-fed rats, and chewing of the gangrenous tails resulted in hemorrhage and death (self-mutilation) in 3 rats of this group during the fourth week. Thrombosis of vessels was evident at the base of these lesions, indicating that stasis of blood may have played a role in their development. Beare-Rogers and Nera (7) did not report similar findings in their study.

In previous studies conducted at room temperature by Hulan, et al., (10), we have observed swollen, scaly, hemorrhagic, and necrotic lesions on the tails of RSO-fed rats, but not on Chow-fed rats. These lesions were not as severe as those observed in the study reported here, and it is possible that the low environmental temperature may have led to vasoconstriction resulting in the production of more severe lesions.

Histological examination of the hearts at 4 weeks revealed focal areas of necrosis as previously described (3,8) in 5 rats fed the Chow diet and in 1 rat fed the RSO-containing diet (Table II). A necrotic lesion was present in the heart of 1 of 3 rats that died during the fourth week as a result of self-mutilation. However, the other two rats showed no evidence of myocardial necrosis.

In spite of the pronounced lipidosis in the heart and developing myocardial necrosis in rats fed RSO and maintained in the cold, the high mortality reported by Beare-Rogers and Nera (7) under the same conditions was not confirmed.

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Relationship Between Erucic Acid and Myocardial Changes in Male Rats

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ABSTRACT

The back and belly fat of pigs fed a diet containing 20% by wt rapeseed oil (22% erucic acid) for 16 weeks was rendered into oil. This rendered pig fat, which contained 5.6% erucic acid, was fed to male rats in three separate experiments at 20% by wt of the diet for 16 weeks. In experiment I rendered pig fat was compared only to *Brassica campestris* var. Span rapeseed oil containing 4.8% erucic acid. In experiments II and III, rendered pig fat was compared to commercial lard containing 0.2% docosenoic acid, commercial lard to which 5.4% free erucic acid was added, and Span rapeseed oil. There was no significant ($P<0.01$) differences observed in the level of erucic acid in the hearts of rats fed diets of rendered pig fat, Span rapeseed oil, or commercial lard plus erucic acid. However, the incidence ($P<0.001$) and severity ($P<0.01$) of cardiac lesions were significantly higher in Span rapeseed oil fed rats compared to rats fed control diets. The number of rats affected or the severity of lesions in the rendered pig fat fed group was not significantly different from controls. The results of this study indicate that the myocardial lesions associated with feeding 20% rapeseed oil diets are not related to the content of erucic acid per se. The possible reasons why rapeseed oil causes cardiac lesions in rats are discussed. It is suggested that a triglyceride imbalance in the oil might play an important role in causing these lesions in rats.

INTRODUCTION

Several investigations in recent years point to erucic acid as the toxic factor responsible for the cardiopathogenicity of rapeseed oil (1,2). Myocardial lesions, similar in nature and intensity to those produced by rapeseed oil, have been found in rats fed diets containing isocaloric amounts of erucic acid as glyceryl trierucin (3). Similar myocardial lesions have also been produced in male rats fed rapeseed oil containing low levels of erucic acid (4-6). The same foci of overt myocardial necrosis have not been found to the same degree in pigs fed such oils (7-9). However, when swine are fed diets containing rapeseed oil, erucic acid is deposited in the adipose tissue (10,11). Thus, it was of interest to investigate if feeding rats a diet that included fat rendered from the belly and back of pigs previously fed rapeseed oil and containing erucic acid would produce necrotic lesions observed in the hearts of rats fed rapeseed oil of comparable erucic acid content.

MATERIALS AND METHODS

The following lipid sources were used: *Brassica campestris* var. Span rapeseed oil (Span RSO) containing 4.8% erucic acid, commercial lard (Canada Packers Ltd., Hull, Quebec), rendered pig fat (RPF) containing 5.6% erucic acid, and a positive control consisting of commercial lard containing 0.2% docosenoic acid to which 5.4% free erucic acid was added (L + EA). The rapeseed oil was processed and stored as described earlier (5). RPF was prepared in our laboratory as follows: The belly and back fat of pigs fed a diet containing 20% regular rapeseed oil (22% erucic acid) for 16 weeks in a separate experiment (9) was collected and rendered into oil on the day of slaughter. The rendered oil was decanted and filtered through six layers of cheesecloth, and antioxidant (5) was added at the rate of 75 mg per kg. The RPF was kept in sealed containers under nitrogen and stored at -20 C. Prior to mixing into the diets, the oils were thoroughly agitated and mixed.

In all experiments, weanling 3-week old male Sprague-Dawley rats (Bio-Breeding Laboratories, Ottawa, Ontario) weighing between 40-50 g were randomly assigned to treatments, individually caged, and fed ad libitum the test rations for 16 weeks. Water was available at all times. The semisynthetic diet, to which the test oils were added at a level of 20% by wt, have been described earlier (12). All rats were individually weighed at the beginning and at 2

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TABLE I

Fatty Acid Composition of the Diets

Fatty acids[b]	Diets[a]			
	Lard	L + EA	RPF	Span RSO
14:0	1.5	1.8	1.1	0.1
16:0	25.6	22.4	12.6	4.8
16:1	2.6	3.1	2.2	0.2
18:0	14.6	14.7	4.8	2.1
18:1	43.2	37.9	39.1	58.6
18:2	9.6	10.4	18.5	19.5
18:3	0.6	0.7	6.1	5.2
20:0	0.1	0.2	0.3	0.6
20:1	1.2	1.4	8.3	3.3
22:0	0.1	0.1	0.1	0.3
22:1	0.2	5.6	5.6	4.8
24:0	tr[c]	tr	tr	0.1
24:1	0.1	0.2	0.1	0.2
S/U	0.73	0.66	0.23	0.08
18:2/18:3	16.0	14.9	3.0	3.8

[a]Lard = Commercial lard; L + EA = commercial lard to which was added 5.4% erucic acid; RPF = rendered pig fat; Span RSO = Span rapeseed oil.

[b]Fatty acid composition is expressed as mole % of total fatty acids. Fatty acids are designated by number of carbon atoms: number of double bonds. S = Saturated fatty acids; U = unsaturated fatty acids.

[c]tr = Trace, <0.05%.

week intervals throughout the experiment.

In Experiment I, two groups of 50 rats were fed, as part of a larger experiment (13), either RPF or Span RSO containing diets for 16 weeks, at which time they were killed and the hearts examined histologically as previously described (14).

In Experiment II, three groups of 36 rats each were fed either the lard, L + EA, or RPF containing diet, and one group of 26 rats was fed a diet containing Span RSO. Four rats from each dietary treatment were killed by stunning and exsanguination after 3 days, 1 week, 8 weeks, and 16 weeks on treatment. The hearts were immediately removed, frozen on dry ice, and stored at -20 C until analyzed. Heart lipids were extracted and analyzed as described previously (5). The remaining rats in this experiment were killed at 16 weeks and the hearts examined histologically (14).

In Experiment III, four groups of 45 rats each were fed diets containing the same oils as were used in Experiment II. After 16 weeks on the various treatments, the rats were killed and the hearts examined histologically (14).

Analyses of variance were determined for all methyl ester analyses. The New Duncan's Multiple Range Test (15) was used to determine significant differences ($P<0.01$) between treatment means. Approximate X^2 statistics, obtained following the approach of Fienberg (16), were used in Experiments II and III to examine the frequency tables, Tables III and IV, for evidence of differences in patterns of incidence and severity of lesions among the various dietary treatments.

RESULTS AND DISCUSSION

The fatty acid composition of the diets is shown in Table I. The fatty acid profile of lard and L + EA were very similar except for the presence of 5.6% 22:1 in the latter. Lard contained high levels of saturates (16:0 and 18:0), about 10% 18:2, and low levels of 18:3 and long chain monoenes (20:1, 22:1 and 24:1). Span RSO and RPF were distinctly different from lard in that they contained low levels of saturates (16:0 and 18:0), twice the amount of 18:2, and appreciable amounts of 18:3, 20:1, and 22:1. However, Span RSO differed from RPF in the level of saturates, i.e., 8% and 18.9%, respectively. RPF was typical of depot fats of animals fed rapeseed oil (10), the relative concentration of saturated fatty acids was higher, and, although 20:1 and 22:1 were incorporated into the adipose tissue, 22:1 was deposited in much lesser amounts compared to 20:1. The rapeseed oil fed to pigs contained 22.3% 22:1 and 12.3% 20:1, whereas the pig adipose tissue contained 5.6% 22:1 and 8.3% 20:1. Similar results were obtained by other investigators with pigs (11) and rats (17) fed rapeseed oil containing diets.

The fatty acid composition of the cardiac lipids in rats fed the various diets for 3 days, 1 week, 8 weeks, and 16 weeks were determined

TABLE II

Fatty Acid Composition[a] of the Hearts of Male Rats Fed These Diets

Fatty acids[b]	Diets[c]	Time			
		3 days	1 week	8 weeks	16 weeks
16:0	Span RSO	9.0	10.0[2]	8.9	10.0[2]
	RPF	10.4	10.9[2]	12.1	10.3[2]
	L + EA	13.6[1d]	12.0[1]	13.9[1]	12.9[1]
	Lard	12.4[1]	11.6[1]	13.7[1]	12.1[1]
18:0	Span RSO	13.8[2,3]	17.1[1]	16.9	16.4[2]
	RPF	13.0[3]	15.4[1]	20.2[1]	15.2
	L + EA	15.5[1,2]	15.0[1]	20.6[1]	18.8[1]
	Lard	16.6[1]	15.9[1]	21.8[1]	18.4[1,2]
18:1	Span RSO	17.7[1]	18.5	18.2[1]	19.7[1]
	RPF	13.4[2]	16.0[1]	16.6[1,2]	17.6[1]
	L + EA	16.5[1]	15.9[1]	17.0[1,2]	17.3[1]
	Lard	13.4[2]	13.0	15.1[2]	18.4[1]
18:2	Span RSO	13.8	15.4	14.5[1]	14.5[1]
	RPF	12.3[1]	14.1	15.4[1]	14.7[1]
	L + EA	12.7[1]	12.3	12.6	10.9
	Lard	18.3	18.1	21.3	20.0
18:3	Span RSO	2.1	1.1[1]	1.5	1.6
	RPF	0.9	0.9[1]	1.0	1.0
	L + EA	0.2[1]	0.1[2]	0.1[1]	0.1[1]
	Lard	0.1[1]	0.2[2]	0.1[1]	0.1[1]
20:1	Span RSO	3.3	2.9	2.1[1]	2.0
	RPF	4.7	6.3	3.9	4.1
	L + EA	1.7[1]	1.6[1]	1.4[1,2]	1.1[1]
	Lard	1.0[1]	1.2[1]	0.6[2]	0.6[1]
20:4	Span RSO	12.9[2]	14.1[1,2]	19.1[1]	18.6[1,2]
	RPF	15.0[1,2]	13.9[2]	16.1[2]	20.0[1]
	L + EA	13.5[2]	16.5[1]	17.0[1,2]	18.0[2]
	Lard	16.8[1]	18.0	19.1[1]	20.0[1]
22:1	Span RSO	5.4[1]	4.3[1]	1.7[1]	0.9[1]
	RPF	4.8[1,2]	5.2[1]	1.8[1]	1.3[1]
	L + EA	4.2[2]	3.3	1.9[1]	1.1[1]
	Lard	0.2[2]	0.1	0.2	0.1
22:4ω6[e]	Span RSO	1.0	0.6	0.7[2]	0.8[2]
	RPF	1.6	0.9	0.7[2]	0.7[2]
	L + EA	1.3	1.6	1.4[1]	1.0[1]
	Lard	1.9	2.2	1.4[1]	1.3[1]
22:5ω6	Span RSO	0.2[2]	0.5[1]	0.4[2]	0.6[1]
	RPF	0.4[1,2]	0.4[1]	0.3[2]	0.4[1]
	L + EA	0.6[1]	1.2	1.9[1]	3.8
	Lard	1.0	2.4	1.9[1]	2.8
22:6ω3	Span RSO	9.9[1]	5.7[1]	8.9	7.0[2,3]
	RPF	12.5	5.5[1]	4.6[1,2]	8.3[1,2]
	L + EA	8.7[1]	9.5	3.4[2]	6.2[3]
	Lard	15.6	14.6	6.0[1]	9.6[1]

[a]Each value represents the mean of four observations.

[b]Fatty acid composition is expressed as mole % of total fatty acids. Fatty acids are designated by number of carbon atoms: number of double bonds. S = saturated fatty acids; U = unsaturated fatty acids.

[c]Lard = Commercial lard; L + EA = commercial lard to which was added 5.4% erucic acid; RPF = rendered pig fat; Span RSO = Span rapeseed oil.

[d]Means with similar superscripts within a column are not significantly different ($P < 0.01$).

[e]ω represents the number of carbon atoms between the terminal double bond and the methyl end of the molecule.

(Table II). In general, the relative concentration of saturated fatty acids (14:0, 16:0, and 18:0) in rat hearts remained constant throughout the feeding trial, and no significant differences

TABLE III

Myocardial Lesions in Male Rats Fed Experimental Diets: Experiment I

Diet[a]	22:1 (%)	Affected/examined	Number of rats[b] 1-2	3-5	6-10	>10
RPF	5.6	10/50	6	4	0	0
Span RSO	4.8	23/50	13	4	6	0
X^2 Analysis[c]		Incidence (d.f.)[d]			Severity (d.f.)	
Between treated		7.801** (1)			5.696 (2)	

[a]RPF = Rendered pig fat; Span RSO = Span rapeseed oil.

[b]Number of rats with lesion scores of 1-2, 3-5, 6-10, or >10 in three sections of heart.

[c]A comparison of rats affected to number of rats examined was used in the analysis of incidence. Only rats with heart lesions were compared in the analysis of severity. Significant differences: *($P<0.05$); **($P<0.01$); ***($P<0.001$).

[d]The degrees of freedom (d.f.) of these analyses were (r-1) (c-1), where r and c are the number of rows and columns, respectively. If an entire column was zero, the degrees of freedom were reduced accordingly.

($P<0.01$) were observed due to diet; rats fed Span RSO appeared to contain less 16:0 and 18:0.

The effect of diet and length of feeding period on the relative concentration of the shorter chain monounsaturated fatty acids 16:1 and 18:1 was not significant. However, considerable differences were observed in the relative concentration of the long chain fatty acids, 20:1 and 22:1. The Span RSO and RPF diets both contained a high concentration of 20:1, which accumulated in the hearts within the first week of feeding these diets but decreased by the 8th and 16th weeks. Rats fed the L + EA diet contained higher levels of 20:1 compared to rats fed the lard diet, presumably due to β-oxidation of erucic acid to eicosenoic acid (18,19).

The Span RSO, RPF, and L + EA diets all contained about the same concentration of 22:1 (Table I), and the level of this acid in the cardiac lipids was very similar throughout the feeding trial (Table II). Erucic acid levels increased wtihin the first week on these diets to ca. 4-5% and then declined to ca. 1% after 16 weeks. This early fat accumulation and gradual decline has been reported by several investigators (1,2,5,6,20). The similarity of erucic acid incorporation into cardiac lipids of rats fed either the Span RSO, RPF, or L + EA diets suggests that these oils were equally absorbed and metabolized, including the 5.4% free erucic acid added to lard.

The relative concentration of 18:2 was significantly ($P<0.01$) higher in the hearts of rats fed the lard diet compared to that in the hearts of rats fed the other diets, and this difference remained throughout the feeding trial (Table II). Apparently, the addition of free erucic acid to the lard diet significantly ($P<0.01$) depressed, the relative concentration of 18:2 in the cardiac lipids.

Significant ($P<0.01$) amounts of 18:3 appeared only in the cardiac lipids of rats fed Span RSO or RPF, diets which contained appreciable amounts of this acid. The relative concentration of 20:4 was remarkably similar in the heart lipids of rats fed the three erucic acid containing diets, and an increase was apparent from the first week to the latter time periods (8th and 16th weeks). Rats fed the lard diet had higher levels of 20:4 only at 3 days and 1 week compared to rats fed the other three diets. The proportion of the long chain polyunsaturates was also affected by diet and length of feeding period. In general, rats fed Span RSO or RPF retained low concentrations of 22:4ω6 and 22:5ω6, whereas the concentration of the latter acid increased significantly ($P<0.01$) in rats fed lard or L + EA. This appeared to indicate that 18:2ω6 was increasingly anabolized in rats fed diets containing lard or L + EA. Furthermore, the relative concentration of 22:5ω3 was not affected by diet, whereas, in general, the abundance of 22:6ω3 in the cardiac lipids decreased with the duration of feeding.

Regardless of the source of oil fed, myocardial lesions characterized by overt myocardial necrosis were observed in a number of male rats from each treatment (Tables III-V). Rats fed the Span RSO containing diet were the most severely affected in all experiments. When RPF was fed for the first time (Experiment I, Table III), only 10 of 50 rats showed evidence of myocardial necrosis compared to 23 of 50 rats fed the Span RSO containing diet. The incidence of lesions observed

TABLE IV

Myocardial Lesions in Male Rats Fed Experimental Diets: Experiment II

Diet[a]	22:1 (%)	Affected/examined	Number of rats[b] 1-2	3-5	6-10	>10
Lard	0.2	6/20	4	1	1	0
L + EA	5.6	3/19	3	0	0	0
RPF	5.6	12/20	8	1	1	2
Span RSO	4.8	6/9	1	4	0	1
X^2 Analysis[c]		Incidence (d.f.)[d]			Severity (d.f.)	
Between controls		1.13 (1)			1.89 (2)	
Between treated		0.12 (1)			7.81* (3)	
Controls vs. treated		10.73*** (1)			4.27 (3)	

[a]Lard = Commercial lard; L + EA = commercial lard to which was added 5.4% erucic acid; RPF = rendered pig fat; Span RSO = Span rapeseed oil.

[b]Number of rats wiht lesion scores of 1-2, 3-5, 6-10, or >10 in three sections of heart.

[c]A comparison of rats affected to number of rats examined was used in the analysis of incidence. Only rats with heart lesions were compared in the analysis of severity. Significant differences: *(P<0.05); **(P<0.01); ***(P<0.001).

[d]The degrees of freedom (d.f.) of these analyses were (r-1) (c-1), where r and c are the number of rows and columns, respectively. If an entire column was zero, the degrees of freedom were reduced accordingly.

TABLE V

Myocardial Lesions in Male Rats Fed Experimental Diets: Experiment III

Diet[a]	22:1 (%)	Affected/examined	Number of rats[b] 1-2	3-5	6-10	>10
Lard	0.2	7/45	6	1	0	0
L + EA	5.6	10/45	8	1	1	0
RPF	5.6	14/45	14	0	0	0
Span RSO	4.8	34/45	12	11	8	3
X^2 Analysis[c]		Incidence (d.f.)[d]			Severity (d.f.)	
Between controls		0.66 (1)			1.13 (2)	
Between treated		19.87*** (1)			22.06*** (3)	
Control vs treated		24.50*** (1)			5.47 (3)	
Span vs controls		43.65*** (1)			11.60** (3)	
RPF vs controls		2.46 (1)			3.96 (2)	

[a]Lard = Commercial lard; L + EA = commercial lard to which was added 5.4% erucic acid; RPF = rendered pig fat; Span RSO = Span rapeseed oil.

[b]Number of rats with lesion scores of 1-2, 3-5, 6-10, or >10 in three sections of heart.

[c]A comparison of rats affected to number of rats examined was used in the analysis of incidence. Only rats with heart lesions were compared in the analysis of severity. Significant differences: *(P<0.05); **(P<0.01); ***(P<0.001).

[d]The degrees of freedom (d.f.) of these analyses were (r-1) (c-1), where r and c are the number of rows and columns, respectively. If an entire column was zero, the degrees of freedom were reduced accordingly.

when RPF was fed was significantly (P<0.01) lower than in animals fed Span RSO and similar to that observed in male rats fed control diets (13). Since both RPF and Span RSO contained the same level of esterified erucic acid, the results of Experiment I suggest that erucic acid per se is not responsible for the production of the myocardial necrosis when rapeseed oil containing 5% erucic acid is fed to rats, contrary to that reported earlier (3).

As indicated in Table IV (Experiment II), the incidence of myocardial lesions in rats fed RPF was similar to that found when Span RSO was fed. This appeared contradictory to the results of Experiment I. However, there was a significant difference (P<0.05) in the severity of lesions between Span RSO and RPF fed rats. The severity of lesions between the control fed or treated fed rats was not significant (P<0.05), while the incidence of myocardial lesions was

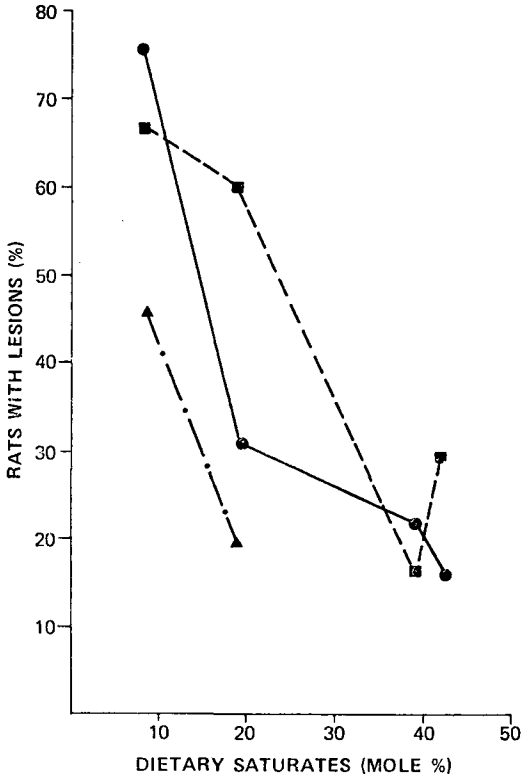

FIG. 1. Effect of level of dietary saturated fatty acids on the incidence of myocardial lesions in rats. ▲—▲ Experiment I; ■— —■ Experiment II; ●——● Experiment III.

significantly ($P<0.001$) higher in rats fed Span RSO and RPF than in rats fed the lard and L + EA diets (Table IV).

Because of the wide discrepancy between Experiments I and II in the incidence of myocardial lesions when diets containing RPF were fed, Experiment III was carried out as a repeat of Experiment II. As indicated in Table V, in Experiment III there were no significant differences ($P<0.05$) observed in the incidence of myocardial lesions in rats fed either of the control diets (lard or L + EA) or the diet containing RPF. The incidence and severity of lesions was significantly higher ($P<0.001$) in rats fed the Span RSO diet than in rats fed the diet containing RPF (Table V). The highly significant ($P<0.001$) difference observed in incidence of lesions between control and treated could be accounted for by the fact that there were significantly ($P<0.001$) more lesions in rats fed Span RSO than in rats fed control diets (lard or L + EA) or RPF (Table V).

As can be observed from the results of Experiment III, the lesion response to the diet containing RPF was comparable to the response observed in Experiment I. The apparent higher incidence of lesions when RPF was fed in Experiment II might be the result of wide animal variation and the small numbers used (13). This type of variation has been a source of concern in a number of experiments done on the lesion response of rats fed dietary oils during the course of the past 3 years. A complete and thorough statistical evaluation of this variability is being undertaken (manuscript in preparation).

The results of Experiments II and III would support the conclusion drawn from Experiment I that erucic acid alone is not responsible for the myocardial necrosis produced by feeding a 5% erucic acid rapeseed oil to rats. This is substantiated by the fact that there were no significant differences ($P<0.01$) observed in the level of this acid in the hearts of male rats fed either of the three erucic acid containing diets (L + EA, RPF, Span RSO), whereas there was a two- to threefold (Experiment III) increase in the incidence of lesions in the Span RSO fed rats over that of rats fed the other two erucic containing diets. These results would also indicate that about 5% erucic acid in the free (L + EA) or esterified form (RPF) does not increase the incidence of myocardial lesions above that observed in control rats.

The differences observed in lesion response (Tables IV and V) when the three erucic acid containing diets were fed to male rats might be explained by the following possibilities: (a) The pigs, from which the RPF was prepared, may have removed some cardiotoxic component from RSO, toxic to male rats (5), but not to pigs (7-9). However, in a previous attempt (21) to reduce Span RSO toxicity by subjecting the oil to exhaustive molecular distillation or adsorption chromatography, we were unable to demonstrate either the presence of a distillable toxic component or show that the highly purified triglycerides were less cardiotoxic than the original oil (13). (b) Triglycerides of rapeseed oil differ from triglycerides of pig adipose tissue in fatty acid composition (Table I) and positional distribution of fatty acids on the glycerol moiety (Hulan et al., unpublished data). (c) A more likely possibility is that an imbalanced fatty acid composition of rapeseed oil is responsible for the myocardial lesions (13). The results reported here suggest that a certain level of saturates in the diet may prevent myocardial necrosis. As indicated in Figure 1, when oils containing ca. 5% erucic acid (Span RSO, RPF, or L + EA) were fed to male rats, the incidence of myocardial lesions decreased as the level of saturate fatty acids in the dietary oils increased. For example, the presence of 5% erucic acid in

a fat such as lard, which contains a high level of saturates (42 moles %), did not increase the incidence of lesions over the control (lard alone). This concept might also explain why mixing equal amounts of Span RSO and a 3:1 lard:corn oil mixture (22) greatly reduced the incidence of myocardial lesions in male rats compared to when only Span RSO was fed.

The results of this study would suggest that feeding an oil properly balanced in fatty acids for the male rat does not increase the incidence of myocardial necrosis above background.

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Phospholipid Exchange Proteins in Rat Intestine

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ABSTRACT

The 105,000 *g* supernatant and pH 5.1 supernatant fractions from rat intestinal homogenates stimulate phosphatidylcholine exchange between [^{32}P]phosphatidylcholine liposomes and beef heart mitochondria. This active fraction shows the characteristics of a protein. Isoelectric focusing of the intestinal pH 5.1 fraction shows two peaks of phosphatidylcholine exchange activity: one at an acidic pH (4.5-5.3), the other in a basic pH range (8-9). The second peak of activity appears to be a new phospholipid exchange protein. The anatomic distribution of phosphatidylcholine exchange activity in intestine has been investigated. Expressed per mg of protein, phosphatidylcholine exchange activity is higher in mucosa than in the intestinal wall. No significant differences have been found between villi and crypts cells or between jejunal and ileal villi. Furthermore, exchange activity per mg of protein in mucosa is unaffected by fasting or by feeding a high fat or high cholesterol diet. This suggests that phospholipid exchange activity in the absorptive cells is not a rate limiting step in the process of fat absorption.

INTRODUCTION

The existence of proteins stimulating the exchange of phospholipids between cellular membranes has been shown in several mammalian tissues, including heart (1), liver (2-4), brain (5), kidney (6), and thyroid (7), and in plants such as cauliflower and potato (8). To date, however, no study has investigated the presence of a similar phospholipid exchange protein in the intestine. Because the presence of such a protein in the absorptive cell could be of great physiological significance, we have sought to determine if there is a phospholipid exchange protein in the small intestine, and, if so, whether it might play a role in the absorption of fat.

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EXPERIMENTAL PROCEDURES

Radioactive Lipids

Labeled phosphtidylcholine was prepared as previously described (9). [^{14}C]Triolein was obtained from Applied Sciences Laboratory (State College, PA) and purified by chromatography on Silica Gel H with hexane:diethylether:acetic acid, 80:20:1.

Tissue Preparation

Male Sprague-Dawley rats (280-350 g) were maintained ad libitum on Agway Rat Mouse diet 5RF, except as specified. They were decapitated before removal of the liver and intestine. Livers and intestines were rinsed thoroughly with ice-cold 0.25 M sucrose containing 1 mM ethylene diaminetetraacetic acid (EDTA) and 0.05 M tris(hydroxylmethyl)aminomethane (Tris) Cl buffer (pH 7.4) to remove blood or alimentary debris. A 20% homogenate was made in the same medium with a Potter-Elvejhem homogenizer. Preparations of jejunal crypts and villi were obtained by differential scraping (10).

The homogenates were centrifuged 20 min at 15,000 *g* average to sediment cell debris and mitochondria. The resulting supernatant fractions were centrifuged 60 min at 105,000 *g* average (type 40 fixed angle rotor, Beckman, Palo Alto, CA) to discard microsomes. The pH 5.1 supernatant fractions were obtained by adjusting the pH of the 105,000 *g* supernatant fraction to 5.1 with 0.1 M HCl and centrifuging 15 min at 15,000 *g* to discard the protein precipitate. The pH 5.1 supernatant fraction was dialyzed overnight against water at 4 C before use.

Liposomes

To [^{32}P]phosphatidylcholine were added 10% butylated hydroxytoluene and a trace amount of [^{14}C] triolein. After the solvent was evaporated under a stream of nitrogen, the lipid was redissolved with diethylether and redried, which left a thin lipid film. Buffer containing 0.25 M sucrose, 1 mM EDTA, and 0.05 M Tris Cl (pH 7.4) was added to make a final concentration of 1.25 mg of phospholipid per ml. The suspension was mixed vigorously 10 min, allowed to stand 1 hr, and then sonicated 30 min at 25 C in a Branson-HD 50 waterbath.

TABLE I

Phosphatidylcholine Exchange Activity in pH 5.1 Supernatants in Rats[a]

	Phosphatidylcholine exchange activity	
	Per g of tissue	Per mg of protein
Liver (n = 5)[b]	27.3 ± 2.5[c]	0.71 ± 0.05
Mucosa wall (n = 3)	3.8 ± 0.9[d,e]	0.24 ± 0.03[d,e]
Villi + crypts (n = 3)	11.7 ± 2.0[f]	0.90 ± 0.15

[a]Rats were 60-80 days old, fed a chow diet, and fasted overnight before sacrifice. Numerical values are nmoles of phosphatidylcholine transferred per min.

[b]n = Number of experiments.

[c]Mean ± standard error.

[d] $P < 0.001$ vs. villi + crypts.

[e] $P < 0.001$ vs. liver.

[f] $P < 0.05$ vs. liver.

Mitochondria

Beef heart mitochondria were prepared according to the procedure of Green et al. (11). They were subsequently washed twice by centrifugation in sucrose-EDTA-Tris Cl (pH 7.4) buffer and stored at -20 C. Upon thawing, they were rewashed once before use.

Assay Method

Exchange activity was determined by measuring the transfer of [^{32}P] phosphatidylcholine from liposomes to mitochondria as previously described (9). Liposomes (5 μg phospholipid P) were incubated with beef heart mitochondria (6.25 mg protein) and an appropriate aliquot of exchange protein in a total volume of 2 ml in 0.25 M sucrose, 1 mM EDTA, and 0.05 M Tris Cl (pH 7.4). Assays were carried out for 40 min at 37 C, during which time the exchange rate remained constant. The loss of ^{32}P from the recovered liposomes at the end of incubation was compared with the recovery of [^{14}C] triolein which was present in the liposomes as a nonexchangeable marker. The recovery of nonexchangeable marker in the supernatant fraction at the end of incubation was usually 90%. The phosphatidylcholine exchange in the absence of exchange protein was less than 2%. The exchange figures reported here were corrected for this blank value. One unit of exchange activity is defined as the transfer of 1 nmole of phosphatidylcholine per min (12). This was calculated on the assumption that the radioactivity in the exchangeable phosphatidylcholine in the liposome, i.e., the outer layer, decreased exponentially during the incubation period.

Analytical Methods

Lipids were extracted with 20 volumes of chloroform:methanol (2:1, v/v) overnight. The extract was washed by the procedure of Folch et al. (13). Lipid phosphorus was determined by the method of Bartlett (14). Protein was determined by the biuret method (15) or by the method of Lowry et al. (16) with bovine serum albumin as standard.

Isoelectric focusing was carried out in a LKB 8101 column (110 ml) maintained at 15 C. Ampholytes (LKB Produkter AB, Bromma, Sweden) were present at a final concentration of 1% (w/v), and electrofocusing continued at 600 V for 24 hr (17).

RESULTS AND DISCUSSION

Presence of Phosphatidylcholine Exchange Activity in the Rat Intestine

The presence of 105,000 *g* supernatant of pH 5.1 supernatant fraction from rat intestine greatly stimulates phosphatidylcholine exchange between ^{32}P-PC liposomes and mitochondria, as has been previously reported with similar fractions from rat liver (18). With 2, 4, and 8 mg of protein from liver or intestine 105,000 *g* supernatant, we find 1.0, 2.0, 3.9 and 0.21, 0.43, 1.0 units, respectively, of phosphatidylcholine exchange. Expressed per mg of protein in the 105,000 *g* homogenate, the exchange activity of intestine is about one-fourth that of liver. The phosphatidylcholine exchange activity of the whole small intestine is estimated to be ca. 12-15% that of whole liver.

Anatomical Distribution of Phosphatidylcholine Exchange Activity

In Table I, phosphatidylcholine exchange activity of intestinal wall and of villi plus crypts from rats fed a chow diet and fasted overnight

TABLE II

Phosphatidylcholine Exchange Activity in pH 5.1 Supernatants in Rats[a]

	Phosphatidylcholine exchange activity	
	Per g of tissue	Per mg of protein
Liver (n = 8)[b]	30.1 ± 2.6[c]	0.86 ± 0.08
Jejunum villi (n = 4)	14.4 ± 4.4[d]	1.20 ± 0.25
Jejunum crypts (n = 6)	12.8 ± 3.4[d]	1.14 ± 0.12
Ileum villi (n = 4)	10.0 ± 4.0[d]	0.80 ± 0.34
Ileum crypts (n = 6)	9.0 ± 1.8[d]	0.98 ± 0.20

[a]Rats were fed a chow diet and not fasted. Numerical values are nmoles of phosphatidylcholine transferred per min.

[b]n = Number of experiments.

[c]Mean ± standard error.

[d]$P < 0.05$ vs. liver.

is compared to that of liver. Per g of tissue of per mg of protein, villi and crypts show a much greater phospholipid exchange activity than intestinal wall. Thus, crypts and villi, which make up 35-40% of the wet intestinal wt, contain 60-70% of the total exchange activity of the intestine. The activity per mg of protein is ca. equal in crypts plus villi and in liver, but, per g of tissue, liver has almost three times as much activity as villi plus crypts.

Phosphatidylcholine exchange activities in crypts and villi of jejunal and ileal intestinal segments are compared in Table II. Rats were not fasted for these experiments.

Expressed per g of tissue or per mg of protein, the exchange activity of villi and crypts is similar and appears to be somewhat higher for jejunal than for ileal tissue, although these differences are not significant at the $P < 0.05$ level. When the results are calculated on the basis of DNA content, the data yielded the same conclusions.

Effect of Fasting and Diet

Expressed per g of tissue or per mg of protein, the exchange activity in liver from fed animals (Table II) does not differ significantly from that for liver of rats fasted overnight (Table I). Similar conclusions can be drawn about the exchange activity in villi plus crypts from fasted and from fed rats. However, if rats are fasted for 3 days, a slight decrease is observed in the exchange activity expressed per mg of protein (30%) in liver, while activity in crypts and villi is unchanged.

To test the possible influence of a high lipid or high cholesterol diet on the exchange activity, groups of three rats (mean wt 278-285 g) were maintained on chow diet (fasted overnight and nonfasted) or chow diet plus 20% Wesson oil and 20% Wesson oil plus 1% cholesterol for 2 weeks. Final wts in the three fed groups of rats were similar (328-343 g) and in the group fasted overnight was slightly less (294 g). The phosphatidylcholine exchange activity in pH 5.1 supernatant of liver and of villi plus crypts obtained from jejunum and ileum is shown in Figure 1.

Again, the exchange activity seems to be slightly less in ileal villi plus crypts than in jejunal villi plus crypts. No differences in phospholipid exchange activity in liver or intestine due to feeding a chow diet or a high lipid or cholesterol diet are observed.

Comparisons between Supernatant and pH 5.1 Fraction from Rat Liver and Intestine

Properties of the phospholipid exchange factor present in the 105,000 *g* supernatant of rat liver have been reported previously by Wirtz and Zilversmit (19). The active factor is a protein because it is nondialyzable, precipitable with $(NH_4)_2SO_4$, temperature sensitive, and digestible by trypsin.

The following characteristics of the exchange factor in intestinal 105,000 *g* and pH 5.1 supernatant fractions indicate the presence of a similar protein. The intestinal factor is nondialyzable and precipitable with $(NH_4)_2SO_4$ at 90% of saturation. When 40 mg of intestinal pH 5.1 supernatant is dialyzed overnight at 4 C in the presence of 6 mg of trypsin, the exchange activity is lost. If the same quantities of supernatant protein are dialyzed in the presence of trypsin plus 12 mg of trypsin inhibitor, the activity is recovered.

Similar effects of temperature on intestinal and hepatic exchange activity are observed. The preincubation of 105,000 *g* and pH 5.1 super-

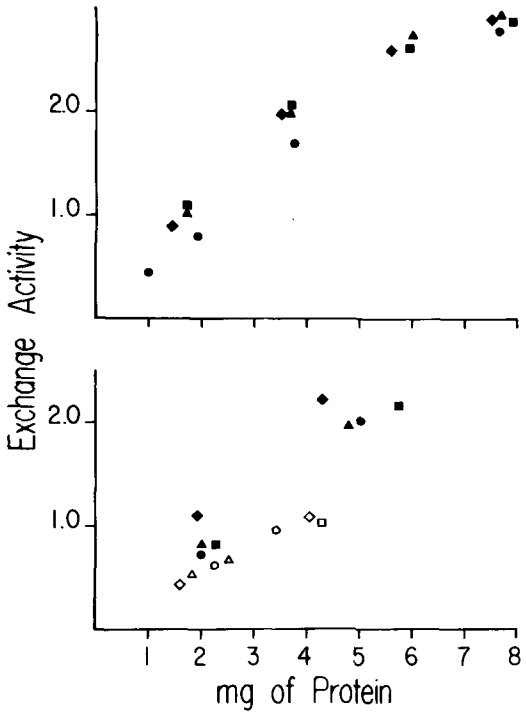

FIG. 1. Phosphatidylcholine exchange activity in pH 5.1 supernatant. Top: liver. Bottom: villi plus crypts from jejunum (closed symbols) and ileum (open symbols). Rats fed a chow diet and fasted overnight (●, ○); rats not fasted (◆, ◇); rats fed a chow diet plus 20% Wesson oil (■, □); rats fed chow diet plus 20% Wesson oil and 1% cholesterol (▲, △). Exchange activity was determined by measuring the transfer of [^{32}P] phosphatidylcholine from [^{32}P]-labeled phosphatidylcholine liposomes (5 μg phospholipid phosphorus) to beef heart mitochondria (6.25 mg protein) and an aliquot of pH 5.1 supernatant in a total volume of 2 ml of SET buffer after 40 min at 37 C.

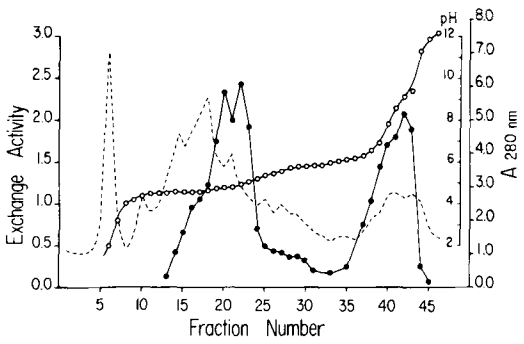

FIG. 2. Isoelectric focusing of pH 5.1 supernatant from rat intestine (244 mg of protein) in a pH gradient 4-9. The column was run for 24 hr at 15 C, 600 V. Fractions of 2½ ml were collected. - - - Absorbance at 280 nm; ●——● phosphatidylcholine exchange activity; ○——○ pH.

natants from intestine at 55, 60, or 65 C for 10 min results in the same decrease in activity (15, 45, and 75%, respectively) as for liver. When 105,000 *g* or pH 5.1 supernatant fraction is stored at 4 C, a small, identical increase in exchange activity is observed in liver and in intestine (about 20% after 2 months).

The electrophoretic behavior of phospholipid exchange protein of liver has been compared with that of intestine; the pH 5.1 supernatant from 10 homogenized rat intestines was dialyzed overnight at 4 C against water and was subjected to isoelectric focusing in a pH gradient from 4 to 9. The results are shown in Figure 2. Two distinct peaks of activity are seen. The first peak appears between pH 4.5 and 5.3 and accounts for about 65% of the recovered activity. The second peak is located between pH 7 and 9.2 (top ca. pH 9.0) and corresponds to 30% of the recovered activity. Total recovered activity accounts for 50% of the activity subjected to isoelectric focusing. With pH 5.1 supernatant from rat liver, we find a similar pattern with two distinct peaks of activity.

All phospholipid exchange proteins which have been purified from mammalian tissues (beef heart, beef liver, beef brain) have shown isoelectric points between 4.7 and 5.8 (20-22). The fractions from intestine and liver with the acidic isoelectric points probably correspond to similar proteins as described in beef heart (20) and beef liver (21). The fraction with an isoelectric point of ca. 9 may be a new protein that stimulates phosphatidylcholine exchange. It is also possible that part of the acidic phosphatidylcholine exchange protein is present in combination with a more basic protein fraction. This possibility is discounted, however, on the basis of unpublished findings in which the mol wt of this fraction differs little, if at all, from the phospholipid exchange protein with the acidic isoelectric point (Lutton and Zilversmit, unpublished data).

Apparently, phosphatidylcholine exchange activity is present in 105,000 *g* and pH 5.1 supernatant fractions of rat small intestine. This activity has been assumed to play a role in the redistribution of newly synthesized phospholipids between subcellular organelles. It seemed possible that phospholipid exchange proteins of intestine might be involved in the transfer of phospholipids to chylomicrons and be rate limiting for fat absorption. In support of this notion, we found that the phosphatidylcholine exchange activity expressed per mg of protein or per g of tissue is much higher in intestinal mucosa than in the rest of the intestinal wall. However, no differences were observed either

between crypts and villi or between jejunal and ileal mucosa. Furthermore, neither fasting nor high fat and cholesterol diets brought about any changes in the phospholipid exchange activity of the mucosa. The anatomical distribution of phosphatidylcholine exchange activity and its response to changes in diet differ from that of fatty acid binding protein recently isolated by Ockner and Manning (23). Our observations do not support the hypothesis that phosphatidylcholine exchange proteins are rate limiting in the process of fat absorption.

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Neutral Lipid Composition of *Culex quinquefasciatus* and *Culex tritaeniorhynchus* Cells at Two Phases of Growth

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ABSTRACT

The mosquito cells *Culex quinquefasciatus* and *Culex tritaeniorhynchus* were grown in spinner cultures. The fatty acid profiles of the neutral lipid classes were analyzed, and comparisons were made between the two species at logarithmic and stationary phases of growth and between the species and the medium. The results from the data suggest an increase in the average chain length of the fatty acids in the free fatty acid and sterol ester fractions of the *Culex quinquefasciatus* cells with aging. From the logarithmic to stationary phase, both Culex cells showed some increase in desaturation of acids in the di- and triglyceride and free fatty acid fractions. The *Culex tritaeniorhynchus* cells also showed some increase in desaturation of acids in the monoglyceride fraction. Differences between the two *Culex* species at the logarithmic phase of growth were observed in the fatty acid profiles of all the neutral lipid fractions examined and at the stationary phase of growth in the free fatty acid, triglyceride, and sterol ester fractions.

INTRODUCTION

It has been shown that the Dipteran order of insects have a unique lipid composition with phosphatidylethanolamine as the major component of the phospholipids. The average chain length of the fatty acids in both neutral and polar lipids is shorter than in organisms in which phosphatidylcholine predominates (1).

Mosquito cells, *Aedes aegypti* species, cultivated in vitro usually maintained an arbovirus infection without cell lysis in contrast to infected mammalian cells (2). Lipids extracted from two *Aedes* species of larval cells grown in a cell culture medium were examined for metabolic changes and/or a lipid-virus interaction (3,4).

Two species of *Culex* ovarian cells, *Culex quinquefasciatus* and *Culex tritaeniorhynchus,* were grown in spinner culture and were harvested at logarithmic and stationary phases of growth. These cells were selected for study to compare the lipids from cells in the genus and to observe lipid changes at the two phases of growth. The fatty acid composition of the neutral lipid classes is reported in this paper.

MATERIALS AND METHODS

Cells

The *C. quinquefasciatus* and *C. tritaeniorhynchus* cell strains were received from S.H. Hsu, U.S. Naval Medical Research Unit No. 2, Taipei, Taiwan (5,6). The cells were cultivated at 28 C in suspension in 250 ml spinner flasks containing 150 ml leafhopper medium (7) supplemented with 20% heat inactivated newborn calf serum. The cells were harvested in the late logarithmic, 48-72 hr, and stationary, 8-10

TABLE I

Distribution of Neutral Lipids

Neutral lipid classes	Percentage of neutral lipids				
	Leafhopper medium	*Culex quinquefasciatus*		*Culex tritaeniorhynchus*	
		Log[a]	Stat[b]	Log	Stat
Monoglycerides	tr[c]	0.8 ± 0.2[d]	0.5 ± 0.0	0.5 ± 0.2	0.7 ± 0.3
Diglycerides	0.5 ± 0.0	2.2 ± 0.5	2.2 ± 0.3	1.0 ± 0.3	3.6 ± 0.4
Triglycerides	19.2 ± 0.4	80.4 ± 1.9	78.8 ± 3.6	80.2 ± 2.4	78.9 ± 3.2
Free fatty acid	15.1 ± 0.1	1.0 ± 0.7	1.0 ± 0.8	1.9 ± 0.3	1.3 ± 0.9
Sterol esters	56.7 ± 0.2	2.5 ± 0.7	1.5 ± 0.9	3.0 ± 0.2	2.1 ± 0.4
Free sterols	8.6 ± 0.4	13.2 ± 2.5	16.2 ± 1.5	13.6 ± 1.7	13.6 ± 1.8

[a]Logarithmic phase of growth.
[b]Stationary phase of growth.
[c]tr = Trace, < 0.5%.
[d]Mean ± differences from the mean.

TABLE II

Constituent Fatty Acids of Monoglycerides[a]

Fatty acid	*Culex quinquefasciatus*		*Culex tritaeniorhynchus*	
	Log[b]	Stat[c]	Log	Stat
12:0[d]			tr[e]	0.6[f] ± 0.2[g]
14:0	0.5 ± 0.1	0.9 ± 0.1	0.6 ± 0.3	1.5 ± 0.8
15:0		2.9 ± 2.8	tr	
16:0	7.5 ± 0.4	8.4 ± 0.4	11.2± 1.4	11.3 ± 3.4
16:1	0.8 ± 0.1	1.2 ± 0.1	1.1± 0.1	2.8 ± 1.6
18:0	7.3 ± 0.3	7.7 ± 2.5	11.0± 2.5	9.2 ± 0.5
18:1	3.2 ± 0.1	5.2 ± 0.2	4.0± 1.4	8.0 ± 4.3
18:2	1.4 ± 0.4	3.2 ± 0.7	0.7± 0.1	1.5 ± 0.5
20:0	37.3± 1.0	34.1 ± 1.7	31.0± 0.8	27.3 ± 5.7
20:2	2.7± 2.7			
20:3	0.8 ± 0.1	1.0 ± 0.1	1.0± 0.7	1.7 ± 0.0
20:4	4.6 ± 0.3	4.0 ± 0.4	5.9± 2.9	6.2 ± 0.8
20:5	1.7 ± 0.7			0.8 ± 0.8
22:0	32.0 ± 2.2	31.6 ± 1.2	32.2± 4.1	28.7 ± 3.7
22:1				0.8 ± 0.8
Saturates	84.6	85.6	86.0	78.6
Monoenes	4.0	6.4	5.1	11.6
Polyenes	11.2	8.2	7.6	10.2

[a]Leafhopper medium contained trace amounts – not analyzed.
[b]Logarithmic phase of growth.
[c]Stationary phase of growth.
[d]Number of carbon atoms in acid:number of double bonds.
[e]tr = Trace, < 0.5%.
[f]Relative percentage of fatty acids.
[g]Mean ± differences from the mean.

days, phases of growth. The cells were combined from the spinner flasks and washed in Hanks' balanced salt solution (8).

Lipid Analysis

The neutral lipid fraction was obtained from the total lipids by fractionation on a silicic acid column (9). The neutral lipids were separated into classes by thin layer chromatography using the method of Freeman and West (10). The bands of lipid were located by spraying with 2', 7' dichlorofluorscein (11) and viewed under ultraviolet light.

Methylation of the classes of neutral lipids, extraction of the esters, and fatty acid analysis by gas liquid chromatography followed the procedures previously reported (4).

Cholesterol was analyzed by R.D. Ellefson, Mayo Clinic Lipids Laboratory (Rochester, MN (12,13).

All reagents were purified as described by Makino et al. (14). Reference materials were obtained from sources previously listed (4). Gravimetric analyses were performed as previously described (14).

RESULTS

The percentage of each class of neutral lipid is presented in Table I. The amount of the classes was the same in the two species at both stages of growth. The main components of the neutral lipids of the *Culex* cells were triglycerides (TGs) (79-80%) and sterols (13-16%). In the growth medium, sterol esters comprised 57%, TGs 19%, and sterols 9% of the total neutral lipid.

Culex quinquefasciatus Cells – Fatty Acid Profiles from Logarithmic and Stationary Phases of Growth

The mono-, di-, and triglyceride fractions of the neutral lipids (Tables II-IV) contained ca. the same amount of saturated acids in both growth phases of the *C. quinquefasciatus* cells. There was a small decrease in the amount of monoenes and a small increase in the amount of polyenes in the di- and triglyceride fractions from logarithmic to stationary phase of growth. The free fatty acid (FFA) and sterol ester fractions (Tables V and VI) had a decrease in the amount of monoenes, with increases or a tendency toward increases in saturated acids and polyenes. Chain elongation occurred in the FFA and sterol ester fractions from the logarithmic to stationary phases.

The neutral lipid fractions that had changes in their fatty acid profiles with aging of the cells were TG, FFA, and sterol ester (Tables IV-VI). The amount of 16:1 acid increased and the amount of 18:1 decreased in the TG frac-

TABLE III

Constituent Fatty Acids of Diglycerides

Fatty acid	Leafhopper medium[a]	*Culex quinquefasciatus*		*Culex tritaeniorhynchus*	
		Log[b]	Stat[c]	Log	Stat
12:0[d]	0.6[e] ± 0.5[f]	1.8 ± 0.4	3.5 ± 1.9		1.2 ± 0.1
14:0	4.3 ± 1.0	2.0 ± 0.0	2.3 ± 0.2	2.1 ± 0.1	2.6 ± 0.5
15:0	0.8 ± 0.5	0.5 ± 0.1	1.3 ± 0.7	tr[g]	0.5 ± 0.1
16:0	35.7 ± 2.6	15.8 ± 0.3	15.0 ± 1.1	20.5 ± 0.9	15.4 ± 3.0
16:1	7.3 ± 3.1	12.4 ± 0.8	12.2 ± 0.2	9.3 ± 0.4	12.9 ± 1.3
17:0	0.5 ± 0.2	0.8 ± 0.3	0.7 ± 0.1	0.6 ± 0.0	0.5 ± 0.1
18:0	7.6 ± 0.4	7.6 ± 0.4	6.7 ± 0.2	12.0 ± 0.8	8.8 ± 0.2
18:1	25.7 ± 1.5	40.4 ± 3.8	35.6 ± 2.8	37.0 ± 2.3	37.0 ± 1.1
18:2	3.7 ± 0.4	9.9 ± 0.4	10.8 ± 0.8	7.8 ± 0.1	9.1 ± 0.4
18:3	tr	1.9 ± 0.1	1.9 ± 0.0	1.2 ± 0.2	1.3 ± 0.1
20:0	1.0 ± 0.7	2.8 ± 0.4	2.8 ± 0.5	2.8 ± 0.2	3.7 ± 1.0
20:3	2.1 ± 1.6	1.6 ± 0.6	3.5 ± 0.1	2.8 ± 0.3	2.1 ± 0.7
20:4	2.2 ± 0.5	2.5 ± 0.3	2.9 ± 0.1	2.3 ± 0.3	3.5 ± 0.1
20:5	3.3 ± 3.3		1.4 ± 0.3	0.6 ± 0.0	0.8 ± 0.1
22[h]	4.3 ± 2.0				
Saturates	50.5	31.3	32.3	38.0	32.7
Monoenes	33.0	52.8	47.8	46.3	49.9
Polyenes	15.6	15.9	20.5	14.7	16.8

[a]Average 1,2, and 1,3 diglycerides.

[b]Logarithmic phase of growth.

[c]Stationary phase of growth.

[d]Number of carbon atoms in acid:number of double bonds.

[e]Relative percentage of fatty acids.

[f]Mean ± differences from the mean.

[g]tr = Trace, < 0.5%.

[h]22 series acids.

TABLE IV

Constituent Fatty Acids of Triglycerides

Fatty acid	Leafhopper medium	*Culex quinquefasciatus*		*Culex tritaeniorhynchus*	
		Log[a]	Stat[b]	Log	Stat
12:0[c]	tr[d]	0.6[e] ± 0.1[f]	0.6 ± 0.1	0.8 ± 0.2	0.8 ± 0.8
14:0	5.5 ± 0.7	2.5 ± 0.5	2.3 ± 0.2	2.2 ± 0.5	3.3 ± 0.5
14:1		0.6 ± 0.2	0.6 ± 0.0	tr	0.8 ± 0.1
15:0	1.2 ± 0.6				
16:0	30.1 ± 4.0	11.8 ± 1.4	14.5 ± 0.6	24.9 ± 0.6	19.4 ± 1.7
16:1	7.0 ± 2.1	12.5 ± 0.2	15.4 ± 0.6	10.0 ± 0.5	16.4 ± 0.4
17:0	0.5 ± 0.4	tr		tr	
17:1	0.6 ± 0.0				
18:0	9.0 ± 0.5	6.6 ± 0.6	4.6 ± 0.5	11.1 ± 1.0	7.8 ± 0.3
18:1	37.0 ± 0.6	47.8 ± 0.3	40.0 ± 3.0	38.4 ± 0.4	34.9 ± 0.3
18:2	3.7 ± 0.2	9.8 ± 0.5	12.9 ± 2.5	7.0 ± 0.3	9.5 ± 0.3
18:3	1.4 ± 0.5	1.7 ± 0.1	1.8 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
20:0	tr	2.1 ± 0.1	2.6 ± 1.0	0.7 ± 0.3	2.5 ± 0.1
20:2	1.1 ± 1.1				
20:3	0.5 ± 0.1	0.5 ± 0.2		0.7 ± 0.1	
20:4	0.9 ± 0.0	1.6 ± 0.0	2.4 ± 0.1	2.0 ± 0.1	2.0 ± 0.1
20:5	tr	0.9 ± 0.1	1.3 ± 0.5	0.6 ± 0.1	0.7 ± 0.0
22[g]	tr				
Saturates	46.3	23.6	24.6	39.7	33.8
Monoenes	44.6	60.9	56.0	48.7	52.1
Polyenes	7.6	14.5	18.4	11.4	13.4

[a]Logarithmic phase of growth.

[b]Stationary phase of growth.

[c]Number of carbon atoms in acid:number of double bonds.

[d]tr = Trace, < 0.5%.

[e]Relative percentage of fatty acids.

[f]Mean ± differences from the mean.

[g]22 series acids.

TABLE V

Constituent Fatty Acids of Free Fatty Acids

Fatty acid	Leafhopper medium	*Culex quinquefasciatus*		*Culex tritaeniorhynchus*	
		Log[a]	Stat[b]	Log	Stat
12:0[c]	0.5[d] ± 0.1[e]	0.6 ± 0.1	3.8 ± 3.8	tr[f]	1.4 ± 0.5
14:0	2.3 ± 0.0	4.6 ± 0.5	4.5 ± 1.6	2.1 ± 0.4	4.3 ± 1.8
15:0	0.8 ± 0.3	0.7 ± 0.1	1.4 ± 1.1		0.7 ± 0.1
16:0	24.3 ± 3.1	27.4 ± 0.1	20.6 ± 0.4	25.6 ± 3.3	26.1 ± 4.1
16:1	7.0 ± 1.4	14.1 ± 1.6	9.6 ± 1.6	9.0 ± 3.8	15.9 ± 0.2
17:0	0.8 ± 0.2	tr	tr	tr	tr
18:0	13.9 ± 0.1	11.3 ± 0.4	17.6 ± 1.8	17.3 ± 2.5	12.8 ± 2.5
18:1	40.5 ± 0.8	26.8 ± 4.1	18.1 ± 1.3	31.1 ± 2.3	20.6 ± 0.7
18:2	5.3 ± 0.0	7.5 ± 0.2	8.6 ± 3.7	6.3 ± 0.5	9.7 ± 1.7
18:3	1.0 ± 0.3	0.7 ± 0.7	0.9 ± 0.9	0.6 ± 0.6	1.0 ± 1.0
20:0		2.2 ± 0.3	2.9 ± 1.2	1.8 ± 0.8	2.1 ± 0.8
20:3	0.7 ± 0.1	3.3 ± 1.4	10.3 ± 7.0	2.4 ± 1.5	3.4 ± 1.5
20:4	2.4 ± 0.1	0.5 ± 0.5	1.2 ± 1.2	1.4 ± 0.3	1.6 ± 1.6
20:5		tr	tr	0.9 ± 0.9	tr
Saturates	42.6	46.8	50.8	46.8	47.4
Monoenes	47.5	40.9	27.7	40.1	36.5
Polyenes	9.4	12.0	21.0	11.6	15.7

[a]Logarithmic phase of growth.
[b]Stationary phase of growth.
[c]Number of carbon atoms in acid: number of double bonds.
[d]Relative percentage of fatty acids.
[e]Mean ± differences from the mean.
[f]tr = Trace, < 0.5%.

TABLE VI

Constituent Fatty Acids of Sterol Esters

Fatty acid	Leafhopper medium	*Culex quinquefasciatus*		*Culex tritaeniorhynchus*	
		Log[a]	Stat[b]	Log	Stat
12:0[c]	0.6[d] ± 0.6[e]				
14:0	2.6 ± 0.8	1.4 ± 0.1	1.3 ± 0.2	1.4 ± 0.0	1.9 ± 0.0
15:0	1.4 ± 0.5	tr[f]	0.7 ± 0.1	tr	0.6 ± 0.0
16:0	13.5 ± 2.2	11.2 ± 0.9	8.0 ± 0.1	15.5 ± 0.3	14.7 ± 0.5
16:1	9.8 ± 2.4	10.5 ± 1.0	10.8 ± 3.0	9.5 ± 0.4	11.6 ± 0.3
17:0		1.0 ± 0.1	0.7 ± 0.7	1.3 ± 0.0	1.2 ± 0.1
17:1	1.5 ± 0.3				
18:0	1.2 ± 0.5	3.9 ± 0.8	5.5 ± 3.2	3.8 ± 0.3	4.2 ± 0.1
18:1	20.4 ± 0.9	49.7 ± 1.3	41.8 ± 7.3	35.7 ± 0.0	37.3 ± 1.2
18:2	30.4 ± 5.8	14.8 ± 0.4	13.2 ± 3.8	22.6 ± 0.5	17.6 ± 0.0
18:3	4.2 ± 0.5	2.1 ± 0.2	2.1 ± 0.2	3.2 ± 0.2	2.5 ± 0.2
20:0	1.1 ± 0.5	1.3 ± 0.1	6.6 ± 4.1	1.1 ± 0.4	1.8 ± 0.7
20:2	0.9 ± 0.9				
20:3	1.9 ± 0.6	2.3 ± 0.7	7.3 ± 4.5	1.7 ± 0.4	3.5 ± 1.2
20:4	8.6 ± 0.6	1.9 ± 0.3	2.7 ± 1.0	4.1 ± 0.1	3.2 ± 0.3
20:5	1.8 ± 0.0				
Saturates	20.4	18.8	22.8	23.1	24.4
Monoenes	31.7	60.2	52.6	45.2	48.9
Polyenes	47.8	21.1	25.3	31.6	26.8

[a]Logarithmic phase of growth.
[b]Stationary phase of growth.
[c]Number of carbon atoms in acid:number of double bonds.
[d]Relative percentage of fatty acids.
[e]Mean ± differences from the mean.
[f]tr = Trace, < 0.5%.

tion from logarithmic to stationary phase. There were decreases in the percentages of 16:0 and 18:1 acids and an increase in the percentage of 18:0 acid in the FFA fraction as the cells aged. In the sterol ester fraction, there was a decrease in the amount of 16:0 acid with aging.

Culex tritaeniorhynchus Cells—Fatty Acid Profiles from Logarithmic to Stationary Phase of Growth

The mono-, di-, and triglyceride fractions (Tables II-IV) showed decreases in the amount of saturated acids, and the amount of polyenes remained ca. equal as the *C. tritaeniorhynchus* cells aged.

The amounts of saturated acids were ca. the same at the two growth phases of the cells in the FFA and sterol ester fractions (Tables V and VI). There was a small increase in the polyenes in the FFA fraction and a small decrease in polyenes in the sterol ester fraction as the cells aged.

In the fatty acid profiles of the fractions, the monoglycerides were ca. the same; the diglycerides (DGs) showed an increase in 16:1 fatty acid and a decrease in 18:0 acid; and the TGs showed increases in amounts of 16:1 and 18:2 acids, decreases in amounts of 16:0 and 18:0, and a suggestion of a decrease in 18:1 acid from the logarithmic to stationary phase of growth of the cells. The percentage of 16:1 acid increased and 18:1 acid decreased in the FFA fraction, and the percentage of 18:2 acid decreased in the sterol ester fraction with aging of the cells.

Comparison of Profiles of Fatty Acids of *Culex quinquefasciatus* and *Culex tritaeniorhynchus* in the Logarithmic and Stationary Phases of Growth

The two species had ca. the same amounts of saturated and unsaturated acids at the logarithmic phase of growth in the monoglyceride fractions (Table II). In the stationary phase of growth, the *C. quinquefasciatus* cells contained a smaller percentage of monoenes and a larger percentage of saturated acids than the *C. tritaeniorhynchus* cells. The fatty acid profile of the monoglycerides of the *C. quinquefasciatus* cells, logarithmic phase, contained less 16:0 acid and more 20:0 acid than the *C. tritaeniorhynchus* cells at the same growth phase. The fatty acid composition of the monoglycerides was about the same for the two species at the stationary phase.

In the DG fraction (Table III), the *C. quinquefasciatus* cells contained less saturated acids and more monoenes at the logarithmic phase than the *C. tritaeniorhynchus* cells, but in the stationary phase the two species had ca. the same amounts of saturated acids, monoenes, and polyenes. In the fatty acid profiles of the DGs, the *C. quinquefasciatus* cells, logarithmic phase, contained less 16:0 and 18:0 and more 16:1 than the *C. tritaeniorhynchus* cells. The fatty acid composition of the DGs was ca. the same for both species in the stationary form.

In the TG fraction (Table IV), the *C. quinquefasciatus* cells contained a smaller amount of saturated acids and a larger amount of unsaturated acids at both phases of development than the *C. tritaeniorhynchus* cells. In the fatty acid profiles of the TGs (Table IV), the *C. quinquefasciatus* cells contained less 16:0 and 18:0 acids and more 18:1 and 18:2 acids, and there was a suggestion of more 16:1 acid in the logarithmic phase than was observed in the *C. tritaeniorhynchus* cells. In the stationary phase, the *C. quinquefasciatus* cells contained less 16:0 and 18:0 acids in the TG fraction than the *C. tritaeniorhynchus* cells.

In the FFA fraction (Table V), the two *Culex* species in the logarithmic phase of growth had ca. the same amounts of saturated acids, monoenes, and polyenes. In the stationary phase, the *C. quinquefasciatus* cells had a smaller percentage of monoenes and a larger percentage of polyenes than the *C. tritaeniorhynchus* cells. In comparison of the fatty acid profile of the FFA fraction (Table V) of the two *Culex* species, the *C. quinquefasciatus* had a smaller amount of 18:0 acid in the logarithmic phase and a smaller amount of 16:1 acid in the stationary phase.

The *C. quinquefasciatus* cells in the logarithmic phase had more monoenes and fewer polyenes and in the stationary phase had ca. the same amounts as the *C. tritaeniorhynchus* cells in the sterol ester fraction (Table VI). The sterol ester fraction (Table VI) of the *C. quinquefasciatus* contained less 16:0 and 18:2 acids and more 18:1 acid in the logarithmic phase and less 16:0 acid in the stationary phase than the *C. tritaeniorhynchus* cells.

There was a suggestion in the data that chain elongation of the fatty acids occurred in the FFA and sterol ester fractions with aging.

Lipids in Mosquito Cells Compared to the Growth Medium

The only exogenous source of lipids provided the cells was the calf serum in the growth medium.

In the DG fractions (Table III), both *Culex* cells at logarithmic and stationary phases contained less saturated acids and more monoenes than the medium. The *C. quinquefasciatus* cells, stationary phase, showed a slight increase in polyenes, and the *C. tritaeniorhynchus* cells had ca. the same amount of polyenes in comparison to the medium. The mosquito cells at the two phases of growth had smaller percentages of 16:0 acids and larger percentages of 18:1 and 18:2 acids than the medium. In addition, the *C. tritaeniorhynchus* cells at the logarithmic phase contained more 18:0 acid than the medium.

The *C. quinquefasciatus* cells at both growth phases and the *C. tritaeniorhynchus* cells at the stationary phase had smaller percentages of saturated acids, and larger percentages of monoenes and polyenes in the TG fraction than found in the medium (Table IV). The *C. tritaeniorhynchus* cells at the logarithmic phase of growth had a smaller percentage of saturated acids and slightly larger percentages of monoenes and polyenes in the TG fraction than in the medium. The fatty acid profile of the TG fraction of the *C. quinquefasciatus* cells, logarithmic and stationary phases, and the *C. tritaeniorhynchus* cells, stationary phase, had less 16:0 acid and more 16:1 and 18:2 acids than the medium. The *C. quinquefasciatus* cells also had more 18:1 acid in the logarithmic phase and less 18:0 acid in the stationary phase than the medium. The *C. tritaeniorhynchus* cells had more 18:1 acid in the logarithmic phase than the medium.

In the FFA fraction (Table V), the *Culex* cells at both growth phases contained a smaller percentage of monoenes than the medium. The amount of polyenes in the cells, logarithmic phase, was ca. the same as the medium, and in the stationary phase showed an increase over the percentage in the medium. The fatty acid profiles of the *Culex* cells at both growth phases contained less 18:1 acid than the medium. In addition, the *C. quinquefasciatus* cells in the logarithmic phase had less 18:0 acid and more 16:1 and 18:2 acids, and in the stationary phase had more 18:0 acid than found in the medium. Also, the *C. tritaeniorhynchus* cells in the stationary phase contained more 16:1 and 18:2 acids than the medium.

Compared to the medium, the sterol ester fraction (Table VI) of the two *Culex* cells at both phases of growth contained more monoenes and less polyenes. The fatty acid profiles of the sterol esters showed the mosquito cells at both phases having larger relative percentages of 18:1 acid and smaller relative percentages of 20:4 acid than the medium. In addition, the *C. quinquefasciatus* cells at both phases and the *C. tritaeniorhynchus* cells at the stationary phase had smaller percentages of 18:2 acid, and the *C. quinquefasciatus* cells, stationary phase, had a smaller percentage of 16:0 acid than the medium.

DISCUSSION

The calf serum provided the lipids in the medium. The fatty acid profile of each neutral lipid class from the cells differed from the fatty acid composition of the same lipid class isolated from the medium. This would indicate that the cells metabolized or exchanged lipids with the medium at different rates dependent upon species and phase of growth.

Age and stage of development are known to influence the composition of lipids of some insects (15). Stephen and Gilbert (16) demonstrated that the rates of lipid synthesis chain lengthening, and desaturation were functions of the developmental stage of the silk moth, *Hyalophora cecropia*. Municio and coworkers (17) found the lipid synthesis rate of the eggs and adults of *Ceratitis capitata* 1/5 to 1/10 of the larva and pupa. Differences have been shown in regulation of fatty acid synthesis using homogenates of larvae and pharate adults of *C. capitata* (18). Larvae homogenates desaturated and elongated labeled compounds according to chain length, whereas those changes by pharate adult homogenates were insignificant. Hayashiya and Harwood (19) determined the fatty acid composition in various developmental stages of two strains of *Anopheles freeborni* and found the closest similarity in adults 10 days old. Different metabolic requirements were necessary at each stage of development. The changes observed between *Aedes* and *Culex* mosquito cells could be associated with the specificity of a particular cell.

The changes in fatty acid profiles that occurred in cells grown in spinner culture were similar to those found in whole insects. A comparative study was made of in vitro and in vivo incorporation of ^{14}C acetate into different classes of lipids by *C. capitata* at larval and adult stages (20). The pattern of labeled lipid obtained depended on stage of development of the insect and on experimental conditions. The lipid metabolism of cells grown in spinner culture may differ from cells grown in monolayer and from those from whole insects, in vitro or in vivo, but still serve as a model to understand the physiology of normal and arbovirus-infected insect cells.

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Comparison of the Lipid Composition of *Culex quinquefasciatus* and *Culex tritaeniorhynchus* Cells Obtained from the Logarithmic and Stationary Phases of Growth

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ABSTRACT

Culex quinquefasciatus and *Culex tritaeniorhynchus* cells were grown in spinner culture medium. The cells were harvested at late logarithmic and stationary phases of growth. The total lipid, total neutral lipid, and total phospholipid contents of the cells were analyzed to determine changes that occurred in the fatty acid profiles of the lipids with aging and between species. There was an increase in the amount of total neutral lipids with a corresponding decrease in amount of total phospholipid from logarithmic to stationary phases of growth of the *Culex quinquefasciatus* cells. Chain elongation and/or desaturation of acids occurred with aging of cells. The fatty acids of the phospholipids had a longer average chain length than the neutral lipids.

INTRODUCTION

Fewer studies have been made on the growth of arboviruses in arthropod cells than in mammalian or avian cells. The cell strains from larvae of *Aedes albopictus* and *Aedes aegypti* (1) were investigated for susceptibility to infection with various arboviruses (2,3). Buckley (4) screened arboviruses for their ability to produce a cytopathic effect or to infect *A. albopictus* and *A. aegypti* cell lines. Igarashi and coworkers (5) studied the growth of Japanese encephalitis virus (JEV) in three kinds of *Aedes* cell strains (1,2,6). Later it was reported (7) that a certain strain of JEV grown in *A. albopictus* cells had the same properties as that grown in mammalian cells. It was found by Fujita et al. (8) that strains of JEV multiplied in cell cultures from *Culex molestus*. Hsu, Mao, and Cross (9) used a cell strain established from the ovaries of *Culex quinquefasciatus* for in vitro studies of arboviruses and other arthropod-borne organisms. The tissue cultures of various mosquito genera could be a useful tool in the clarification of the interaction between arboviruses and mosquito cells.

The study of lipids of the host cells might provide information on metabolic changes that occur in infected cells. Diptera have an active lipid metabolism (10); only sterols (11,12) and polyunsaturated 18 carbon fatty acids (13) are dietary requirements. The lipids of Diptera differ from those of mammalian cells in fatty acid profiles and phospholipid composition, and thus, with infection, changes in lipids between the two cells might be noted. However, changes have been observed in lipid composition of insects at different stages of metamorphosis (14) and of insect cells grown in cultures with aging (15). It would be necessary to determine the lipid composition of the cells grown in tissue culture at a particular phase of development and to observe changes that occurred during development before studying the effect of arbovirus infection on lipid content of the cells.

A. aegypti and *A. albopictus* larvae cells have been grown in spinner culture (15,16). The fatty acid profiles of the total lipid, total neutral lipids, and total phospholipids were determined at logarithmic and stationary phases of growth. This study revealed that differences in the fatty acid patterns were observed in the aging of the cells, but not between the species (15).

C. quinquefasciatus and *Culex tritaeniorhynchus* ovarian cells were obtained (9,17) and grown in the same type of medium as *Aedes* cells and harvested at the same phases of development. The fatty acid analyses of the lipid classes were made to determine if similarities in composition existed between two species of *Culex* mosquito cells.

MATERIALS AND METHODS

Preparation of Cells

The *C. quinquefasciatus* and *C. tritaeniorhynchus* cells isolated from ovarian tissue of adult mosquitos were obtained from S.H. Hsu, Naval Medical Research Unit No. 2, Taipei, Taiwan (9,17). The cells were cultivated at 28 C in suspension in 250 ml spinner flasks containing 150 ml of leafhopper medium (18) sup-

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TABLE I

Growth of *Culex quinquefasciatus* and *Culex tritaeniorhynchus* Using Leafhopper Medium in Spinner Cultures at 28 C

Culex quinquefasciatus		*Culex tritaeniorhynchus*	
Time (hr)	Cells x 10^5/ml	Time (hr)	Cells x 10^5/ml
0	5.2	0	5.0
24	7.0	24	5.6
48	10.0	48	11.0
72	13.0	72	19.0
96	20.0	96	16.0
120	23.0	120	17.0
144	29.0	144	16.0
168	32.0	168	16.0
192	31.0	192	15.0
216	29.0		
240	28.0		

TABLE II

Lipid Content of *Culex tritaeniorhynchus* and *Culex quinquefasciatus* in Different Phases of Growth

Lipids	Leafhopper medium	*C. quinquefasciatus*		*C. tritaeniorhynchus*	
		Log[a]	Stat[b]	Log	Stat
Total lipids mg	370 346	85 108	85 120	93 132	100 143
Total lipids (% of gravimetric wt)	1.2 ± 0.0[c]	32.3 ± 2.2	35.1 ± 1.1	30.9 ± 3.2	40.7 ± 5.6
Neutral lipids (% total lipids)	56.5 ± 4.0	37.6 ± 2.1	47.7 ± 1.4	44.2 ± 1.4	48.8 ± 2.7
Phospholipids (% total lipids)	43.5 ± 4.0	62.4 ± 2.1	52.3 ± 1.4	55.8 ± 1.4	51.2 ± 2.7
Ratio NL:PL	1.30	0.60	0.91	0.79	0.95

[a]Logarithmic phase of growth.

[b]Stationary phase of growth.

[c]Mean ± differences from the mean.

plemented with 20% heat inactivated newborn calf serum. The cells were harvested in the late logarithmic and stationary phases of growth: 72 hr and 10 days, respectively, for *C. quinquefasciatus* cells; 48 hr and 8-10 days, respectively, for *C. tritaeniorhynchus* cells. The cells were combined from the spinner flasks and washed in Hanks' balanced salt solution (19).

Lipid Analysis

The lipids were extracted from the cells, fractionated by column and thin layer chromatography, methylated, and analyzed by gas liquid chromatography by the methods reported in previous publications (15,20).

The methyl esters were analyzed on a Victoreen Model 4000 gas chromatograph at 185 C using a flame ionization detector and vibrating reed electrometer. An aluminum column, 8 ft x 1/8 in. inside diameter (244 cm x 0.32 cm), containing 15% EGSS-X on Gas Chrom P, 100/120 mesh, was used for the analyses. Gravimetric determinations of the lipids were made on an electronic Cahn Balance, Model G (Cahn Instrument Co., Paramount, CA).

Standards

The methods used for solvent purification were reported by Makino et al. (20) and Townsend et al. (21). Methyl ester standards were purchased from the Lipids Preparation Laboratory of The Hormel Institute (Austin, MN) and Nu-Chek-Prep, Inc. (Elysian, MN).

RESULTS

The growth of the *Culex* species is shown in Table I and was the basis for determination of late logarithmic and stationary phases of growth. The number of cells of *C. quinquefasciatus* was ca. double that of *C. tritaeniorhyn-*

TABLE III

Fatty Acid Profiles of the Total Lipids of *Culex* Species Cultivated In Vitro in Logarithmic and Stationary Phases of Growth

Fatty acid	Leafhopper medium	*C. quinquefasciatus*		*C. tritaeniorhynchus*	
		Log[a]	Stat[b]	Log	Stat
14:0[c]	1.9[d] ± 0.1[e]	1.5 ± 0.2	1.8 ± 0.1	1.3 ± 0.1	2.0 ± 0.5
15:0	0.6 ± 0.4			tr[f]	tr
16:0	21.7 ± 0.1	14.4 ± 0.3	14.7 ± 0.3	16.6 ± 0.5	14.4 ± 2.9
16:1	5.9 ± 1.8	11.0 ± 0.7	11.9 ± 0.2	8.2 ± 0.3	12.6 ± 1.7
17:0	1.0 ± 1.0	0.7 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.0
17:1	0.9 ± 0.2				
18:0	11.5 ± 0.2	8.1 ± 0.3	7.8 ± 0.9	12.0 ± 0.2	9.0 ± 0.2
18:1	30.7 ± 0.3	37.9 ± 2.6	34.5 ± 2.9	39.3 ± 0.5	30.7 ± 2.2
18:2	13.6 ± 1.1	9.2 ± 0.1	12.7 ± 1.8	9.6 ± 1.0	9.4 ± 0.1
18:3	1.4 ± 0.0	1.6 ± 0.2	1.9 ± 0.3	1.3 ± 0.1	3.9 ± 0.8
20:0	tr	5.2 ± 1.4	5.8 ± 0.9	3.4 ± 0.7	1.3 ± 0.1
20:3	1.7 ± 0.4	3.2 ± 0.8	3.4 ± 0.6	0.9 ± 0.3	4.8 ± 2.6
20:4	5.1 ± 0.5	2.8 ± 0.2	3.2 ± 0.1	3.2 ± 0.2	4.7 ± 1.6
20:5	tr	0.8 ± 0.1	1.0 ± 0.2	0.8 ± 0.1	1.2 ± 0.6
22:0		tr		2.3 ± 0.3	
22:5	0.9 ± 0.0	0.7 ± 0.1			1.2 ± 0.6
22:6	0.6 ± 0.2				0.9 ± 0.2
Saturates	36.7	29.9	30.7	36.1	27.2
Monoenes	37.5	48.9	46.4	47.5	43.3
Polyenes	23.3	18.3	22.2	15.8	26.1

[a]Logarithmic phase of growth.

[b]Stationary phase of growth.

[c]Number of carbon atoms in acid: number of double bonds.

[d]Relative percentage of fatty acid.

[e]Mean ± difference from the mean.

[f]tr = Trace, <0.5%. Trace amounts of 12:0, 14:1, 20:1, 20:2, 22:3, and 22:4 are not listed.

chus cells at 168 and 192 hr.

Table II shows the lipid content of the growth medium and the *C. quinquefasciatus* and *C. tritaeniorhynchus* cells at logarithmic and stationary phases of growth. The percentage wt of the total lipid was similar in the two species at both phases of development. In the *C. quinquefasciatus* cells, there was an increase in the amount of total neutral lipids with a corresponding decrease in total phospholipids with aging of the cells.

The ratio of neutral lipids to phospholipids in the growth medium was 1.3. As the mosquito cells aged, there was a change in the ratio from 0.6 to 0.9 in the *C. quinquefasciatus* cells and from 0.8 to 1.0 in the *C. tritaeniorhynchus* cells.

The fatty acid profiles of the total lipids, total neutral lipids, and total phospholipids of the mosquito cells at logarithmic and stationary phases of growth and the growth medium are reported in Tables III-V.

Culex quinquefasciatus Cells - Fatty Acid Profiles from Logarithmic and Stationary Phases of Growth

The amounts of saturated acids and monoenes were ca. equal in the total lipid from the logarithmic to stationary phase of growth of the cells (Table III). The percentage of polyenes increased (14.7-20.4) and saturated acids decreased (29.0-23.7) in the total neutral lipid fraction with aging of the *C. quinquefasciatus* cells (Table IV). The total phospholipid fraction showed a decrease in monoenes (50.9-43.9) from 3 to 10 days of growth (Table V). The relative percentages of the individual fatty acids were ca. equal in the total neutral lipid fractions as the mosquito cells aged. There was an increase of 20:0 fatty acid in the total phospholipid fraction and a slight increase of 18:2 fatty acid in the total lipid from the logarithmic to stationary phase of growth of the cells. Also, there was an increase in the average chain length of the fatty acids of the total phospholipids with aging of the cells.

Culex tritaeniorhynchus Cells - Fatty Acid Profiles from Logarithmic and Stationary Phases of Growth

There was a decrease in the percentage of saturated acids and an increase in polyenes in the total lipids of the cells with aging (Table III). The total neutral lipid fraction showed a decrease in saturated acids (Table IV) from the logarithmic to stationary phase of growth. The amounts of saturated and unsaturated acids were ca. the same in the total phospholipid

TABLE IV

Fatty Acid Profiles of the Total Neutral Lipids of *Culex* Species Cultivated In Vitro in Logarithmic and Stationary Phases of Growth

Fatty acid	Leafhopper medium	*C. quinquefasciatus*		*C. tritaeniorhynchus*	
		Log[a]	Stat[b]	Log	Stat
14:0[c]	3.0[d] ± 0.4[e]	3.5 ± 1.3	1.8 ± 0.3	2.6 ± 0.1	2.8 ± 1.3
14:1		tr[f]	0.7 ± 0.1	tr	1.0 ± 0.1
15:0	1.0 ± 0.4			tr	
16:0	22.6 ± 0.5	16.1 ± 0.2	14.3 ± 0.5	24.1 ± 0.0	20.0 ± 0.9
16:1	7.6 ± 2.0	12.1 ± 1.6	15.1 ± 0.6	9.5 ± 0.2	16.7 ± 0.9
17:0	0.9 ± 0.1	0.8 ± 0.1		0.7 ± 0.0	
18:0	6.4 ± 0.2	6.4 ± 0.9	5.0 ± 0.3	11.3 ± 0.7	7.6 ± 0.4
18:1	31.7 ± 1.4	43.6 ± 0.2	39.9 ± 3.2	37.9 ± 0.2	34.4 ± 0.0
18:2	15.5 ± 1.5	9.8 ± 1.0	13.1 ± 1.7	8.0 ± 0.1	9.8 ± 0.2
18:3	1.9 ± 0.1	1.9 ± 0.3	2.2 ± 0.3	1.5 ± 0.1	1.4 ± 0.0
20:0	tr	2.2 ± 0.4	2.6 ± 0.7	1.0 ± 0.1	2.2 ± 0.1
20:3	1.1 ± 0.5	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.0	0.6 ± 0.0
20:4	6.1 ± 2.7	1.5 ± 0.1	2.1 ± 0.1	1.9 ± 0.0	1.8 ± 0.1
20:5	1.3 ± 0.5	0.8 ± 0.0	1.0 ± 0.2	0.5 ± 0.0	0.7 ± 0.1
22:5			0.6 ± 0.1		tr
22:6			0.7 ± 0.7		tr
Saturates	33.9	29.0	23.7	39.7	32.6
Monoenes	39.3	55.7	55.7	47.4	52.1
Polyenes	25.9	14.7	20.4	12.6	14.3

[a]Logarithmic phase of growth.

[b]Stationary phase of growth.

[c]Number of carbon atoms in acid: number of double bonds.

[d]Relative percentage of fatty acid.

[e]Mean ± difference from the mean.

[f]tr = Trace, <0.5%. Trace amounts of 12:0, 20:1, 20:2, 22:3, and 22:4 are not listed.

TABLE V

Fatty Acid Profiles of the Total Phospholipids of *Culex* Species Cultivated In Vitro in Logarithmic and Stationary Phases of Growth

Fatty acid	Leafhopper medium	*C. quinquefasciatus*		*C. tritaeniorhynchus*	
		Log[a]	Stat[b]	Log	Stat
14:0[c]	0.6[d] ± 0.1[e]	1.4 ± 0.2	1.5 ± 0.1	0.8 ± 0.1	tr[f]
15:0	0.8 ± 0.8				
16:0	20.7 ± 0.5	14.8 ± 0.3	15.6 ± 0.3	15.1 ± 1.1	12.9 ± 0.4
16:1	2.2 ± 1.1	11.0 ± 0.9	9.4 ± 0.9	9.1 ± 0.4	11.8 ± 0.2
17:0	0.9 ± 0.1	0.7 ± 0.1		0.6 ± 0.1	
17:1	0.7 ± 0.4				
18:0	21.4 ± 2.3	10.5 ± 0.4	10.6 ± 0.6	13.1 ± 0.5	13.0 ± 0.5
18:1	30.4 ± 0.7	39.9 ± 2.8	34.5 ± 1.9	40.4 ± 0.5	34.4 ± 1.2
18:2	8.1 ± 0.8	8.0 ± 0.8	9.0 ± 0.9	7.7 ± 1.0	10.0 ± 0.5
18:3	0.6 ± 0.1	1.1 ± 0.1	1.2 ± 0.2	1.2 ± 0.1	1.0 ± 0.1
20:0	tr	4.0 ± 0.4	8.0 ± 0.6	2.8 ± 0.6	5.8 ± 0.9
20:2		tr		0.9 ± 0.6	
20:3	4.0 ± 0.6	2.9 ± 0.3	5.4 ± 0.1	2.5 ± 0.5	4.9 ± 0.5
20:4	6.8 ± 0.8	3.1 ± 0.5	2.9 ± 0.4	3.5 ± 0.2	4.0 ± 0.2
20:5	0.5 ± 0.5	0.7 ± 0.5	0.9 ± 0.1	0.5 ±-0.0	1.2 ± 0.2
22:5	0.8 ± 0.8	0.8 ± 0.1	0.6 ± 0.1	0.9 ± 0.1	tr
22:6	0.6 ± 0.6	0.6 ± 0.1	0.7 ± 0.3	0.7 ± 0.1	tr
Saturates	44.4	31.4	35.7	32.4	31.7
Monoenes	33.3	50.9	43.9	49.5	46.2
Polyenes	21.4	17.2	20.7	17.9	21.1

[a]Logarithmic phase of growth.

[b]Stationary phase of growth.

[c]Number of carbon atoms in acid: number of double bonds.

[d]Relative percentage of fatty acid.

[e]Mean ± difference from the mean.

[f]tr = Trace, <0.5%. Trace amounts of 14:1, 20:2, 22:3, and 22:4 are not listed.

fraction (Table V) at the two growth phases.

The fatty acid profiles in the stationary phase of growth in comparison to the logarithmic phase showed decreases in 18:0 and 18:1 fatty acids and an increase in 16:1 in the total lipid. There were decreases in the amounts of 16:0 and 18:0 acids, a slight decrease in 18:1 acid, an increase in the amount of 16:1 acid, and a slight increase in 18:2 acid with aging of the cells in the total neutral lipid fraction. In the total phospholipid fraction there was a decrease in the amount of 18:1 acid and an increase in 16:1 acid with aging.

Comparison of *Culex quinquefasciatus* and *Culex tritaeniorhynchus* Cells at Logarithmic and Stationary Phases of Growth

In the total lipid, the *C. quinquefasciatus* cells in the logarithmic phase had less 18:0 fatty acid and more 16:1 acid, and in the stationary phase had more 20:0 acid and a tendency for more 18:2 fatty acid than was found in the corresponding growth phase of the *C. tritaeniorhynchus* cells. The fatty acid profile of the total neutral lipid of the *C. quinquefasciatus* cells had less 16:0 and 18:0 fatty acids and more 18:1 acid in the logarithmic phase, and less 16:0 and 18:0 acids and slightly more 18:1 and 18:2 acids in the stationary phase than the *C. tritaeniorhynchus* cells. In the total phospholipid fraction, the *C. quinquefasciatus* cells at both growth phases contained smaller amounts of 18:0 acid and in the stationary phase contained a larger amount of 16:0 acid and a slightly smaller amount of 16:1 acid than the *C. tritaeniorhynchus* cells.

In both species of *Culex* cells, as the cells aged, the 16:0, 18:0, and 18:1 acids were ca. the same in amount or, if a change occurred, it tended toward a decrease or was a definite decrease in relative percentage in the lipid fractions examined. The 16:1 and 18:2 acids remained ca. equal or showed an increase in amount in the three lipid fractions of the *Culex* cells from the logarithmic to stationary phase.

Lipids in Mosquito Cells Compared to Growth Medium

The total lipids from *C. quinquefasciatus* cells, logarithmic phase, showed relative increases in monoenes and decreases in amounts of saturated acids and polyenes in comparison to the growth medium. The total lipids from the *C. tritaeniorhynchus* cells, logarithmic phase, had more monoenes and a smaller percentage of polyenes than the medium. Both species of cells in the stationary phase of growth in the total lipids showed increases in the amount of monoenes and decreases in saturated acids in comparison to the medium.

The total lipid fatty acid profiles (Table III) of the two *Culex* species in the logarithmic phase of growth, in comparison with the fatty acid pattern of the medium, had larger amounts of 18:1 and 20:0 acids and smaller amounts of 16:0 and 18:2 acids. The *C. quinquefasciatus* cells in the logarithmic phase also contained a larger percentage of 16:1 and a smaller percentage of 18:0. Differences were observed between the profiles of total lipid fatty acids of the stationary phase of *C. quinquefasciatus* cells and the medium. There were larger relative percentages of 16:1 and 20:0 acids and smaller amounts of 16:0 and 18:0 acids in the mosquito cells. The *C. tritaeniorhynchus* cells at 10 days of growth had a larger amount of 16:1 and smaller amounts of 16:0, 18:0, and 18:2 than the medium.

The total neutral lipid fraction of the two species of *Culex* cells, at both phases of growth, contained more monoenes and smaller amounts of polyenes than the medium. In addition, the *C. quinquefasciatus* cells at the stationary phase had a smaller percentage of saturated acids than found in the medium, and the *C. tritaeniorhynchus* cells at the logarithmic phase had more saturated acids than the medium.

Differences in the fatty acid profile of the neutral lipid fraction between the two types of cells and the medium were observed (Table IV). The *C. quinquefasciatus* cells at both growth phases had more 18:1 acid and less 16:0 than the medium, in the logarithmic phase they contained less 18:2 acids, and at the stationary phase they contained more 16:1 acid than the medium. The *C. tritaeniorhynchus* cells at both growth phases contained less 18:2, at the logarithmic phase they had more 18:0 and 18:1 acids, and at the stationary phase they contained more 16:1 acid than the medium in the total neutral lipid fraction.

The total phospholipid fraction of the mosquito cells at both phases of growth had a larger amount of monoenes and a smaller amount of saturated acids than the medium. The *Culex* cells, logarithmic and stationary phases, had more 16:1 acid and less 16:0, 18:0, and 20:4 acids than the medium and, in addition, in the logarithmic phase contained more 18:1 acid than the medium in the total phospholipid fraction. There appeared to be an increase in 20:0 acid in the logarithmic phase and an increase in amount in the stationary phase of both *Culex* cells in comparison to the medium.

DISCUSSION

Good reproducibility of data was obtained

from two individual experiments using a different starting number of cells for the lipid extraction.

In previous studies, it was suggested that *A. aegypti* cells had an independent metabolism (22,23). The fatty acid profile of the leafhopper medium differed from the *Culex* cells in the stationary phase in the amounts of 16:1 and 18:0 acids in total lipid and total phospholipids. As in the *Aedes* cells (15), the differences in 16 and 18 carbon chain acids would suggest that independent metabolism of the lipids from the medium took place in the cells.

It has been shown that all insects have a requirement for sterols in the diet (11,12). Several species of insects have no other dietary requirement for lipids than sterols, and inclusion of lipids in the diet may have detrimental effects (24). A number of insects require a dietary source of polyunsaturated 18 carbon acids for successful metamorphosis from larvae to adults (13,24). Our observations from studies of mosquito cells of the *Culex* genus as well as the *Aedes* genus (15) suggest that insect cells cultivated in vitro may not need preformed lipids.

The *Culex* cells showed increases in chain length and/or desaturation with aging. However, the differences were not as pronounced as in the study with *Aedes* cells (15). The *Culex* cells were ovarian cells from adult insects and the *Aedes* cells were larval cells. In work reported by Municio et al. (25), each insect (Diptera) developmental stage – egg, pupa, larva, adult – possessed a quite different biosynthetic capability of producing fatty acids. The adults and eggs showed incorporation of acetate ^{14}C only 1/5 to 1/10 as great as found in other developmental stages in synthesizing fatty acids. In later work from the same laboratory (26), it was found that in vitro elongation and desaturation reactions of labeled fatty acids were different at the larval and pharate adult stages. If insect cells grown in culture reflect the same metabolic changes as those of insect homogenates, then the differences in fatty acid between *Aedes* and *Culex* cells could be due to the stage of insect development from which the cells were isolated.

In these studies, the cells were grown in suspension culture, not in monolayers as reported in previous studies of growth of mosquito cells (3,4,8-10). Differences could occur because of dissimilar techniques used in growing cells.

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Cholesterol and Fatty Acid Synthesis in Swine[1]

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ABSTRACT

In incubation studies with swine tissue slices, acetate-1-^{14}C or glucose-U-^{14}C as substrates were incorporated more readily into fatty acids and cholesterol in adipose tissue than other tissues tested. Cholesterol and fatty acid synthesizing activity was substantial in the small intestine. When acetate was available, liver, small intestine, and adipose tissue were important sites for cholesterol synthesis. Heart and aortic tissue had marginal levels of cholesterol synthesizing ability. Lipogenesis in adult swine liver, heart, and aortic tissue was extremely low. As in tissue slices, incorporation of acetyl-1-^{14}C CoA into fatty acids by adipose homogenates indicated high lipogenic activity. Subcellular fractionations of heart and aortic tissue indicated that the heart microsomal fraction had the highest lipogenic activity as measured by the incorporation of acetyl-1-^{14}C CoA into fatty acids. In adult swine adipose tissue, the incorporation of glucose-U-^{14}C into fatty acid was higher than its incorporation into glyceride-glycerol. The synthesis of glyceride-glycerol from glucose-U-^{14}C or acetate-1-^{14}C in liver was higher than for fatty acid synthesis. The activity of acetyl CoA carboxylase, fatty acid synthetase, citrate cleavage enzyme, nicotinamide adenine dinucleotide phosphate-malate dehydrogenase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase was considerably higher in adipose tissue than in other tissues tested, paralleling its high lipogenic capacity.

INTRODUCTION

Using both in vivo and in vitro techniques, it became apparent that the relative contribution of tissue sites to overall cholesterol and fatty acid synthesis varies appreciably depending on the animal species studied. In the rat and mouse, adipose tissue is the major site of lipogenesis (1-3). However, chick liver contribution to total fatty acid and cholesterol synthesis is far greater than that of adipose tissue (4-7). In swine, it has been found that adipose tissue is the major site for fatty acid synthesis (8,9). Dietschy and Siperstein (10) demonstrated that rat liver has the highest rate of cholesterol synthesis, and gastrointestinal tract ranked second. It has also been found that more than 90% of total cholesterol synthesis occurs in the liver and intestine of the squirrel monkey (11). Romsos et al. (9) indicated that liver and adipose tissue were the most important sites in swine for incorporation of acetate-1-^{14}C into cholesterol in vivo. Glucose, rather than acetate, is the more important physiological precursor of fatty acid and cholesterol synthesis. However, the relative contribution of tissue sites to cholesterol synthesis with glucose as a substrate in swine is not known, and no estimates of the contribution of other tissue to total cholesterol and fatty acid synthesis in this species has been made. The importance of lipid to arterial tissue in aging and atherosclerosis has long been recognized, and reactions in which the aorta metabolizes lipids are of great interest. It seemed important to investigate the role of different tissues in fatty acid and cholesterol synthesis in swine. Because the mitochondrial fraction represents the major site of fatty acid synthesis in heart and aortic tissue, the synthesis of fatty acids in subcellular fractions in these tissues was also investigated. Furthermore, such information would be important for the study of lipogenic and cholesterogenic adaptations to dietary treatment.

MATERIALS AND METHODS

Animals and Diets

Crossbred weanling swine (Yorkshire x Hampshire) were used in Experiment 1, while Hampshire weanling swine were used in Experiments 2 and 3. Swine in Experiment 1 were fed to 6 months of age the basal diet containing 3% fat and 14.3% protein as furnished by 1,745 lb of ground yellow corn, 200 lb of solvent extracted soybean meal, and 55 lb of a premix of multiple vitamins and minerals per ton of basal ration. (The premix consisted of 5% lysine, 20%

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calcium [calcium carbonate, dicalcium phosphate], 9% phosphorus, 15% sodium and potassium chloride, 0.004% calcium iodate, 0.018% zinc, 0.18% iron, 0.14% manganese as oxides or carbonates, and the following vitamines per lb: 40 mg riboflavin, 100 mg d-pantothenic acid, 300 mg niacin, 2,000 mg choline, 0.32 μg vitamine B_{12}, a minimum of 60,000 United States Pharmacopeia units of vitamin A palmitate, 60,000 international units (IU) of vitamin D_3, and 165 IU of vitamin E). In Experiments 2 and 3, the basal diet was supplemented with 200 million units of vitamin D_3 per ton of basal ration for 6 weeks, after which time the vitamin D_3 was withdrawn. The basal diet was then fed for 3 months until the swine were killed. Food and water were available ad libitum.

Tissue Incubation Procedures

For the enzymatic and in vitro incubation studies, liver, adipose, heart, intestine, and aortic tissue samples were obtained immediately after slaughter and placed in 0.9% NaCl during transportation to the laboratory. Adipose tissue samples were obtained from the subcutaneous layer of abdominal fat. The medial and intimal layers of aortic tissue and the mucosal and submucosal layers of small intestine were used for tissue incubation and enzyme assays. Liver, heart, small intestine, and aortic tissue were kept on ice while the adipose tissue was kept at room temperature. Slices of tissues (100-200 mg) were prepared using a Stadie-Riggs hand microtome. The incubation and isolation procedures were similar to the methods described by O'Hea and Leveille (5). Tissue slice incubations were carried out at 37 C in 3 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) under 95% O_2 and 5% CO_2 in a metabolic shaker for 2 hr. Substrates were dissolved in a buffer to a concentration of 10 mM acetate and/or 5 mM glucose. Each flask also contained 0.9 μCi acetate-1-^{14}C or glucose-U-^{14}C and 0.3 unit insulin.

Enzyme Studies

The tissue samples remaining after the preparation of slices were homogenized in four volumes of ice-cold homogenization buffer (pH 7.4) which contained 0.3 M sucrose, 30 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM glutathione. The homogenate was centrifuged 60 min at 105,000 x g, and the supernate fraction (cytosol) was collected and used for the enzyme assay. Enzyme activity measurements were conducted at 37 C in the linear range of activity.

6-Phosphogluconate dehydrogenase (EC 1.1.1.44) was assayed as described by Glock and McLean (12) using the following components: 58 mM glycylglycine buffer (pH 7.4), 10 mM $MgCl_2$, 0.30 mM nicotinamide adenine dinucleotide phosphate, oxidized form ($NADP^+$), and 1.4 mM 6-phosphogluconate.

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was assayed by the double substrate method of Glock and McLean (12). The reaction mixture contained 67 mM glycylglycine buffer (pH 7.4), 10 mM $MgCl_2$, 0.3 mM $NADP^+$, 1.4 mM 6-phosphogluconate, and 1.4 mM glucose-6-phosphate.

Nicotinamide adenine dinucleotide phosphate (NADP)-malate dehydrogenase (EC 1.1.1.40) was assayed according to the method of Ballard and Hanson (13). Component concentrations were 34 mM Tris buffer (pH 7.3), 1.5 mM $MnCl_2$, 0.3 mM $NADP^+$, and 0.75 mM sodium malate.

Citrate cleavage enzyme (EC 4.1.3.8) was assayed by coupling with nicotinamide adenine dinucleotide (NAD)-malate dehydrogenase according to the method of Takeda et al. (14). The concentrations of reactions components were 200 mM Tris buffer (pH 8.4), 10 mM $MgCl_2$, 10 mM mercaptoethanol, 20 mM citrate, 2 units malate dehydrogenase, 0.2 mM reduced nicotinamide adenine dinucleotide (NADH), 10 mM adenosine triphosphate (ATP), 0.2 mM CoA, and 0.3 mM dithoitreitol.

Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) was assayed according to the method of Gudjarnason et al. (15) using 50 mM triethanolamine-5 mM EDTA, (pH 7.5), 0.2 mM NADH, and 1.0 mM dihydroxyacetone phosphate.

Acetyl CoA carboxylase (EC 6.4.1.2) was assayed by the method of Ingle et al. (16). The cytosol fraction (0.24 ml) was preincubated 30 min at 37 C in 0.01 ml of bovine serum albumin (240 mg/ml), and 0.25 ml of a stock solution (40 mM Tris buffer [pH 7.4], 0.4 mM EDTA, 0.4 mM glutathione, 40 mM $MgCl_2$, and 40 mM sodium citrate) in stoppered centrifuge tubes. After preincubation, 0.5 ml of an incubation medium comprised of stock solution, sodium ATP, potassium bicarbonate, and acetyl CoA was added to the reaction vials. The final reaction volume was 1.0 ml (pH 7.4), and component concentrations were 20 mM Tris buffer, 0.2 mM EDTA, 0.2 mM glutathione, 20 mM $MgCl_2$, 20 mM sodium citrte, 2.0 mM sodium ATP, 0.2 mM acetyl CoA, 20 mM potassium bicarbonate (3 μCi/ml), and bovine serum albumin (2.4 mg/ml). The reaction was stopped with 0.2 ml of 6 N hydrochloric acid after a reaction time of 10 min at 37 C. After centri-

TABLE I

Relative Rates of Utilization of Acetate-1-^{14}C by Swine Tissue Slices In Vitro[a]

Metabolite measured	Tissue			
	Liver[b]	Adipose[b]	Heart[c]	Aortic[b]
$^{14}CO_2$	300 ± 15	185 ± 17	176 ± 34	87 ± 25
Fatty acids	1.75 ± 0.30	176 ± 6	0.10 ± 0.01	<0.05
Cholesterol	1.45 ± 0.20	3.0 ± 1.0	<0.1	<0.05

[a]Results are expressed as nmoles of substrate converted to the product indicated per 100 mg tissue per 2 hr.

[b]Mean ± SEM for five swine (average wt 110 kg).

[c]Mean ± SEM for three swine (average wt 117 kg).

fugation, an aliquot (0.6 ml) of supernatant solution was transferred to scintillation vials and counted in a liquid scintillation counter with 15 ml of counting fluid (7 parts toluene containing 0.4% w/v omnifluor: 6 parts Triton X-100).

Fatty acid synthetase was assayed according to the method of Chang et al. (17). The incubation medium (1 ml) contained 100 mM potassium phosphate buffer (pH 6.8), 0.05 mM acetyl CoA, 0.1 mM malonyl CoA, 1.16 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 3 mM EDTA, 2 mM dithiothreitol, and 0.03 μCi malonyl-CoA-1,3-$^{14}C_2$. The reaction was allowed to proceed 5 min at 37 C for liver and 10 min for other tissues and was stopped with 0.5 ml of 70% $HClO_4$. Ethanol (1 ml) was added, and the fatty acids were extracted three times with 5 ml portions of petroleum ether (bp 60-68 C) which were transferred to scintillation vials. The combined extracts were dried, and 10 ml of toluene scintillation fluid were added, the samples were counted in a Packard liquid scintillation spectrometer.

In Experiment 3, homogenized samples of heart, adipose, and aortic tissues and subcellular fractions of heart and aortic tissue were used for incubation studies. Samples from adipose and aortic tissues were minced and homogenized in the same homogenization buffer in an omnimixer while samples from heart were homogenized in a Thomas glass homogenizer kept in ice. The homogenates were centrifuged at 1000 x g for 15 min in a refrigerated International Equipment Co. PR-J centrifuge, and the debris was discarded. The top layer was used for incubation studies. Subcellular fractionation of heart and aortic tissue was accomplished as follows: The 1,000 x g supernate was centrifuged 30 min at 12,000 x g to yield a pellet containing the mitochondria. The supernate, after removal of the mitochondria, was centrifuged 60 min at 100,000 x g to yield the microsomal pellet. the supernate from this spin yielded the high speed supernate (HSS). The incubation mixture used was a modification of the method of Howard (18). All tubes contained 131 mM glycylglycine (pH 7.4), 6.6 mM glutathione, 0.2 mM NADPH, 1.2 mM NADP, 6.6 mM glucose-6-phosphate, 0.33 units/ml glucose-6-phosphate dehydrogenase, 1.6 mM NADH, 12.9 mM potassium isocitrate, 2.5 mM niacinamide, 5.2 mM $MgCl_2$, 6.5 mM sodium ATP, 0.65 mM $MnCl_2$, 6.5 mM creatine phosphate, 0.6 units/ml creatine phosphokinase, 20 mM glycerol-3-phosphate, and 33 mM sodium bicarbonate. Bovine serum albumin (4 mg/ml) was added to the HSS and nonsubcellular fraction tubes. Coenzyme A (0.3 mM) was included in the HSS tube containing acetate-1-^{14}C and also in all tubes of microsomes and mitochondria containing acetate-1-^{14}C or acetyl-1-^{14}C CoA. When acetate was used as the substrate, 6.3 mM acetate-1-^{14}C (1 μCi) was added to the HSS, microsomes, and mitochondria. However, when acetyl CoA was used as the substrate, 0.1 mM acetyl-1-^{14}C CoA (0.5 μCi) was added to the HSS, microsomes, mitochondria, and nonsubcellular fractions. The final volume was 1.0 ml, and incubation was carried out at 37 C with constant shaking for 10, 20, and 60 min in adipose, heart, and aortic tissue, respectively. Protein was determined according to the method of Schaterle and Dollack (19) by the modification of Lowry et al. (20).

RESULTS

Experiment 1

Adipose tissue, with a synthetic rate of 176±6 nmoles per 100 mg tissue per 2 hr, had the highest lipogenic activity of the four different assayed tissues (Table I). Swine liver had a limited capacity for converting acetate to fatty acids, which agrees with the results originally obtained by O'Hea and Leveille (8). The results also show that the ability of adipose tissue slices to convert acetate-1-^{14}C to fatty

TABLE II

Relative Rates of Utilization of Glucose-U-^{14}C by Swine Tissue Slices In Vitro[a]

Metabolite measured	Tissue[b]		
	Liver	Adipose	Aortic
$^{14}CO_2$	2.2 ± 0.1	112 ± 16	8.8 ± 3.7
Fatty acids	<0.1	138 ± 14	<0.05
Cholesterol	<0.05	3.0 ± 0.8	<0.05

[a]Results expressed as nmoles of substrate converted to the product indicated per 100 mg tissue per 2 hr.

[b]Mean ± SEM for five swine (average wt 110 kg).

TABLE III

Activity of Pentose Pathway Dehydrogenases and NADP[a]-Malate Dehydrogenase in Certain Swine Tissues[b]

Enzyme assayed	Tissue			
	Liver[c]	Adipose[c]	Heart[d]	Aortic[c]
Glucose-6-P dehydrogenase	9.4 ± 1.7	161 ± 27	12 ± 2	23 ± 3
6-P-gluconate dehydrogenase	32 ± 3	89 ± 14	12 ± 3	13 ± 4
NADP-malate dehydrogenase	9.9 ± 2	186 ± 12	11 ± 1	9.7 ± 0.3

[a]NADH = Reduced nicotinamide adenine dinucleotide.

[b]Activity expressed as nmoles of substrate utilized per min per mg protein at 37 C.

[c]Mean ± SEM for five swine (average wt 110 kg).

[d]Mean ± SEM for three swine (average wt 117 kg).

acid is about 100 times that of liver slices when expressed on an equal wt basis. As also shown in Table I, heart and aortic tissue had a fatty acid synthetic rate considerably lower than that of adipose tissue. The incorporation of acetate-1-^{14}C into cholesterol was 3.0 and 1.45 nmoles per 100 mg tissue per 2 hr in adipose tissue and liver, respectively. Heart and aortic tissue had marginal levels of cholesterol biosynthesis. Acetate oxidation to CO_2 was much greater in heart and aortic tissue than to fatty acid and cholesterol synthesis. However, the incorporation of glucose-U-^{14}C into CO_2 was much lower than that of acetate-1-^{14}C in liver and aortic tissue (Table II). The data revealed that the amount of glucose-U-^{14}C oxidized to $^{14}CO_2$ was lowest in liver slices; incorporation of glucose-U-^{14}C to $^{14}CO_2$ was only 2% in adipose tissue. Incorporation of glucose-U-^{14}C into fatty acids (Table II) selected the same pattern but was much lower than that of acetate-1-^{14}C incorporation into fatty acids. These results indicated that both the rate of CO_2 production and fatty acid synthesis were considerably greater in adipose tissue than in liver and aortic tissue when glucose was used as the substrate.

The results also show that adipose tissue, with a synthetic rate of 3.0 nmoles per 100 mg tissue per 2 hr, was the most active tissue for cholesterol synthesis when either glucose-U-^{14}C or acetate-1-^{14}C was used as the substrate.

The activities of pentose pathway dehydrogenases and NADP-malate dehydrogenase was determined in liver, adipose, heart, and aortic tissues; the data are presented in Table III. Of these four tissues, adipose tissue had the highest dehydrogenase enzymatic activity. Both the pentose pathway dehydrogenases and NADP-malate dehydrogenase were active in adipose tissues. This implies that both pathways are important sources of NADPH in swine adipose tissue. In contrast to adipose tissue, the activity of three dehydrogenase enzymes in liver, heart, and aortic homogenates was extremely low.

Experiment 2

Results of this second experiment confirm previous findings and indicate that swine liver, heart, and aortic tissue have a limited capacity to convert glucose to CO_2, fatty acids, and cholesterol.

The production of CO_2 from glucose was higher in the small intestine and adipose tissue slices (Fig. 1) than in the other tissues. These data also show that the amount of glucose-

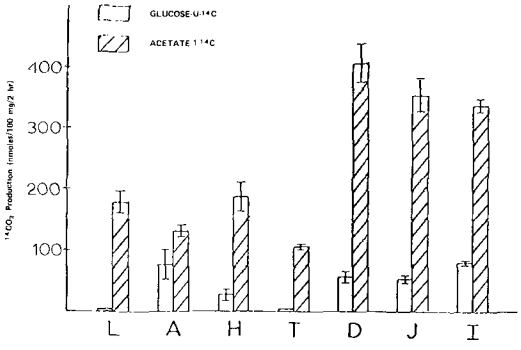

FIG. 1. Incorporation of glucose-U-^{14}C and acetate-1-^{14}C into $^{14}CO_2$ by certain swine tissue slices: L = liver, A = adipose, H = heart, T = aortic, D = duodenum, J = jejunum, I = ileum.

TABLE IV

Incorporation of Glucose-U-^{14}C and Acetate-1-^{14}C into Fatty Acids by Certain Swine Tissue Slices[a]

Tissue	Substrate[b] Glucose-U-^{14}C	Acetate-1-^{14}C
Liver	<0.05	0.73 ± 0.04
Adipose	69 ± 7	82 ± 5
Heart	<0.1	0.11 ± 0.05
Aorta	<0.05	<0.1
Duodenum	2.09 ± 0.39	17.2 ± 5.3
Jejunum	1.77 ± 0.12	25.3 ± 2.4
Ileum	1.34 ± 0.14	16.0 ± 0.7

[a]Results are expressed as nmoles of substrate converted to the product indicated per 100 mg tissue per 2 hr.

[b]Mean ± SEM for three swine (average wt 99 kg).

TABLE V

Incorporation of Glucose-U-^{14}C and Acetate-1-^{14}C into Cholesterol by Certain Tissues of Swine[a]

Tissue	Substrate Glucose-U-^{14}C[b]	Acetate-1-^{14}C[b]
Liver	<0.05	2.10 ± 0.49
Adipose	3.79 ± 0.91	5.80 ± 2.70
Heart	<0.05	<0.05
Aorta	<0.05	<0.05
Duodenum	0.49 ± 0.11	2.25 ± 0.50
Jejunum	0.70 ± 0.10	3.05 ± 0.11
Ileum	0.90 ± 0.36	3.36 ± 1.28

[a]Results are expressed as nmoles of substrate converted to the product indicated per 100 mg tissue per 2 hr.

[b]Mean ± SEM for three swine (average wt 99 kg).

U-^{14}C oxidized to $^{14}CO_2$ by all tissues except adipose tissue slices was only a fraction of that of acetate-1-^{14}C. The oxidation of acetate-1-^{14}C to $^{14}CO_2$ was highest in the small intestine slices (duodenum, jejunum, and ileum).

The relative values for incorporation of glucose-U-^{14}C and acetate-1-^{14}C into fatty acids by various swine tissue slices are summarized in Table IV. As in Experiment 1, the incorporation of acetate-1-^{14}C into fatty acids was much greater than that of glucose-U-^{14}C. Adipose tissue synthesized the highest amount of fatty acid of all tissues tested. Fatty acid synthetic activity was substantial in small intestinal slices, especially in the case of acetate-1-^{14}C. Lipogenesis in liver, heart, and aortic tissue was marginal, especially with glucose as the substrate. A comparison of acetate with glucose as a substrate for cholesterol synthesis is presented in Table V. It was found that the incorporation of glucose-U-^{14}C into cholesterol was much higher in adipose tissue than in any other tissues. Samples from small intestinal slices had substantially higher rates of cholesterol synthesis, especially in the case of acetate. Of the three segments tested, the ileum had the highest rate of cholesterol synthesis. Acetate-1-^{14}C was readily incorporated into liver cholesterol, whereas the incorporation of glucose-U-^{14}C into liver cholesterol was not detected. These data also revealed that the incorporation of acetate-1-^{14}C into heart cholesterol was very low and there was no cholesterol synthesis in heart muscle when glucose was used as the substrate; neither glucose-U-^{14}C or acetate-1-^{14}C were incorporated into cholesterol in aortic tissue.

Data on the incorporation of glucose-U-^{14}C and acetate-1-^{14}C into glyceride-glycerol by various swine tissue slices are presented in Figure 2. Slices from all tissues were found to actively synthesize glyceride-glycerol. In adipose tissue, glucose was the substrate of choice for glyceride-glycerol synthesis. However, the incorporation of acetate-1-^{14}C into glyceride-glycerol was higher than that of glucose-U-^{14}C in liver, heart, and the small intestine; glyceride-glycerol synthesis was about the same in heart and small intestine slices. The incorporation of glucose-U-^{14}C or acetate-1-^{14}C into glyceride-glycerol was the lowest in aortic tissue. However, as compared to fatty acid and cholesterol synthesis in aortic tissue, glyceride-glycerol synthesis was extremely high.

The activity of acetyl CoA carboxylase, fatty acid synthetase, citrate cleavage enzyme, and glycerol-3-phosphate dehydrogenase in various swine tissues differed substantially (Table VI). The activity of acetyl CoA carboxylase, fatty acid synthetase, and citrate cleavage enzyme were considerably higher in adipose tissue. These three key enzymes for lipogenesis, as well as pentose pathway dehy-

drogenases and NADP-malate dehydrogenase, paralleled fatty acid synthesis. Citrate cleavage enzyme, presumably involved in the production of acetyl CoA in the cytoplasm, showed the lowest activity in swine liver homogenates. The activities of citrate cleavage enzyme and NADP-malate dehydrogenase (Table III) were low in swine liver, whereas the activity of glycerol-3-phosphate dehydrogenase was extremely high.

Experiment 3

The rate of incorporation of acetyl-1-^{14}C CoA into fatty acids by homogenates of adipose, heart, and aortic tissues is shown in Table VII. Adipose tissue homogenates, like the tissue slices, possessed the highest lipogenic activity of the three tissues tested. Heart homogenates, with a synthetic rate of 4.56 nmoles per mg protein per hr, had a higher rate of incorporation of acetyl-1-^{14}C CoA into fatty acids than aortic homogenates. When measuring lipogenic activity on an equal wt basis, heart homogenates showed a tremendously high incorporation of acetyl-1-^{14}C CoA into fatty acids as compared to lipogenesis in heart tissue slices using acetate-1-^{14}C as the substrate.

Acetyl CoA incorporation into fatty acids was considerably higher in heart microsomes than in aortic tissues (Table VIII). Fatty acid synthesis in the aortic microsomal fraction was slightly higher than that in the high speed supernatant (HSS) and mitochondrial fractions. The synthesis of fatty acids from acetate or acetyl CoA by the HSS or mitochondria in heart and aortic tissue was very low.

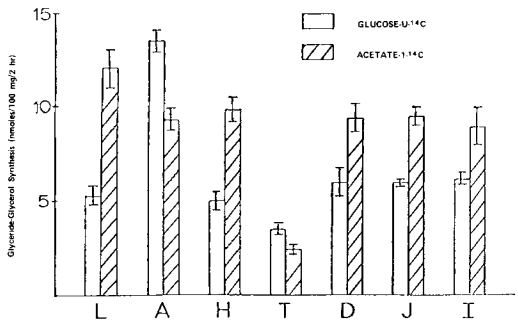

FIG. 2. Incorporation of glucose-U-^{14}C and acetate-1-^{14}C into glyceride-glycerol by certain swine tissue slices: L = liver, A = adipose, H = heart, T = aortic, D = duodenum, J = jejunum, I = ileum.

TABLE VI

Activity of Acetyl CoA Carboxylase, Fatty Acid Synthetase, Citrate Cleavage Enzyme, and Glycerol-3-Phosphate Dehydrogenase in Certain Swine Tissues[a]

	Liver	Adipose	Heart	Aorta	Duodenum	Jejunum	Ileum
Acetyl CoA carboxylase	0.68	2.56	0.11	0.020	0.20	0.22	0.17
Fatty acid synthetase	0.76	2.32	0.19	0.044	0.15	0.15	0.17
Citrate cleavage enzyme	0.99	19	1.6	2.4	3.9	--[b]	3.7
Glycerol-3-P dehydrogenase	389	119	224	1.1	9.5	3.6	27

[a]Activity expressed as nmoles of substrate utilized per min per mg protein at 37 C. Values represent average of triplicate determinations on pooled tissue cytosol from three animals. See text for details of assay conditions.
[b]Not tested.

TABLE VII

Incorporation of Acetyl-1-^{14}C CoA into Fatty Acids by Homogenates of Adipose, Heart, and Aortic Tissues[a]

Tissue	Fatty acid synthesis	
	nmoles/mg protein/hr	nmoles/100 mg/2 hr
Adipose	76	85
Heart	4.6	23
Aorta	0.009	0.016

[a]Values represented average of triplicate determinations on pooled tissues from three animals. See text for details of incubation conditions.

DISCUSSION

O'Hea and Leveille (8) have suggested that in vivo more than 99% of de novo synthesis of fatty acids occurs in the adipose tissue of swine when glucose-U-^{14}C is used as the substrate. However, they studied only the liver and adipose tissue. The results presented here agree with the findings of O'Hea and Leveille (8) that adipose tissue is the major site of fatty acid synthesis with both glucose-U-^{14}C and acetate-1-^{14}C as substrates in swine tissue slices. When acetate-1-^{14}C was used as a substrate, the small intestine accounted for a great percentage of overall lipogenesis. This is in contrast to in vivo results obtained with young swine intestines where the fatty acid synthesis represented only a small fraction (1-6%) of the estimated total lipogenesis (9). In vivo studies indicate that young swine liver accounted for 25-30% of the

TABLE VIII

Incorporation of Different Substrates into Fatty Acids by Subcellular Fractions of Swine Heart and Aorta[a]

Tissue	Substrate	Concentration (mM)	HSS[b]	Microsomes	Mitochondria
Heart	Acetate-1-^{14}C	6.3	9	37	20
	Acetyl-1-^{14}C CoA	0.10	10	11,940	10
Aorta	Acetate-1-^{14}C	6.3	12	33	33
	Acetyl-1-^{14}C CoA	0.10	8	18	1

[a]Activity expressed as μμmoles of substrate utilized per mg of protein per hr at 37 C. Values represent average of triplicate determinations on pooled tissue from three animals. See text for details of incubation conditions.

[b]HSS = High speed supernatant.

newly synthesized fatty acids (synthesis occurring in liver and adipose tissue only) when acetate-1-^{14}C was injected into the vena cava (8). However, in these studies, the synthesis of fatty acids in adult swine liver was very low, even when acetate was used as the substrate. The synthesis of fatty acids in heart and aortic tissue was also very low with both glucose-U-^{14}C and acetate-1-^{14}C as substrates. Subcellular fractionations of heart and aortic tissue possessed high fatty acid synthetic activity in the microsomal fraction. Because the microsomal elongation system is well developed in the rat and rabbit, it seems possible that, in swine heart and aortic tissue, the elongation system is more important than de novo synthesis of fatty acids.

The NADPH produced mainly in the pentose pathway dehydrogenases and NADP-malate dehydrogenase is used to reduce acetyl CoA for fatty acid biosynthesis (21). The activity of these enzymes in swine adipose tissue is high, paralleling its high lipogenic capacity. This implies that they may serve an important function in the production of reducing equivalents for fatty acid synthesis in this tissue. In contrast, the low activity of the three dehydrogenase enzymes in liver, heart, and aortic tissue may be due to their limited capacity to generate NADPH.

The activity of acetyl CoA carboxylase and fatty acid synthetase were highest in swine adipose tissue of ca. equal activity in all of the tissues observed in this study. The activities of citrate cleavage enzyme, NADP-malate dehydrogenase, and pentose pathway dehydrogenase were higher in adipose tissue than any other tissues tested, which is in agreement with the concept that adipose tissue is the primary site of fatty acid biosynthesis. Activity assayed as fatty acid synthetase and acetyl CoA carboxylase was found in all tissues. The activity could represent chain elongation activity normally found in mitochondrial and microsomal fractions inasmuch as tissue homogenization conditions were extreme, especially in the case of tough aortic tissue.

In adult swine, adipose tissue appears to be an important site for cholesterol synthesis; the small intestine ranked second, while liver, heart, and aortic tissue possessed marginal levels of cholesterol synthesis when glucose was used as a substrate. When acetate is available, liver, small intestine, and adipose tissue are important sites for cholesterol synthesis. This is in contrast to the results obtained with young swine in vitro where the intestine contributed only about 4% of the total cholesterol synthesized from acetate-1-^{14}C, whereas liver and adipose tissue contributed 67 and 29%, respectively (9). In the small intestine, the ileum seems to be higher in cholestrol synthesizing ability than the duodenum and jejunum. This is in agreement with the results obtained with rat, monkey, and human intestine where sterol synthesis was greater in the ileum than in the jejenum (10,11,22). However, this is in contrast to in vivo results obtained from young swine; the upper three segments of the small intestine contributed more to intestinal cholesterol synthesis than the lower segment (9).

Although glucose cannot be converted to cholesterol in swine liver slices, a substantial amount of acetate-1-^{14}C was incorporated into liver cholesterol. Apparently, free acetate is required for cholesterol synthesis in swine liver. It has been reported that organic acids such as acetic, propionic, and lactic acid are produced by microorganisms in the digestive tract of swine (23,24), appear in the blood stream (25,26), and are removed by the liver (26). Swine liver possesses an active acetyl CoA synthetase enzyme (27) which could activate acetate of endogenous or exogenous origin and make it available for the synthesis of cholesterol.

Glucose is also an important source of glycerol-3-phosphate for lipid synthesis in adipose tissue (28). The present results indicate that there was a higher incorporation of glucose-U-^{14}C and acetate-1-^{14}C into fatty acids than glyceride-glycerol in adult swine adipose tissue. This agrees with the findings of Mersmann et al (29) that glucose was incorporated equally into fatty acids and glyceride-glycerol before weaning, with a shift toward fatty acids as the primary product after weaning, whereas acetate was mainly incorporated into fatty acids at all ages in swine adipose tissue.

The greatest production of CO_2 from acetate by small intestine preparations in swine probably reflected the highest mitochondrial activity. Although fatty acid synthesis is low in aortic tissue, it might have high mitochondrial activity as reflected by the high CO_2 production from acetate.

When glucose-U-^{14}C is used as the substrate for fatty acid and cholesterol biosynthesis in swine, citrate cleavage enzyme is necessary for the conversion of citrate to acetyl CoA. The very low activity of citrate cleavage enzyme in swine liver presumably led to the marginal levels of cholesterol and fatty acid synthesis using glucose as precursor. Because glucose is the main substrate absorbed by nonruminant animals, the results obtained with glucose-U-^{14}C may more nearly reflect the actual situation than results obtained when acetate-1-^{14}C was used as the substrate for fatty acid and cholesterol biosynthesis in swine.

Subcellular fractionation indicated that microsomal fractions in heart and aortic tissue had higher fatty acid synthesizing ability than the high speed supernate and mitochondrial fractions. Similar results were also found in squirrel monkey aorta when acetyl-1-^{14}C CoA was used as the substrate (18). However, Whereat (30) and Whereat et al. (31) demonstrated that the mitochondria were the most important sites of synthesis in rabbit heart and aorta for the incorporation of acetate-1-^{14}C into fatty acids. Low fatty acid synthesis in aortic tissue slices and homogenates indicated that swine aortic tissue was not an important site for lipogenesis.

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Effect of Dietary Polyunsaturated Pork on Plasma Lipids and Sterol Excretion in Man

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ABSTRACT

Pork, enriched in linoleic acid content, was compared with conventional pork in the diet of three human subjects with respect to the plasma cholesterol concentration and the excretion in feces of neutral sterols and bile acids. Since the fatty acids in pork glyceride have an unusual positional distribution, the redistribution that might occur during the absorption and disposition of a fat meal was also studied. The plasma cholesterol was lower with polyunsaturated pork, the difference, 14 mg/100 ml plasma, being of the order expected from the change in polyunsaturated to saturated fatty acid ratio. On average, the excretion of neutral sterols was 57% greater with polyunsaturated than with conventional pork in all three subjects, and in this respect the results resembled the findings with polyunsaturated ruminant fats. During the absorption of pork fat, the high proportion of palmitate in the 2 position of lard triglyceride served as a useful marker, since human triglyceride carries mainly unsaturated fatty acids in that position. There were stepwise changes in the fatty acid composition at the 2 position of triglyceride as the fat was absorbed, transported through, and cleared from plasma, the palmitate being gradually replaced by oleate and linoleate. By contrast, the total fatty acid profile in the triglyceride changed relatively little, implying selective reacylation with palmitate at the 1 and/or 3 position. During the clearing of dietary triglyceride, the porcine triglyceride was thus converted to the form occurring in humans.

INTRODUCTION

Dietary polyunsaturated fatty acids generally lower the plasma cholesterol concentration in man (1). These polyunsaturated fatty acids are usually derived directly from vegetable oils and are consumed in the form of oils, margarine, etc. Recently, we demonstrated that polyunsaturated ruminant fats, eaten as beef, lamb, and dairy products, also reduce the plasma cholesterol concentration in man while increasing the excretion of fecal sterols (2,3). In studies carried out by Ahrens et al. using relatively unsaturated pork fats as the source of polyunsaturated fatty acids, the expected cholesterol lowering effect was not seen (1).

The triglycerides (TGs) of porcine adipose tissue have a structural arrangement of fatty acids which is different from that in most other animal and plant TGs; the 2, or beta, position of the pork TG is largely esterified with palmitic acid, and the 1 and 3 positions contain the unsaturated moieties and stearic acid (4,5). This arrangement of fatty acids is the reverse of that normally present in plant and animal TGs where palmitic acid occupies the 1 position and the unsaturated fatty acids occupy position 2 and, to a lesser extent, position 3 (4,5).

The intestinal digestion of TG leads to the formation and direct absorption of 2-monoglyceride. Thus, the fatty acid occupying the 2 position (palmitic acid in the case of lard) may not be significantly diluted by other endogenous fatty acids prior to incorporation into chylomicron lipid (6). In addition, the absorption of dietary cholesterol from the small intestine appears to be influenced by the presence of 2-monoglyceride produced by pancreatic lipase hydrolysis (7), and the nature of the fatty acid esterified to the monoglyceride, e.g., 16:0 with pig fat, may be important.

With the knowledge obtained from feeding polyunsaturated ruminant products to human subjects, and with the possiblity that the structural arrangement of fatty acids in the TG molecule may influence lipid metabolism, it seemed desirable to reevaluate the role of polyunsaturated pork as a possible influence on plasma cholesterol level and sterol excretion. The unusual fatty acid distribution in porcine glyceride also enabled us to follow any redistribution of fatty acids within serum TGs during digestion and metabolism of dietary fat.

Although the present study was carried out in only three subjects, the uniformity of the results suggests that polyunsaturated pork fat, in comparison with conventional pork fat, does enhance the excretion of sterols and may lead to a lower plasma cholesterol level.

TABLE I

Daily Cholesterol Intake and the Fatty Acid Composition of Dietary Fat

Diet	Cholesterol[a] (mg/day)	Triglyceride fatty acid[b]					
		14:0	16:0	16:1	18:0	18:1	18:2
Conventional pork	549	1	25	5	12	45	12
Polyunsaturated pork	535	1	17	4	9	33	36

[a]Mean for three subjects.

[b]Percentage distribution by wt of major fatty acids in aliquots of the total diet (fatty acids identified according to number of carbon atoms and number of double bonds).

METHODS

Diets

Three healthy male university students, aged 20 years, were studied for 56 days; subject #3 was overweight and midly hypertriglyceridemic. A normal pattern of eating was followed, and calorie intake was adjusted to maintain a relatively constant body wt. Protein, carbohydrate, and fat provided 15%, 41%, and 44% of dietary calories, respectively. There were two 28-day experimental periods in which two types of pork were compared: the first in which meat was obtained from pigs fed conventional barley and sorghum based rations, and the second in which meat was obtained from animals fed similar rations supplemented with sunflower seeds (30% by wt of diet). The dietary fat consumed by the human subjects was derived predominantly from pork, i.e., as lard, bacon, ham, chops, roasts, and sausages. The fatty acid compositions of the two types of fat are shown in Table I. Both sources of meat contained similar amounts of cholesterol (69 mg/100 g for the polyunsaturated and 73 mg/100 g for the conventional meats). Only a portion (ca. 300 mg/day) of the daily intake of cholesterol was, however, derived from pork; the balance was provided from egg yolk powder to give a total of 500-600 mg (Table I). This resembled the intake during studies with polyunsaturated ruminant fats previously carried out in the same three subjects. The findings with the ruminant fats were part of a larger study (3) and have been reported separately.

Laboratory Investigations

Blood was drawn three times weekly after an overnight fast and heparin was used as an anticoagulant. Plasma total cholesterol and TG concentrations were measured with a Technicon II autoanalyzer.

The fatty acid composition of several classes of plasma lipids was estimated during the last week of each dietary treatment. TG, esterified cholesterol, and lecithin were separated by thin layer chromatography. Solvent systems were hexane:diethyl ether:methanol:acetic acid (180:40:6:4) for the separation of neutral lipids, and chloroform:methanol:water (140:60:10) for the isolation of lecithin. The 2-monoglyceride was derived from TG by the method of Luddy et al. (8). The fatty acids in the 1 and 2 positions of lecithin were isolated by the method of Christie and Moore (9) with minor modifications. The fatty acid composition was determined by gas chromatography of the methyl esters using 13% ethylene glycol adipate as the stationary phase.

During the final week of each diet, blood was also drawn 3 hr following a standard meal, and the TG fatty acid compositions were determined in the plasma lipoproteins of Sf>400 ("chylomicrons") and Sf 20-400 (the very low density lipoproteins, or VLDL). These lipoproteins were separated ultracentrifugally as described by Gustafson (10).

The excretion of sterols in feces was measured during the last 8 days of each diet. Four 2-day pools were assayed for neutral and acidic sterol content. The neutral sterols comprised cholesterol, coprostanol, and coprostanone, whereas the acidic sterols comprised bile acids and their degradation products. Separation of the sterol classes and their quantification by gas chromatography was carried out as described by Miettinen et al. (11) and by Grundy et al. (12). Chromium oxide was taken orally by the subjects throughout the study and served as a marker for daily fecal flow (13).

RESULTS

Plasma Lipids

The plasma cholesterol concentration was lower with the polyunsaturated pork in each subject, and this difference was significant ($P<0.05$) in two of the subjects comparing the last six values in each dietary period (Table II). The overall mean difference in plasma cholesterol concentration was only 8% (14 mg/100 ml

TABLE II

Plasma Cholesterol and Triglyceride Concentrations with Diets of Conventional Pork (CP) and Polyunsaturated Pork (PP)

Subject	Cholesterol[a] (mg/100 ml)		Triglyceride[a] (mg/100 ml)	
	CP	PP	CP	PP
1	181 ± 7.7	167[b] ± 8.1	86 ± 4.7	73 ± 6.0
2	194 ± 7.7	182[b] ± 6.8	79 ± 9.9	93 ± 5.7
3	180 ± 14.3	165 ± 5.4	293 ± 158	206 ± 44

[a]Mean of 6 measurements during last 12 days of each diet (± standard deviation).
[b]PP significantly lower than CP ($P<0.05$).

plasma), but the magnitude of this difference was close to the theoretical difference (18 mg/100 ml) calculated from the ratios of linoleate to saturated (palmitate and myristate) fatty acids in the respective diets (14,15). In the comparison between polyunsaturated and conventional ruminant fats in the same three subjects (3), the mean difference in plasma cholesterol was 20 mg/100 ml, which was identical to the theoretical difference based on the changed polyunsaturated to saturated fatty acid ratio. Plasma TG values, however, were not changed in a consistent manner (Table II).

The percentage distribution of the major TG fatty acids, those comprising 1% or more of the total, was determined for several classes of plasma lipids (Table III). Plasma used in these studies was obtained following an overnight fast. The polyunsaturated pork diet resulted in increased proportions of linoleate in TG and lecithin, but not in cholesterol esters. There were also increased proportions of arachidonate in lecithin from subjects consuming the polyunsaturated pork, and this fatty acid, together with linoleate, was almost exclusively located at the 2 position of the lecithin molecule. The linoleate of plasma TG was also preferentially located on position 2, but this specificity was less than that observed in the lecithin. The enhanced proportions of linoleate and arachidonate at position 2 of the lecithin were associated with decreased proportions of palmitate and oleate at this position. There was no effect of diet on the fatty acid composition at position 1 of lecithin.

The enhanced proportion of linoleate in the fasting plasma TGs of those subjects consuming the polyunsaturated pork was associated with a reduced proportion of oleate but no marked change in the other major fatty acids. The dietary induced differences in the plasma TG fatty acid composition of fasted subjects tended to reflect the differences in the fatty acid composition of the respective dietary fats (Tables I and III), though the relatively high content of stearic acid in lard was not reflected in plasma TG.

Comparison of the fatty acid distributions at the 2 position of the respective TGs demonstrated a marked difference between the diet and the plasma from fasted subjects (Table III). The high proportions of palmitate and the low proportions of linoleate at position 2 of the triglycerides from both types of pork were the reverse of that observed in plasma TG from the fasted subjects (Table III).

To obtain some insight into the manner in which this redistribution of fatty acids occurred, we analyzed the TGs of plasma chylomicrons and VLDLs during absorption of the respective diets (Table IV). The total fatty acid composition of chylomicron TG (Table IV) resembled that of the respective dietary lipid (Tables I and III), but this resemblance was more pronounced with the conventional than with the polyunsaturated pork. The fatty acid distribution pattern at position 2 of the chylomicron TGs (Table IV) also tended to resemble the corresponding fatty acid distribution at position 2 of the respective dietary TGs (Table III). Chylomicron and dietary TGs both showed a preferential location of palmitate at position 2 and oleate at positions 1 and/or 3. By contrast, the linoleate of the plasma chylomicrons appeared to be randomly distributed between the 1 (3) and the 2 positions of the TG molecule, whereas the linoleate of dietary TG was preferentially esterified to the 1 (3) position (Tables III and IV).

The plasma Sf 20-400 lipoproteins obtained from subjects actively absorbing fat probably represent a mixture of lipoproteins of intestinal and hepatic origin and, although the TG fatty acid pattern in this class of lipoprotein resembled very closely that of fasting whole plasma, the fatty acids in the 2 position were quite different (Table IV). Furthermore, the fatty acid distribution at position 2 of these

Sf 20-400 lipoproteins was also different from that at position 2 of dietary and chylomicron TGs. The general trend in the 2 position of these VLDL TGs was for the proportion of palmitate to decrease and that of oleate and linoleate to increase relative to the chylomicron TGs (Table IV). To facilitate comparison of the interpositional specificity of location of the various major fatty acids, we calculated the proportions of the individual fatty acids which are located at position 2, expressed as a percentage of the total proportion of the respective fatty acid available for esterification at any given position, assuming complete randomization (Fig. 1). There was a progressive change in the interpositional specificity from pork fat through chylomicrons and small alimentary particles, or VLDL, to TGs of predominantly hepatic origin, with the palmitate at position 2 of dietary pork TG being gradually replaced by oleate and linoleate.

Sterol Excretion

Table V shows the excretion of neutral and acidic sterols by the subjects consuming the polyunsaturated or the conventional pork. These results are based on four measurements during the final 8 days of each diet. In all three subjects, the net output of sterols (total fecal excretion minus cholesterol intake) was significantly greater with polyunsaturated than with the conventional pork ($P<0.05$). The mean difference in net sterol output was 57% for the three subjects. The corresponding difference with ruminant fats was 67% for the same three subjects at the end of 4 week periods comparing polyunsaturated with conventional products (3).

DISCUSSION

Recently we showed that linoleate-enriched ruminant fats in the diet lower the plasma cholesterol level and stimulate sterol excretion in man (2,3). These polyunsaturated ruminant foods are produced by including in the diet of sheep and cattle specially prepared oil or oilseed based supplements in which the constituent polyunsaturated fatty acids are resistent to microbial hydrogenation in the rumen (16). Pigs, in contrast to ruminants, do not hydrogenate dietary fatty acids prior to intestinal digestion and absorption, and their adipose tissue can therefore be readily enriched with linoleic acid.

The purpose of these preliminary studies was two fold: first, to determine whether the interrelationships between dietary linoleate-enriched pork and cholesterol metabolism of man dif-

TABLE III

Fatty Acid Composition[a] of Plasma Lipids and Dietary Lard with Conventional and Polyunsaturated Pork Diets

	Conventional pork								Polyunsaturated pork							
Plasma lipid	14:0	16:0	16:1	18:0	18:1	18:2	20:3	20:4	14:0	16:0	16:1	18:0	18:1	18:2	20:3	20:4
Cholesterol ester[b]		3	3		19	65		10		7	2		18	67		6
Triglyceride[b]																
Total	1	24	5	2	53	15			1	24	7	4	35	29		
2 Position	1	14	5	3	53	24			1	11	6	1	34	47		
Lecithin[b]																
1 Position		52		31	14	3				55		31	11	3		
2 Position		9	3	3	26	42	7	9		5	1	3	12	54	5	20
Lard (dietary)																
Total	3	27	5	11	44	10			2	18	3	9	33	35		
2 Position	6	68	8	2	13	3			4	58	3	2	13	20		

[a]Percentage distribution by wt of fatty acids comprising at least 1% of total.
[b]Mean values for three students, measured in fasting samples of plasma during the third week of each diet.

TABLE IV

Glyceride Fatty Acid Composition in Fasting and Post-prandial Plasma

Plasma lipid[a]	14:0	16:0	16:1	18:0	18:1	18:2
	(Wt %)					
Conventional pork						
Fasting whole plasma						
Triglyceride	1	24	5	2	53	15
2-Monoglyceride	1	14	5	3	53	24
Post-prandial chylomicrons[b]						
Triglyceride	1	27	4	12	48	8
2-Monoglyceride	1	48	8	6	29	8
Post-prandial VLDL[c]						
Triglyceride	1	23	4	5	51	16
2-Monoglyceride	1	30	7	4	40	18
Polyunsaturated pork						
Fasting whole plasma						
Triglyceride	1	24	7	4	35	29
2-Monoglyceride	1	11	6	1	34	47
Post-prandial chylomicrons[b]						
Triglyceride	1	23	4	8	37	27
2-Monoglyceride	2	39	6	4	22	27
Post-prandial VLDL[c]						
Triglyceride		26	4	7	36	27
2-Monoglyceride		22	4	4	32	38

[a]Means for three students; studies carried out during third week of each diet.

[b]Chylomicrons: Sf > 400 plasma lipoproteins 3 hr after fat meal.

[c]Very low density lipoprotein: Sf 20-400 plasma lipoproteins 3 hr after fat meal.

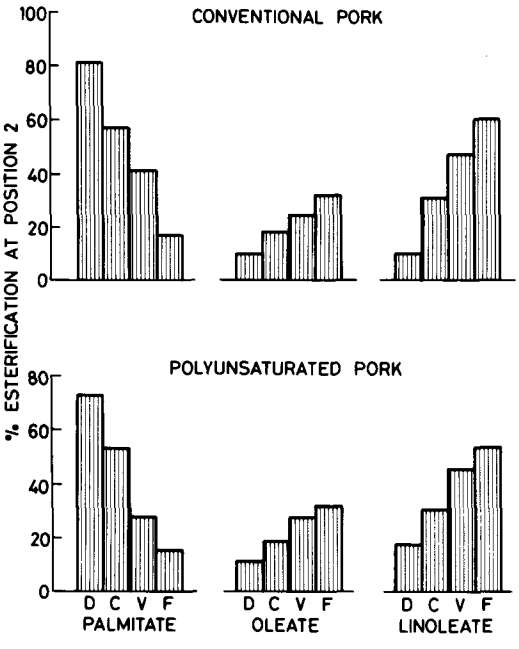

FIG. 1. Proportion of fatty acids esterified at position 2 of triglycerides. D = Diet; C = plasma chylomicrons (Sf>400) 3 hr after meal; V = plasma very low density lipoprotein (Sf 20-400) 3 hr after meal; F = whole plasma from fasted subjects.

fered from those of other dietary polyunsaturated fats and, secondly to exploit the unusual TG structure of pork fat in order to define the redistribution of glyceride fatty acids during the disposition of absorbed fat.

The lowering of the plasma cholesterol concentration and the increase in fecal sterol excretion brought about by substituting polyunsaturated for conventional pork were of the same order as with the comparison of the two varieties of ruminant meats carried out in the same three subjects and under conditions similar to those of the present investigation (3). Furthermore, the observed reductions in plasma cholesterol concentration attributable to the increase in the polyunsaturated to saturated fatty acids ratios were, in both comparisons, similar to the changes predicted from the equations derived by Keys et al. (14,15). The mean difference in net sterol excretion between the two pork diets was 57% compared with 67% between the two ruminant diets.

We have consistently observed an initial enhancement of sterol excretion when polyunsaturated fats are eaten by healthy individuals (2,3). Although this response appears to persist only as long as the plasma cholesterol concentration is falling, it is sufficient to account for the loss from plasma. In the present

TABLE V

Sterol Excretion (mg/day) with Conventional Pork (CP) and Polyunsaturated Pork (PP) Fat

	Neutral sterol		Bile acid		Total sterol		Net output[a]	
Subject	CP	PP	CP	PP	CP	PP	CP	PP
1	866[b]	1216	260	194	1126	1410	605	901
2	582	741	236	268	818	1009	264	475
3	1043	1332	197	255	1240	1587	662	1026

[a]Total sterol excretion - dietary cholesterol.

[b]Each value is the mean of four 2-day pools.

study, the polyunsaturated pork diet, in comparison to the conventional pork diet, caused an increase in the net fecal sterol excretion of 290 mg/day, whereas the overall reduction in the plasma cholesterol pool was ca. 400 mg. Such calculations do not take into account the transfer of cholesterol between tissues and plasma or the rate of cholesterol flux through plasma. However, the net total sterol loss via fecal excretion over a period of weeks is clearly much greater than the total net loss from the plasma. We have discussed elsewhere (3) the similarity of our findings with those of other short term studies using healthy subjects, such as those reported by Moore et al. (17) and Connor et al. (18), where vegetable oils were used as the direct source of dietary polyunsaturated fat.

Because of its effect on plasma cholesterol levels and on sterol excretion, polyunsaturated pork may have some place in the dietary management of hypercholesterolemia. In the classic paper of Ahrens et al. (1) which established the relationship between dietary fatty acid saturation and serum lipid levels, several varieties of lard with differing iodine values were tested; no distinction was observed in the cholesterol lowering properties of lards with iodine values of 52 and 89. The iodine values of the present fats were 67 for the conventional and 99 for the polyunsaturated varieties, respectively.

Pig fat is different from most plant and animal fats in that the 2-position of the TG is largely esterified with palmitic acid, whereas the 2-position of TGs in most other animal species and plants generally is esterified with unsaturated fatty acids (4,5). A comparison of the present results obtained by feeding pork and those obtained by feeding ruminant meats (3) suggests that in humans the nature of the fatty acid esterified at the 2-position of the dietary TG probably does not influence the metabolism of cholesterol.

The high content of palmitate at the 2-position of lard TG also served as a useful marker of glyceride fatty acid redistribution during the absorption and transport of fat. TGs are hydrolyzed by pancreatic lipase and the absorbed products are resynthesized to form chylomicron TG, which is in turn subjected to the action of lipoprotein lipase and hepatic TG lipase. These lipases generally show a preferential hydrolysis of fatty acids at the 1 (3) positions, with a tendency for the fatty acid at position 2 to remain intact (19-22). Although this accounts for the relative stability of the fatty acid pattern in the 2-position during fat absorption and transport, some hydrolysis of this ester bond probably occurs through the activity of monoglyceride hydrolase (20,23).

The present studies have shown progressive changes in the relative location of fatty acids at the 2-position of TG as dietary fat was absorbed and transported through plasma (Table IV, Fig. 1). The progressive reduction in the relative proportion of palmitate at position 2 of the lipoprotein TGs was associated with corresponding increases in the relative proportions of the unsaturated fatty acids (oleic and linoleic acids). This gradual replacement of palmitate with unsaturated fatty acids at position 2 not only reflects the activity of TG lipases and monoglyceride hydrolases, but probably also reflects the specificity of the enzymes involved in the reacylation of TGs via the phosphatidic acid pathway. The very much smaller changes in the total fatty acid profile of the TG molecule implied selective reacylation at either the 1 and/or 3 positions. Within the intestine, where direct esterification of 2-monoglyceride is the preferred pathway for TG formation (6,24), the esterification of palmitate at the 1 and 3 positions probably exceeded that of other fatty acids in order to maintain the similarity of lard and chylomicron TG. Lymphatic rather than plasma chylomicrons would have provided a better comparison. The specificity of lipoprotein lipase for the 1 and 3 positions (21,22) suggests that some 2-monoglyceride might be available to the liver for TG synthesis, although this does not seem to be quantitatively impor-

tant, at least in the rat (25). The alternative pathway involves the acylation of glycerol-3-phosphate which appears to be the major initial specific reaction in hepatic glycero-lipid synthesis (26). This leads to the formation of phosphatidate, which, upon futher acylation, gives rise to diglyceride and finally to TG. The relatively small difference in the palmitate content of conventional lard (27%) to that in fasting plasma TG (24%), in the face of corresponding values of 68% and 14% for palmitate in the 2-position, indicates extensive and probably specific utilization of palmitate in the formation of TG in the liver. The preferential esterification of palmitate in the 1-position of phosphatidic acid and diglyceride has been demonstrated in rat liver (27), whereas linoleic acid is almost exclusively located at the 2-position of hepatic phosphatidic acid shortly after the injection of the radio-labeled fatty acid into the portal vein of rats (28). These findings in the rat were therefore in accord with our interpretation of the present studies with pork fat.

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Effect of Dietary Fat Supplementation on the Composition and Positional Distribution of Fatty Acids in Ruminant and Porcine Glycerides

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ABSTRACT

Dietary fats which were protected from ruminal metabolism were fed to ruminants, and the constituent fatty acids subsequently appeared in the glycerides of tissues and secretory products. These dietary fat induced alterations in tissue lipid composition were particularly apparent when the fat source was enriched with linoleic acid. Similarly, when pigs were fed linoleic-enriched fats, the linoleic acid was incorporated into the adipose tissue triglycerides. Stereospecific analyses were carried out on triglycerides from various tissues and secretory products obtained from animals fed control or linoleate-enriched diets. The analysis of adipose tissue triglycerides showed that linoleate and oleate were preferentially esterified to positions 2 and 3 (cattle and sheep), and positions 1 and 3 (pigs). Of the other major adipose tissue fatty acids, palmitate was preferentially esterified at position 1 (ruminants) and position 2 (pigs), and stearate was preferentially esterified at positions 1 and 3 (ruminants), and position 1 (pigs). Stereospecific analysis of high mol wt milk triglycerides showed that linoleate was either evenly distributed on all three positions (goats), or predominantly on position 3 (cows). Furthermore, the incorporation of this linoleate did not markedly alter the positional specificity of the other major milk triglyceride fatty acids. Of these fatty acids, the short and medium chain length acids (butyrate-laurate) were mainly on position 3, myristate and palmitate on positions 1 and 2, and stearate and oleate evenly distributed. Thoracic duct lymph triglycerides from sheep tended to show preferential incorporation of linoleate at position 3, palmitate at position 2, and stearate at position 1 and 3; oleate, on the other hand, tended to be evenly distributed on all three positions of the lymph triglyceride. The stereospecific arrangement of fatty acids in sheep liver triglycerides was similar to that of lymph triglycerides, and this may reflect the uptake of intact or partially hydrolysed chylomicron and/or very low density lipoprotein triglycerides by the liver. There were also some analogies in the stereospecific arrangement of fatty acids on ruminant lymph and milk triglycerides and this may reflect an incomplete hydrolysis of chylomicron and/or very low density lipoprotein triglycerides prior to uptake by the mammary gland. An unusual feature of lymph from sheep fed linoleate was the presence of phospholipids which contained large amounts of linoleate in ca. equal proportion at both positions 1 and 2 of the phospholipid molecule.

INTRODUCTION

The fatty acid composition of tissue triglycerides (TGs) from monogastric animal species is influenced by the composition of the dietary fat, and the component fatty acids within the tissue TGs of animal species generally show a characteristic positional distribution (1). This distribution is such that the saturated fatty acids are generally esterified to position 1 of the glyceride molecule, and the unsaturated fatty acids are generally esterified to position 2 and to a lesser extent to position 3. (The carbon atoms of the glycerol skeleton are numbered stereospecifically but for convenience the *Sn* prefix is omitted.) The pig is a notable exception in that the adipose tissue TGs from this species show the "reverse structure," i.e., the principal saturated fatty acid, palmitic acid (16:0), is located on position 2, while the unsaturated fatty acids are on positions 1 and 3 (1).

The concentrations of linoleic acid (18:2) in adipose tissue of monogastric species can be markedly increased by feeding this acid, and studies with mice and rats have shown that the 18:2 is preferentially esterified at position 2 of the TGs (2,3). These studies, however, were carried out using pancreatic lipase, and by this method alone it is not possible to differentiate between the fatty acids at positions 1 and 3 of

TABLE I

Fatty Acid Composition of Dietary Lipids

Oil or Lipid supplement	Fatty acids (% by wt)									
	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	Others[a]
Palm oil	-	1	1	43	-	4	41	10	-	-
Tallow	-	-	2	24	3	25	45	1	-	-
Safflower oil	-	-	-	7	-	2	14	77	-	-
Sunflower oil	-	-	-	6	-	3	31	60	-	-
Safflower seeds[b]	-	-	-	8	-	2	12	78	-	-
Sunflower seeds[c]	-	-	-	10	-	8	22	60	-	-
Protected sunflower seed supplements[d]	-	-	1	8	-	8	17	66	-	-
Control cows' milk[e]	3	4	11	26	4	15	26	5	3	3
18:2-enriched[e] cows' milk	3	3	7	18	2	13	25	21	1	7

[a]Principally short chain fatty acids.
[b]Safflower seeds (40% oil).
[c]Sunflower seeds (50% oil).
[d]Protected sunflower seed supplements (45% oil) were prepared by using procedures previously reported (8).
[e]Control and 18:2-enriched cows' milk both contained ca. 4.5% fat.

the glyceride molecule.

Other techniques have been developed whereby the fatty acids at the 1 and 3 positions may be separated and quantified (4,5). One of these techniques, Brockerhoff's stereospecific analysis (4), has been used in the present studies to evaluate the effects of dietary fat supplementation on the positional distribution of fatty acids in the tissue TGs of two classes of animal, ruminants and pigs.

Because of the microbial metabolism and hydrogenation of dietary fatty acids in the stomach of ruminants (6), it has been necessary with these animals to feed "protected" lipid supplements. These lipid supplements are effectively protected from ruminal metabolism, are digested and absorbed from the small intestine, and the constituent fatty acids subsequently appear in milk and tissue glycerides (7,8). Because of the particular role these protected lipid supplements may play in ruminant and human nutrition (8,9), we have considered it necessary to examine in some detail the effects of varying the nature of the protected dietary fatty acid on the fatty acid composition and positional distribution of fatty acids in a variety of ruminant organs, tissues, and secretory products.

MATERIALS AND METHODS

Lipid Supplements

The fatty acid compositions of the lipid supplements fed to ruminants or pigs are given in Table I. Other details pertaining to diets, type of animal, period of feeding, etc., are described along with the relevant results. The fat content of basal hay and grain diets was ca. 2-3% in all studies.

Collection of Samples and Extraction of Lipids

Lymph (20 ml) was collected from sheep by cannulation of the thoracic duct and cellular material was removed by centrifugation at 2000 x g for 10 min. Bile (5 ml) was obtained from the gall bladder of sheep, and adipose tissue and liver samples were removed from sheep, cattle, and pigs at slaughter. The lymph, bile, and tissues were extracted with chloroform:methanol (2:1, v/v) (10). Milk samples (100 ml) were obtained after morning milking of cows and goats, and lipids were extracted (11).

Phospholipids were removed from the total lipid extracts by shaking the lipid (400 mg) in chloroform (10 ml) for 10 min with silicic acid (500 mg). The silicic acid was removed by filtration in a sintered glass funnel and was washed with chloroform. The filtrate and

washings (containing the neutral lipids) were evaporated to dryness in vacuo. When necessary, the phospholipids were recovered from the silicic acid by elution with methanol. The purity of each preparation was checked by thin layer chromatography (TLC). The fatty acid composition of the samples was determined by gas liquid chromatography after methylation of the free fatty acid (12) except for milk TG, which was analyzed after preparation of butyl esters using the procedure of Parodi (13).

Stereospecific Analysis of Triglyceride

Tissue and lymph triglyceride: The intrapositional distribution of fatty acids on the TG from adipose tissue, liver, and lymph was determined using the Grignard reagent method described by Brockerhoff (4) as modified by Christie and Moore (14).

Milk triglyceride: It proved difficult to separate the resultant diglycerides (DGs) when milk TGs were directly reacted with the Grignard reagent (15). A modified procedure (16) was therefore used to isolate the higher mol wt TGs from milk and then to use this fraction for stereospecific analysis.

The milk TGs (150-300 mg) were partitioned into fractions of different mol wt by passing the samples through a glass column (300 x 30 mm ID) containing Sephadex LH 20 (Pharmacia Fine Chemicals, Inc., (Uppsala, Sweden) suspended in chloroform (16). The TGs were eluted with chloroform using a flow rate of 0.15-0.20 ml/min and the fatty acid composition was determined on portions of the eluate (13). On the basis of the fatty acid composition, the eluates were pooled to form three major fractions (fractions I, II, and III in order of decreasing mol wt) (Fig. 1). The TG from fraction I was subjected to stereospecific analysis (4,14). The TGs from fractions II and III were also subjected to the Grignard reagent, but the resultant 1,2 and 1,3 DGs were not resolved effectively by TLC. These lower mol wt TGs were subsequently treated with pancreatic lipase (17) to ascertain the distribution of fatty acids on position 2.

Lymph and biliary phospholipids: The positional distributions of fatty acids in the lymph and biliary phospholipids were determined after reaction with phospholipase A_2 (EC 3.1.1.4) (18).

RESULTS

RUMINANTS

Adipose Tissue Triglyceride

Fatty acid composition: The feeding to

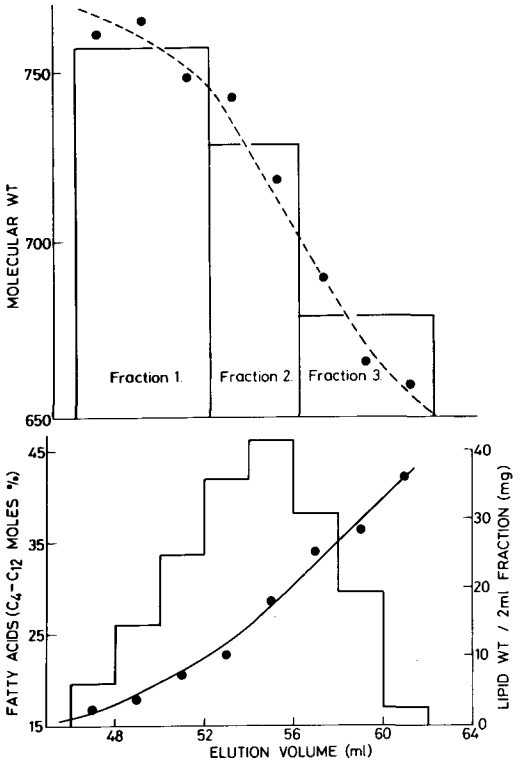

FIG. 1. Sephadex LH-20 separation of goats' milk triglycerides into three arbitrary mol wt fractions. The lower graph shows the relationship between elution volume, wt of lipid (histogram), and the proportion of C_4-C_{12} fatty acids (•—•). The upper graph shows the relationship between elution volume, arbitrary fractions (histogram) and mol wt of triglycerides (•- - -•). Chloroform was the eluting solvent; other details of the method are given in the text.

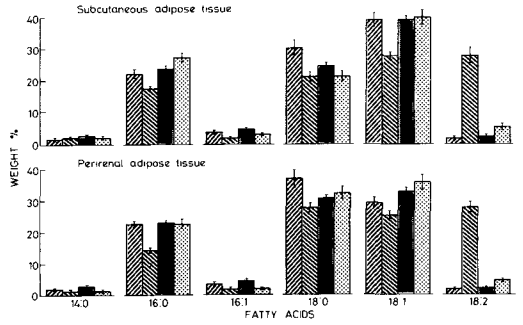

FIG. 2. Effect of feeding protected fats on the fatty acid composition of triglycerides from adipose tissues of lambs. ▨ Control, ▧ safflower oil, ▩ tallow, ⬚ palm oil. The lambs (8-10 weeks of age) were fed either 800 g per day chopped lucerne and crushed oats (1:1) (control diet) or other diets containing lucerne (350 g), oats (140 g), and formaldehyde treated oil: casein (2:1, w/w) supplements (210 g) (7). Lambs were slaughtered after 8 weeks and tissues were immediately transferred to chloroform: methanol (2:1, v/v). Means and standard errors for four lambs.

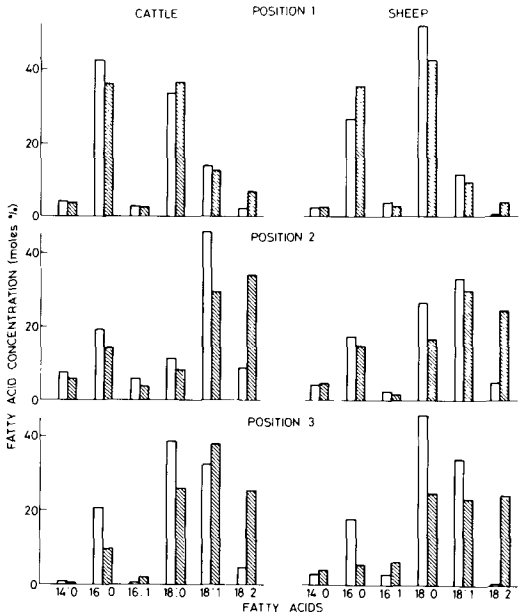

FIG. 3. Intrapositional distribution of major fatty acids in the triglycerides from perirenal adipose tissue from cattle and sheep which had received unsupplemented (□) and 18:2 supplemented (▨) diets. The protected sunflower seed supplements (8) were fed to mature sheep and cattle for 8-10 weeks prior to slaughter. The basal diets contained lucerne and oats (1:1, w/w), and supplemented diets contained 30% by wt of lipid supplement. Each value represents a single animal.

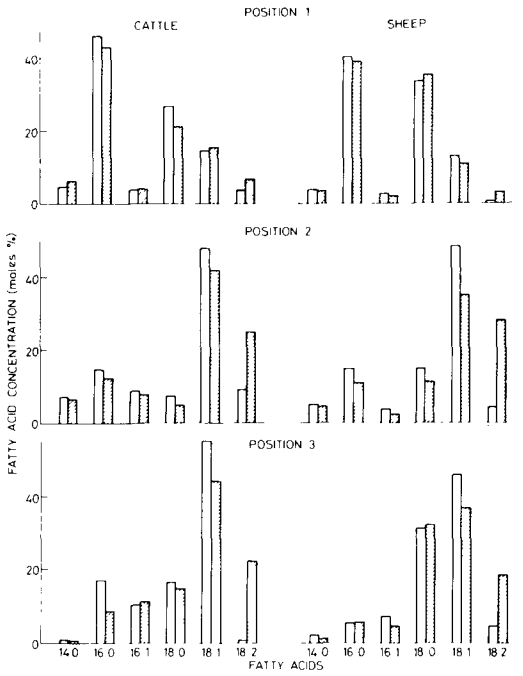

FIG. 4. Intrapositional distribution of major fatty acids in the triglycerides from subcutaneous adipose tissue from cattle and sheep which had received unsupplemented (□) and 18:2 supplemented (▨) diets. The protected sunflower seed supplements (8) were fed to mature sheep and cattle for 8-10 weeks prior to slaughter. The basal diets contained lucerne and oats (1:1, w/w), and supplemented diets contained 30% by wt of lipid supplement. Each value represents a single animal.

lambs of protected safflower oil containing large proportions of 18:2 led to a clear increase in the proportion of 18:2 in the subcutaneous and perirenal adipose tissue TGs (Fig. 2). This was associated with a decline in the proportions of 16:0, 18:0, and 18:1. The feeding of protected tallow or palm oil caused lesser alterations in the fatty acid composition of adipose tissue TG (Fig. 2). The proportion of 18:2 was increased on feeding the protected palm oil, and there were also increases in the proportion of 16:0 (subcutaneous) and 18:1 (perirenal) on this diet. In addition, the feeding of protected palm oil and tallow caused reductions in the proportion of 18:0 in the fat from both tissue sites (Fig. 2).

Positional distribution of fatty acids: Figure 3 shows the positional distribution of fatty acids in the perirenal adipose tissue TG from mature sheep and cattle fed conventional diets or similar diets supplemented with protected 18:2. Figure 4 shows corresponding results for the subcutaneous adipose tissue TG. The 18:2 which was protected against ruminal hydrogenation was incorporated predominantly at positions 2 and 3 of the TGs from both adipose tissue sites. Furthermore, this incorporation of 18:2 did not consistently alter the interpositional specificity of the other major fatty acids, with the possible exception of 16:0, which tended to be replaced more effectively by 18:2 on position 3 than on position 2, particularly in perirenal tissue (Fig. 3). Examination of the positional distribution of fatty acids in perirenal adipose tissue from sheep fed protected palm oil revealed that the interpositional specificity of the major fatty acids was not altered, with the possible exception of position 1, where 16:0 was slightly enhanced and there was a decrease in the proportion of 18:0 (S.C. Mills, unpublished study, 1974).

Milk Triglyceride

Fatty acid composition: Table II shows the effect of feeding protected 18:2 on the fatty acid composition of the three TG fractions separated from cows' milk by Sephadex LH-20 chromatography. The protected 18:2 was found in all three fractions, but the greatest amounts were present in the highest mol wt

TABLE II

Fatty Acid Composition (in Moles %) of Milk Triglyceride Fractions from Cows Fed Control Diets or Similar Diets Supplemented with Protected 18:2

Fatty acid	Control[a]				18:2 supplemented[a]			
	Original triglyceride	Fractions[b] I	II	III	Original triglyceride	Fractions[b] I	II	III
4:0	8.1	7.2	8.0	8.4	5.3	8.4	10.3	13.4
6:0	4.2	2.0	5.1	4.5	5.1	3.1	3.1	5.0
8:0	1.7	0.7	1.9	2.8	2.4	1.2	3.5	4.1
10:0	2.9	1.5	3.0	3.8	4.3	2.8	5.1	5.5
12:0	2.7	1.8	2.6	3.4	3.7	2.7	4.0	4.1
14:0	7.9	6.0	7.9	8.9	8.3	6.8	8.7	8.3
16:0	20.3	18.3	20.3	20.4	15.9	14.7	15.8	14.8
16:1	3.0	3.4	2.7	2.8	1.5	1.6	1.0	1.3
18:0	13.9	15.8	13.4	11.6	12.3	14.2	11.3	10.0
18:1	26.1	29.9	25.2	21.4	19.1	21.8	17.6	15.6
18:2	5.0	5.1	5.9	2.9	18.1	18.6	16.6	13.4
18:3	1.2	2.2	0.9	0.5	1.5	1.6	0.8	0:4

[a]The control and basal diets contained lucerne and oats (1:1, w/w). The formaldehyde treated supplement, safflower oil:casein (2:1, w/w) (7), was fed at 800 g per day.

[b]Fractions I, II, and III were isolated by gel filtration of milk triglyceride on Sephadex LH-20 using procedures described in the text.

fraction (fraction I). This fraction also contained the greatest concentrations of the other long chain fatty acids, e.g., 18:0 and 18:1.

Positional distribution of fatty acids: Figure 5 shows the positional distribution of fatty acids in the high mol wt fraction (fraction I) of milk TG from cows fed conventional diets or diets supplemented with protected 18:2. The characteristic features of these milk TGs are (a) a preferential esterification of short and medium chain length fatty acids (C_4-C_{12}) at position 3: (b) a preferential esterification of 16:0 and 14:0 at positions 1 and 2; (c) ca. random esterification of 18:0 and 18:1, i.e., these acids are located in ca. equal proportions on all three positions; and (d) the protected 18:2 incorporated at all three positions, with the highest proportion at position 3.

Table III shows the positional distribution of fatty acids in the high mol wt fraction (fraction I) of milk TG from goats fed conventional diets or diets supplemented with protected 18:2. The positional specificity of the shorter chain acids (8:0 and 10:0), 16:0, 18:0, and 18:1 was similar to that in cows' milk TGs (Fig. 5), but the 18:2 was distributed more evenly in the goat TGs.

The lower mol wt TG fractions from cows' milk (fractions II and III) were reacted with pancreatic lipase. The reaction products were isolated and their fatty acid compositions determined (Table IV). Although in this case it is not possible to distinguish between the fatty acids on position 1 and those on position 3 of the glyceride molecule, it is possible, by comparing the concentrations of particular fatty acids at the respective 2 and 1(3) positions, to conclude that these lower mol wt TGs probably have a positional distribution of fatty acids which is similar to that of the high mol wt fraction (Fig. 5). This is indicated by (a) the greater concentrations of the shorter chain length acids (4:0 - 8:0) at the 1(3) position, (b) the greater concentration of 16:0 at the 2 position, and (c) the ca. equal proportions of 18:2 at the 2 and the 1(3) positions.

Lymph Triglyceride

Figure 6 shows the positional distribution of fatty acids in TG from the thoracic lymph duct of sheep fed control diets or similar diets supplemented with protected 18:2. A feature of these results is the pronounced interpositional specificity of 16:0; this fatty acid was located predominantly on position 2. Similar results were obtained by Garton and Duncan (19) using pancreatic lipase to isolate the 2-monoglycerides. Of the other principal fatty acids, 18:0 was preferentially esterified to positions 1 and 3, 18:1 was located on all three positions in ca. equal proportions, and 18:2 was preferred at position 3.

The incorporation of protected 18:2 into the lymph TG caused slight alterations to the interpositional locations of the other major fatty acids, i.e., 18:2 substituted for 18:0 on position 3, 16:0 on positions 1 and 2, and 18:1 on positions 1 and 2 (Fig. 6).

Lymph and Biliary Phospholipids

Figure 7 shows the positional distribution of

TABLE III

Intrapositional Distribution (in Moles %) of Fatty Acids in the High Mol Wt Triglycerides from Goats' Milk[a]

Fatty acids	Control			Supplemented		
	Position			Position		
	1	2	3	1	2	3
8:0	1.1	0.2	8.0	2.0	0.9	7.3
10:0	3.5	11.8	14.3	10.4	3.4	14.7
12:0	3.1	6.1	4.3	7.3	3.5	3.9
14:0	5.7	12.8	6.1	16.3	6.8	4.2
16:0	31.0	26.5	5.7	17.4	24.4	10.1
18:0	9.7	6.5	9.2	6.5	13.2	6.1
18:1	18.8	18.0	27.8	15.9	23.0	21.7
18:2	0.7	2.1	4.9	18.9	15.9	13.5

[a]Goats were fed diets of lucerne and oats or similar diets supplemented with formaldehyde-treated safflower oil/casein (7). High mol wt triglyceride fraction I was prepared by gel filtration as described in the text.

TABLE IV

Composition (in Moles %) of Fatty Acids at Position 2 and at Position 1(3) of Triglycerides from Control and 18:2-Enriched Bovine Milk[a]

Fatty acid	Fraction II[b]				Fraction III[b]			
	Control		18:2-enriched		Control		18:2-enriched	
	Position[c]							
	2	1(3)	2	1(3)	2	1(3)	2	1(3)
4:0	3.2	10.4	6.1	13.7	0.9	12.5	2.9	17.7
6:0	2.4	6.4	5.7	1.8	3.1	5.5	2.0	7.5
8:0	1.4	2.2	2.4	3.9	1.4	3.7	2.8	4.6
10:0	2.8	3.1	5.3	4.9	4.9	3.6	5.5	5.6
12:0	3.0	2.4	5.3	3.4	4.6	3.1	6.0	3.3
14:0	8.5	7.6	9.4	8.1	11.3	8.4	11.4	6.9
16:0	24.4	18.3	16.7	15.0	26.8	19.0	19.1	12.9
16:1	4.6	1.8	2.2	-	4.1	2.3	2.4	0.9
18:0	12.7	13.8	9.7	11.7	11.4	12.5	10.5	9.8
18:1	24.5	25.6	15.6	18.1	23.9	21.9	16.7	15.0
18:2	4.6	6.6	15.4	16.7	3.7	2.8	14.8	12.8

[a]The control and basal diets contained lucerne and oats (1:1; w/w). The formaldehyde-treated supplement, safflower oil:casein (2:1, w/w) (7), was fed at 800 g per day.

[b]Fractions II and III were prepared by gel fractionation of milk triglycerides on Sephadex LH-20 as described in the text.

[c]The 2-monoglycerides and free fatty acids were isolated by thin layer chromatography after pancreatic lipase hydrolysis of respective fractions (17). This procedure does not allow the separate isolation of fatty acids on positions 1 and 3, i.e., 1(3).

fatty acids in the biliary and lymph phospholipids from sheep fed conventional diets or similar diets supplemented with protected 18:2. The biliary phospholipids from lambs fed conventional diets showed a positional distribution of fatty acids which is characteristic for most phospholipids (1), i.e., the proportion of saturated fatty acids (16:0 and 18:0) was highest on position 1 and that of the unsaturated fatty acids (18:1 and 18:2) was highest on position 2 (Fig. 7). This marked degree of positional specificity was also evident for the biliary phospholipids obtained from lambs fed protected 18:2 (Fig. 7). The proportion of 18:2 in the biliary phospholipids was considerably enhanced by feeding the protected lipid supplement, and this fatty acid was predominantly located at position 2, mainly at the expense of 18:1.

The positional specificity of fatty acids in the lymph phospholipids was different from that in the bile. 16:0 and 18:1 were distributed equally between positions 1 and 2. 18:0 showed a slight preference for position 1 in the control situation, but this was reversed by feeding the protected 18:2. 18:2 itself showed a

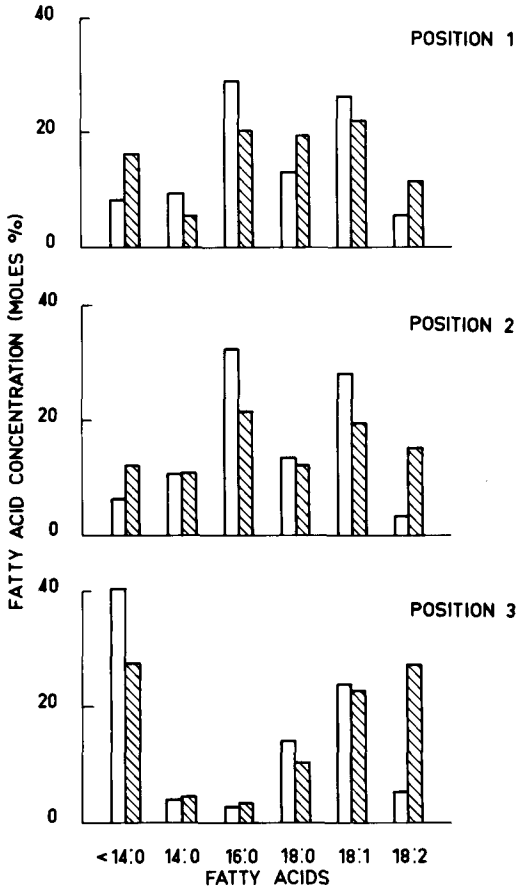

FIG. 5. Intrapositional distribution of fatty acids in high mol wt triglycerides from cows' milk. Cows were receiving unsupplemented (□) or 18:1 supplemented (▨) diets. The 18:2 supplement was formaldehyde treated safflower oil: casein (2:1, w/w) (7) and was fed at 800 g per day; the balance of the ration was lucerne and oats (1:1, w/w). The unsupplemented diet consisted solely of lucerne and oats (1:1, w/w).

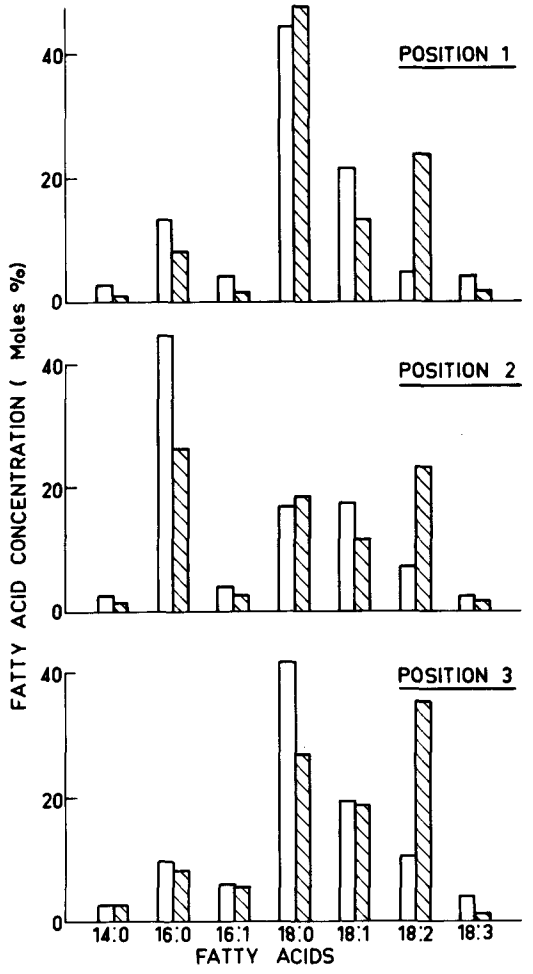

FIG. 6. Intrapositional distribution of fatty acids in thoracic lymph triglycerides from a 1 year old sheep which had received an unsupplemented (□) diet and a similar sheep fed an 18:2 supplemented (▨) diet. The control and basal diets were lucerne and oats (1:1, w/w). The lipid supplement of 200 g per day formaldehyde-treated safflower oil:casein (2:1, w/w), was fed for 3 weeks prior to thoracic duct cannulation. Cannulation and collection of lymph was carried out under general anaesthesia at ca. 12 hr post feeding.

preference for the 2 position in the lymph phospholipids from sheep fed control diets, but this also tended to be reversed by feeding the protected 18:2 supplements (Fig. 7).

Liver Triglyceride

The positional distribution of fatty acids in TG from the livers of sheep fed conventional diets or similar diets supplemented with protected 18:2 is shown in Figure 8. Octadecenoic acid (18:1) showed a preference for positions 2 and 3 in the control sheep, but no marked positional specificity at all for the supplemented animals. Myristic and palmitic acids were located preferentially on positions 1 and 2, while 18:0 was preferentially on positions 1 and 3. The location of 18:2 was largely restricted to positions 2 and 3 for the lipid supplemented animal, and the proportions of all other fatty acids, except 14:0 and 18:3, were reduced at these sites.

PIGS

Pig Adipose Tissue Triglyceride

Fatty acid composition: Pigs were fed 18:2 in the form of (a) crushed safflower seeds, (b) crushed sunflower seeds, (c) formaldehyde treated homogenized sunflower seed based sup-

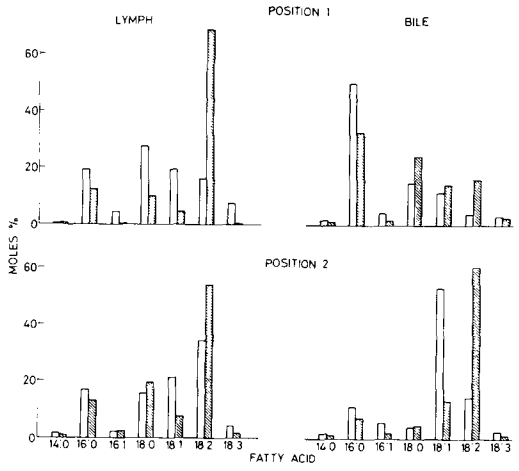

FIG. 7. Intrapositional distribution of fatty acids in biliary and lymph phospholipids from sheep which had received unsupplemented (☐) or 18:2 supplemented (▨) diets. The biliary phospholipids were obtained from young lambs, as in Figure 2, whereas the lymph phospholipids were obtained from older sheep as in Figure 6.

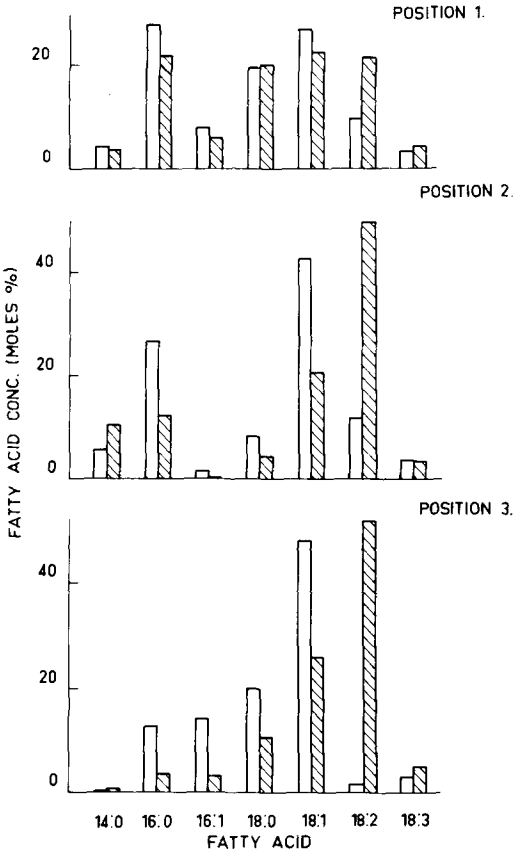

FIG. 8. Intrapositional distribution of fatty acids on liver triglycerides isolated from sheep receiving unsupplemented (☐) and 18:2 supplemented (▨) diets. The protected sunflower seed supplements (8) were fed to mature sheep for 8-10 weeks prior to slaughter. The basal diets contained lucerne and oats (1:1, w/w), and supplemented diets contained 30% by wt of lipid supplement. Each value represents a single animal.

plements prepared for ruminant feeding (8), or (d) 18:2-enriched cows' milk.

Figure 9 shows the fatty acid composition of subcutaneous adipose tissue TG from these pigs and from other pigs fed control diets. Feeding the oilseeds, oilseed supplements or 18:2-enriched cows' milk caused substantial increases in the proportion of 18:2 and reductions in the proportions of 16:0 and 18:1. The proportion of 18:2 in the tissues from pigs fed 18:2-enriched cows' milk was similar to that in the milk diet (Table I).

Positional distribution of fatty acids: Stereospecific analyses of perirenal adipose tissue TGs from pigs fed 18:2-enriched cows' milk showed that the 18:2 was incorporated specifically onto positions 1 and 3, and there were no marked effects of this incorporation on the interpositional specificity of the other major fatty acids, with the possible exception of 18:1 in position 3 (Fig. 10). The positional specificity, however, was markedly different from that in ruminant adipose tissues (Figs. 3 and 4), particularly because position 2 of pig TG was occupied predominatly by 16:0. Stearic acid (18:0) was mainly on position 1, and the other major fatty acid (18:1) was distributed with a preference similar to 18:2, i.e., on positions 1 and 3. Myristic acid (14:0), like 16:0, was placed mainly on position 2.

DISCUSSION

The tissue fatty acid composition of ruminants can be altered by feeding fats which are protected from ruminal hydrogenation (7,8,20). The feeding of such fats containing polyenoic fatty acids, e.g., 18:2, will result in elevated concentrations of the respective fatty acids in the lipids of various organs and tissues (7,8,20) (Fig. 2, Table II). This incorporation of protected polyenoic fatty acids into ruminant tissue lipids is equivalent to that observed when polyenoic fats are fed to monogastric species (Fig. 9).

The feeding of protected nonpolyenoic fats, containing saturated or monoenoic fatty acids, to ruminants also increases the concentration of the major component fatty acids in the respective tissue lipids (Fig. 2). The extent of this alteration is, however, less than that observed when protected polyenoic fats are fed, and this

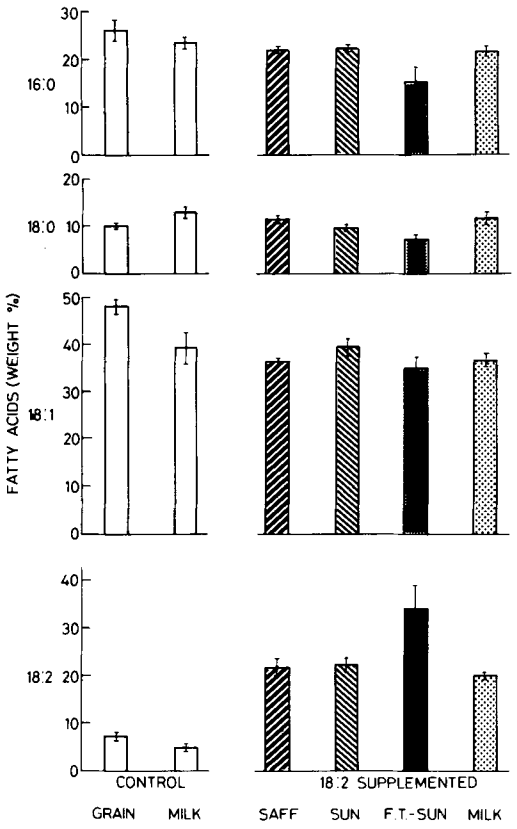

FIG. 9. Effect of feeding different dietary fats on the fatty acid composition of pig subcutaneous adipose tissue. Values are the mean ± standard error for three animals. Saff = safflower seeds, Sun = sunflower seeds, F-T Sun = formaldehyde-treated sunflower seed supplements (8). 18:2 – enriched cows' milk was obtained by feeding F-T Sun to lactating cows. The milk diets were fed as the sole diet for periods of 3-12 months prior to slaughter. The basal grain diet contained sorghum, barley and meat meal. This diet was supplemented with oilseeds or oilseed supplements (ca. 30% by wt of diet) and fed ad libitum for 12 weeks prior to slaughter.

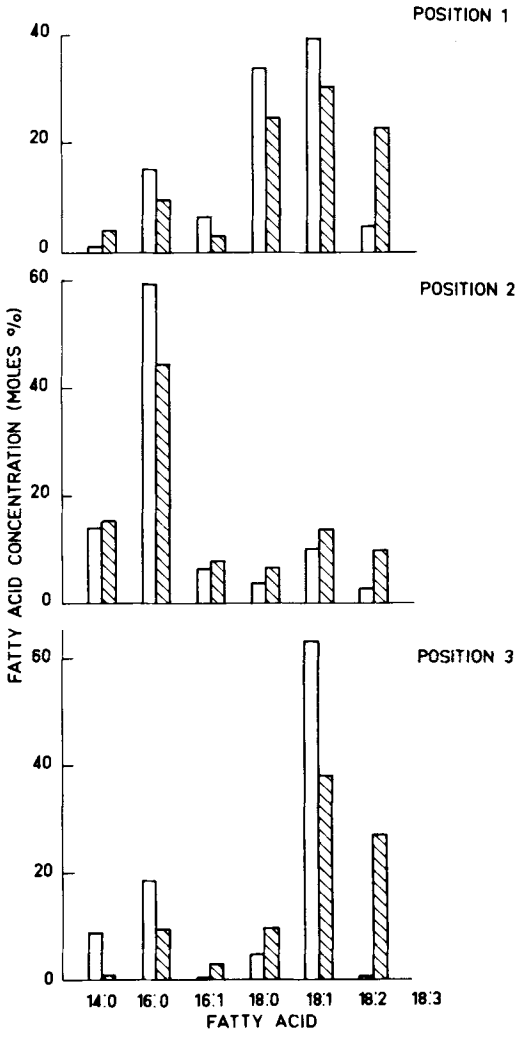

FIG. 10. Intra positional distribution of fatty acids in the perirenal adipose tissue of pigs which had received conventional cows' milk (□) or 18:2-enriched cows' milk (▨). The milk diets, as in Figure 9 were fed for 3 months prior to slaughter. The pigs were introduced to the milk diets at 2-4 weeks of age.

is probably due to the metabolic transformation of the component acids, e.g., 16:0 and 18:0, prior to their acylation to form TG (21,22). It is also possible that these non-polyenoic fatty acids do not suppress the normal endogenous synthesis of tissue fatty acids to the same extent as do the polyenoic fatty acids (23). This latter effect may also partially explain the reduction in the proportions of 16:0, 18:0, and 18:1 as a consequence of 18:2 incorporation into adipose tissue TGs of ruminants (Fig. 2) and pigs (Fig. 9).

The incorporated 18:2 was specifically located on positions 2 and 3 of ruminant adipose tissue TG (Figs. 3 and 4) and on positions 1 and 3 of porcine adipose tissue TG (Fig. 10). This distribution of 18:2 confirms the established observations pertaining to the disposition of unsaturated fatty acids in the respective TGs and highlights the important differences that exist between pigs and other species (1).

The feeding of protected linoleic acid not only enhances the proportion of 18:2 in ruminant adipose tissue TGs, but also increases the proportion of this acid in milk TGs from these species (7,8,20) (Table II). Stereospecific analyses of a high mol wt milk TG fraction showed that this 18:2 was present in ca. equal

proportions for all three positions of the goats' milk TGs (Table III), but tended to be preferentially located on position 3 of the cows' milk TGs (Fig. 5).

There were also positional specificities for the other component fatty acids of the high mol wt milk TGs, and these specificities are in general agreement with previously published analyses carried out on milk TGs from cows, sheep, goats, and humans (15,24-26). The shorter chain length acids ($< C_{14}$) were predominantly on position 3; 16:0 was predominantly on positions 1 and 2; and 18:1 was rather evenly distributed. The lack of any marked preference for 18:0 on position 1 is in contrast to the previous observations (15,24-26). We also did not show any marked preference of 14:0 for the 2 position of milk TGs (26). These different observations may be due to the different methods of isolating high mol wt TG fractions, i.e., Sephadex LH-20 versus silicic acid chromatography (26).

Ruminant lymphatic TGs show a marked specificity for 16:0 at position 2 (19) (Fig. 6); and this possibly results from the competition between 18:0 and 16:0 for acylation at positions 1 and/or 3 (27). Considerable amounts of 18:0 are produced by ruminal hydrogenation of the conventional dietary lipids, and this fatty acid is presented to the small intestine in the nonesterified form (28). In addition, 16:0 from basal dietary components would also exist as nonesterified fatty acid in the intestinal lumen (28). On the other hand, protected dietary TGs are not hydrolyzed during passage through the rumen (20). Thus, the feeding of protected lipid supplement would result in an intestinal mixture of nonesterified fatty acids, derived from the basal diet, and intact TGs, derived from the supplement.

The lack of any specific location of 18:2 at position 2 of lymphatic TGs from sheep fed protected safflower oil (Fig. 6) is in contrast to the specific location of 18:2 at this position in safflower oil and other linoleic enriched seed oils (1,29). This nonretention of the dietary 2-monoglyceride structure is not consistent with results obtained in nonruminants (30,31) and may suggest that, in ruminants, the protected dietary TGs are more extensively hydrolyzed prior to absorption, or that intestinal monoglyceride hydrolases (32,33) are more active than in nonruminants. Any such conclusion, however, must be considered cautiously in view of the presence in the ruminant intestine of large amounts of nonesterified fatty acids derived from ruminal hydrolysis and hydrogenation of basal dietary lipids.

An analogy exists between the stereospecific arrangement of fatty acids on TG from ruminant milk (Fig. 5, Table III) and lymph (Fig. 6) (15,19,25,26). TGs from both secretory products contain relatively large proportions of the 16:0 located at position 2. This analogy may reflect the importance of lymph TGs as a source of milk TGs (34-36). The lymph TG enters the circulatory system as chylomicrons or very low density lipoproteins (VLDL), and the TG may be partially hydrolyzed by mammary gland lipoprotein lipase (37,38). The resultant 2-monoglycerides, containing relatively large proportions of 16:0, could then serve as precursors for mammary gland TG biosynthesis, a pathway similar to that present in the intestine (39,40). Such a pathway in the mammary gland has been previously considered (36,41), but conclusive evidence is not yet available to fully support this idea (35,42).

Adipose tissue lipoprotein lipase (43), like mammary gland lipoprotein lipase (37,38), also has been shown to specifically hydrolyze the 1 and 3 positions of TGs. Accordingly, since chylomicrons and/or VLDL provide a source of fatty acids for adipose tissue TG biosynthesis (44), one might also expect to find a specific location of 16:0 at the 2 position of these TGs. That this is not the case for ruminants could suggest a rearrangement of fatty acids on the TG molecule (45), possibly involving adipose tissue monoglyceride hydrolase (32,33).

That the stereospecificity of ruminant liver TGs (Fig. 8) does bear some relationship to that of ruminant lymph TGs (Fig. 6) may be due to hepatic uptake of intact chylomicron and/or VLDL triglycerides (44,46).

TGs from tissues and secretory products other than ruminant liver, milk, and lymph also show a preferential location of 16:0 at position 2, e.g., TGs from human milk (24), pig milk (47,48), and pig adipose tissue (Fig. 10) (1,4,5). Whether these observations can also be related to a specific location of 16:0 at position 2 of the respective chylomicron of VLDL TGs awaits further investigation. These investigations must be carried out with due regard to the diet and nutritional status of the animal (24,47,49,50). In addition, the question of the stereospecificity of chylomicron TGs is only satisfactorily resolved by analyzing lymphatic chylomicrons and not serum chylomicrons, owing to the rapid stereospecific rearrangements that take place during lipoprotein transport and metabolism (see accompanying paper by Nestel et al.).

With most tissue phospholipids, as with most tissue TGs, there is a characteristic positional distribution of fatty acids. This distribution is

such that the unsaturated fatty acids are generally located at position 2 and the saturated fatty acids at position 1 (1).

The lymph phospholipids from ruminants fed control diets tend to show this specific positional location for 18:0 and 18:2 (Fig. 7), but this specificity was not apparent for 16:0 and 18:1. The feeding of protected 18:2 resulted in a substantial elevation in the 18:2 content of lymph phospholipids, but this 18:2 tended to be evenly distributed between positions 1 and 2 of the phospholipids (Fig. 7). (The concentration of 18:2 at position 1 was actually greater than at position 2.)

Biliary phospholipids from sheep fed protected 18:2 also showed an enhanced concentration of this acid, but the 18:2 was specifically located at position 2 (Fig. 7). This would indicate that biliary phospholipids are not the direct precursor of the linoleate-enriched lymphatic phospholipids. As a corollary, these linoleate-enriched lymphatic phospholipids are probably synthesized de novo by the intestinal cells in response to the enhanced absorption of TG.

Feeding linoleate-enriched oils to rats results in the appearance of a dilinoleyl phosphatidylcholine in lymph chylomicrons (51). Furthermore, it has been shown that this phospholipid is synthesized de novo and is not derived from biliary phospholipid (51).

We did not attempt to separate the individual lymph phosphoglycerides from sheep fed protected 18:2, but the high concentration of 18:2 at position 1 (69%) and at position 2 (54%) would suggest the presence of considerable amounts of dilinoleyl phosphoglyceride, presumably synthesized de novo by the intestinal mucosa as in rats fed safflower oil (51).

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Turnover of Fatty Acids in Rat Liver Cardiolipin: Comparison with Other Mitochondrial Phospholipids

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ABSTRACT

Following intraperitoneal administration of 1-^{14}C-linoleic acid or 2-^{3}H-acetate to rats, the specific radioactivities of both liver cardiolipin and other mitochondrial phospholipids after different time intervals were measured. Comparison of the data obtained with those from another stock of rats treated with ^{32}P-phosphate or 2-^{3}H-glycerol showed that the fatty acids of cardiolipin, like those of other phospholipids, exhibit an independent turnover with respect to the remaining parts of the molecule. The half-life of acyl moieties of cardiolipin is ca. 20% higher than that of the same components of other mitochondrial phospholipids. Moreover, it appears that, in both cardiolipin and other phospholipids, linoleyl residues turn over faster than nonessential fatty acids. Discussion is made as to whether this characteristic can be related to the role of phospholipids in the functioning of some enzymes bound to the inner mitochondrial membrane.

INTRODUCTION

In mammalian tissues, cardiolipin (diphosphatidylglycerol) occurs exclusively in mitochondria (1). This compound, contrary to other phospholipids which are supplied by microsomes (2-4), is synthesized by mitochondrial enzymes (5,6). Recently, Stanacev et al. (7) and Hostetler et al. (8) have pointed out that the main mechanism for the formation of cardiolipin in rat liver is different from the one operating in bacteria (9,10).

The finding that cardiolipin is tightly bound to cytochrome oxidase suggests that this phospholipid could be an important structural component of the respiratory chain (11,12). As a marker of mitochondrial structure, the concentration of this compound, as well as its turnover, has been followed in order to study both the biogenesis of mitochondria and the development of their inner membrane to see whether or not these particles turn over as a unit (13,14).

It has been demonstrated that, within a given membrane fraction, individual phospholipid classes are degraded at different rates (15). In addition, several reports have indicated that, in rat liver, single molecular species of phospholipids have different turnover rates and, moreover, that each portion of various phospholipid molecules exhibits a different behavior (16-19).

Little or no information concerning cardiolipin has been accumulated on the above characteristics found in other phospholipids. The present study was undertaken to evaluate the turnover of acyl moieties of this compound in comparison with that of its other molecular components. The results reported in this paper indicate that the fatty acids of cardiolipin, like those of other phospholipids, turn over faster than the remaining parts of the molecule. Discussion is made as to whether this characteristic can be related to the specific role of cardiolipin in the maintenance of normal functioning in some enzymes of the inner mitochondrial membrane. A preliminary account of this work has been given elsewhere (20).

EXPERIMENTAL PROCEDURES

Materials

1-^{14}C-Linoleic acid, sodium 2-^{14}C-acetate, sodium ^{3}H-acetate, 2-^{3}H-glycerol, 2-^{14}C-glycerol, and ^{32}P-potassium phosphate were purchased from New England Nuclear Corp. (Boston, MA). Standard fatty acid methyl esters were supplied by Applied Science Laboratories, Inc. (State College, PA). Silicic acid (minus 325 mesh, lipid chromatography grade) and reference lipids for thin layer and column chromatography were from Sigma Chemical Co. (St. Louis, MO). Silica Gels H and G were obtained from Merck (Darmstadt, West Germany). The other products used were reagent grade.

Animals and Diets

Adult male Wistar rats weighing 200-250 g were used in these experiments. The animals received a standard laboratory diet and were allowed continuous access to food and water until killed. When the turnover of different components of both cardiolipin and other phos-

pholipids was determined by a single isotope administration method, each rat was injected intraperitoneally with 0.2 ml of polyethylene glycol 400 containing 12.5 mM 1-^{14}C-linoleic acid (sp act, 0.4 μCi/μM) or with 0.4 ml of sodium ^{3}H-acetate (containing 0.4 μmoles and 80 μCi) dissolved in 0.9% (w/v) NaCl. The scheme for intraperitoneal administration of labeled glycerol or ^{32}P was similar to that described for acetate, except that 0.2 ml of 10 mM 2-^{3}H-glycerol (sp act, 200 μCi/μM) or 0.3 ml of 2.5 mM dipotassium ^{32}P-phosphate (sp act, 1 μCi/μM) were used. Pairs of animals were killed at the indicated time intervals after injection.

When the rats received a double isotope administration, they were injected intraperitoneally with 0.1 ml sodium 2-^{14}C-acetate (0.68 μmoles and 40.8 μCi) and then 5.5 days later with 0.2 ml sodium 2-^{3}H-acetate (0.2 μmoles and 40.0 μCi). The animals were killed 1.5 days after receiving the ^{3}H label. For studying the relative turnover of other phospholipids, the same amount of tritium labeled acetate was injected 3.5 days later and the animals killed 18 hr after receiving the ^{3}H label. In this type of experiment, other rats were injected intraperitoneally with 0.2 ml 2-^{14}C-glycerol (2 μmoles and 20 μCi) and then 6.5 days later with 0.2 ml 2-^{3}H-glycerol (0.2 μmoles and 40 μCi). The animals were killed 1.5 days after receiving the ^{3}H label. When the relative turnover of other phospholipids was studied, the same amount of tritium labeled glycerol was injected 5.0 days later; the animals were then killed 12 hr after receiving the ^{3}H label. Both ^{14}C and ^{3}H-labeled glycerol were dissolved in 0.9% NaCl.

Homogenate Preparation and Mitochondria Isolation

After the animals were sacrificed by decapitation, the pooled livers from two rats were immediately removed, chilled in ice cold 0.25 M sucrose, cut into small pieces, washed and homogenized in a glass-teflon homogenizer. Mitochondria were then isolated as described elsewhere (21).

Analytical Methods

Total lipids were extracted from these particles by the procedure of Bligh and Dyer (22). Phospholipids were then separated from neutral lipids by precipitation with acetone in the presence of $MgCl_2$ (23). Cardiolipin was separated from other phospholipids by silicic acid column chromatography. A glass tube (34 cm long x 2 cm internal diameter) containing 50 g silicic acid was used. After washing the column with chloroform:methanol (9:1,v/v), 50 to 60 mg total phospholipids as solution were applied and chromatography was performed with the same solvent system. The first fraction of 50 ml was discarded while the second fraction of 250 ml, containing almost all the cardiolipin, was utilized. The identification and purity of cardiolipin in this fraction was checked by thin layer chromatography on Silica Gel H slurried with 1 mM Na_2CO_3 solution (developing system, chloroform:methanol: acetic acid, 65:25:8, v/v/v). Lipid phosphorus analysis was assayed by the Nakamura method (24). The results reported in Figures 1-3 concerning other mitochondrial phospholipids were calculated after subtracting from the total phospholipid extract the data relative to cardiolipin following its separation by column chromatography. The methyl esters of cardiolipin fatty acids were obtained by the method of Stoffel et al. (25), then fractionated by a two-step development on Silica Gel H thin layer chromatoplates containing 5% $AgNO_3$. In the first step, the solvent n-hexane:diethyl ether (95:5, v/v) was used; in the second, chloroform: ethanol (99:1, v/v). The methyl esters were visualized by charring with 15% H_2SO_4. Identification was made by reference to standard run simultaneously. The area corresponding to each compound was scraped off and radioactivity counted after suspension in 15 ml scintillation solution.

Radioactivity Measurements

Following solvent evaporation under a N_2 stream, radioactivity was counted in a Packard spectrometer model 3380 after the addition of 15 ml of a scintillation mixture consisting of 0.5% 2,5-diphenyloaxazole and 0.03% 1,4-bis(4-methyl-5-phenyloaxazolyl)-benzene in toluene. The distribution of radioactivity among products of cardiolipin deacylation was assayed after saponification and fatty acid extraction (21).

RESULTS

Figure 1A reports the specific radioactivity of liver cardiolipin and other mitochondrial phospholipids at various time intervals after intraperitoneal injection of 1-^{14}C-linoleic acid to rats. From the rate of decline in the specific radioactivity of cardiolipin after one day, it can be calculated by graphical extrapolation that the half-life of linoleyl moieties of this compound in liver is equal to 2.7 days. From this figure it also appears that, by using the same labeled precursor, other hepatic mitochondrial phospholipids exhibit a different behavior, with their specific radioactivity showing a fall 12 hr

after administration. For the essential fatty acid components of these latter compounds, an average half-life of ca. 2.2 days can be calculated, a value slightly less than that found for cardiolipin. It must be specified that for studying the turnover of cardiolipin acyl groups, linoleic acid was chosen because, in liver, it constitutes more than 60% of the fatty acids present in this compound (26).

The decay in the radioactivity of nonessential fatty acids esterified into liver cardiolipin and other mitochondrial phospholipids was followed by intraperitoneal injection with sodium ^{3}H-acetate. The rats were killed after the interval periods indicated in Figure 1B, from which it is readily evident that the specific radioactivity increases up to 12 hr after administration of the labeled precursor and then decreases both in cardiolipin and other phospholipids. However, also in this case, the half-life of nonessential fatty acids esterified into cardiolipin appears to be slightly longer than that of other phospholipids (3.1 vs. 2.5 days, respectively). These observations were confirmed in the experiment performed with the double-labeled precursor (27). In fact, the relative turnover rates of cardiolipin and other mitochondrial phospholipids, determined by using acetate labeled with ^{14}C and ^{3}H as the lipid precursor, were found to be 2.3 and 2.8, respectively, values which fit in well with those obtained by single isotope administration.

As a control, Tables I and II show that radioactivity associated with cardiolipin is due principally to esterified fatty acids either at short intervals (0.5 days) or after longer periods

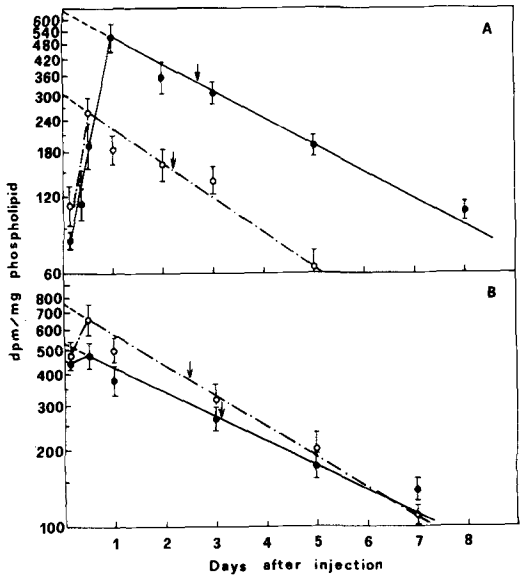

FIG. 1. Changes in specific radioactivity of rat liver mitochondrial cardiolipin and other phospholipids at intervals after intraperitoneal injection of 1-^{14}C-linoleic acid (A) or sodium ^{3}H-acetate (B). Each point is the mean of the results from three (A) or four pairs (B) of animals. The bars give standard deviation of mean. Arrows denote half-life times based on graphical extrapolation; ●—● = cardiolipin; ○—○ = other phospholipids.

(5 days) following 1-^{14}C-linoleic acid injection. It is also evident that linoleic acid contains more than 80% of the total radioactivity.

Previous reports, following ^{32}P-phosphate injection to rats, suggest a slower turnover for liver cardiolipin than for other mitochondrial

TABLE I

Distribution of Radioactivity Among Products of Cardiolipin Deacylation at Various Time Intervals after Intraperitoneal Injection of 1-^{14}C-Linoleic Acid

Precursor	Time after injection (days)	Petroleum ether phase (A) (cpm)	Aqueous phase (B) (cpm)	B/A (%)
1-^{14}C-Linoleic acid	0.5	1060	140	13.2
1-^{14}C-Linoleic acid	1	1470	130	8.8
1-^{14}C-Linoleic acid	5	810	90	11.1

TABLE II

Distribution of Radioactivity among Fatty Acids Esterified into Cardiolipin (%)

Precursor	Time after injection (days)	Saturated	Monoenoic	Dienoic	Tri, tetra, polyenoic
1-^{14}C-Linoleic acid	0.5	5.2	4.7	83.7	6.4
1-^{14}C-Linoleic acid	1	3.8	5.0	84.3	6.9
1-^{14}C-Linoleic acid	5	4.1	5.1	82.7	8.1

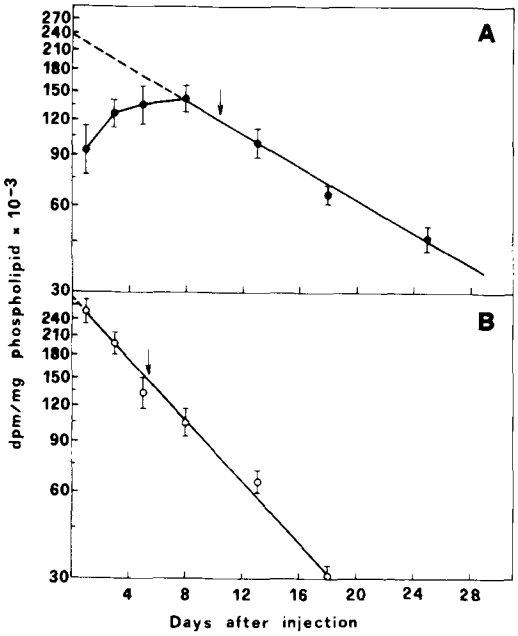

FIG. 2. Changes in specific radioactivity of rat liver mitochondrial cardiolipin (A) and other phospholipids (B) at intervals after intraperitoneal injection of ^{32}P-phosphate. Each point is the mean of the results from four pairs of animals. The bars give standard deviation of mean. Arros denote half-life times based on graphical extrapolation.

phospholipids (2,14,28). Similar behavior was detected in our experiments by intraperitoneal administration of the same isotope. Indeed, the results reported in Figure 2A indicate a half-life of 10.4 days for rat-liver mitochondrial cardiolipin; in identical experimental conditions, other rat-liver mitochondrial phospholipids exhibit an average half-life of 5.4 days (Fig. 2B).

Figure 3A shows the variation with time in the specific radioactivity of cardiolipin derived from liver mitochondria following intraperitoneal rat injection of 2-^{3}H-glycerol. After an initial increase for up to 1 day, a steady decrease of incorporation into cardiolipin is evident, from which a half-life of this component equal to 5.2 days can be calculated by extrapolation.

Regarding other mitochondrial phospholipids (Fig. 3B), by using the same labeled precursor, the appearance of two different decay slopes probably suggests either a heterogeneity of turnover rates among the various phospholipid classes (17,29) or the possibility of a number of metabolic pools of phospholipid components (30,31). The half-life of the fraction exhibiting the faster turnover is <1 day

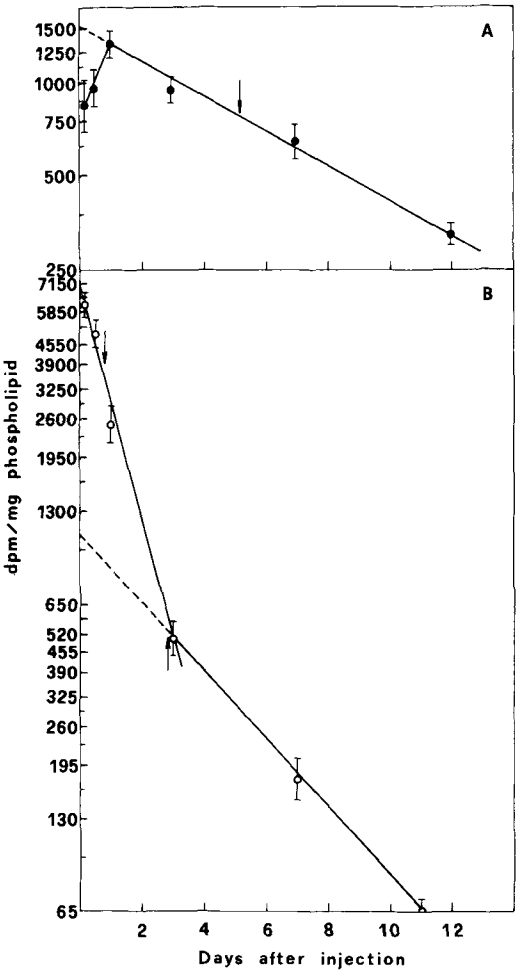

FIG. 3. Decay of radioactivity from rat liver mitochondrial cardiolipin (A) and other phospholipids (B) after intraperitoneal administration of 2-^{3}H-glycerol. Each point represents the mean ± standard deviation of the results from four pairs of animals. Arrows denote half-life time based on graphical extrapolation.

(0.8 days), while that of the slower fraction is about one half that of cardiolipin (2.8 days).

This behavior is more evident from results describing the relative turnover rates of cardiolipin and other phospholipids following injection of glycerol labeled with ^{14}C and ^{3}H as the lipid precursor (27). Because the ^{3}H:^{14}C ratio relative to phospholipids was found to be 19.1, whereas that of cardiolipin was equal to 2.9, it is quite evident that in mitochondria the former have a faster turnover than the latter.

DISCUSSION

The results presented in this paper indicate that, in liver, fatty acids of cardiolipin have a

substantially faster turnover rate than that of the remaining molecular parts. Moreover, the data in Figure 1 indicate that the linoleyl residues of cardiolipin have a half-life slightly lower than that of nonessential fatty acids and that both these components exhibit a slower turnover in respect to the same fatty acids esterified into other mitochondrial phospholipids.

There is now good evidence that, in all membrane phospholipids, a fatty acid exchange (acylation-deacylation) occurs in the maintenance of the specific fatty acid pattern (32,33). This phenomenon has been held responsible for the rapid turnover of these portions of phospholipid molecules (34,35). Many authors, however, have reported that the positional specificity of phospholipid fatty acids reflects the specificity of the enzyme that acylates glycerophosphate; in the de novo synthesis of these compounds, the presence of a saturated fatty acid in the 1-position and a saturated fatty acid in the 2-position occurs initially as a result of specific acylation by enzymes which preferentially localize the saturated fatty acid in the 1-position and the usaturated fatty acid in the 2-position of intermediate phosphatidic acid (36-40).

The results of Figures 1-3 show that acyl moieties of cardiolipin exhibit a much faster turnover rate than that of the remaining molecular components. Since this phospholipid does not present a definite positioning of its esterified fatty acids, which are randomly distributed between α and β position (41,42), this characteristic is probably due to other causes. The independent turnover of acyl groups of cardiolipin and other phospholipids could be considered of special interest because of its effects on the physico-chemical state of lipid membranes, which can be influenced by a change in their fatty acid pattern.

Even though the exact mechanism for cardiolipin degradation in mammalian systems is still unclear (43,44) and marked differences in both the localization and biosynthetic mechanism exist (3-8) in respect to other phospholipids, there appears to be a dynamic relationship between their acid components. In this context, it is worth pointing out the role of polyunsaturated fatty acids in mitochondrial membrane functioning. Indeed, it has recently been reported that the decrease in the linoleic acid content of cardiolipin strongly influences the physical properties of the mitochondrial inner membranes (45) with a concomitant loss of adenosine triphosphatase (46) and phosphorilative ability (47). Therefore, a possible interpretation of the faster turnover of fatty acids of cardiolipin could represent an auxiliary mechanism for preserving the selective pattern of its acyl components.

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Biosynthesis of Polyunsaturated Fatty Acids in the Developing Brain: I. Metabolic Transformations of Intracranially Administered 1-^{14}C Linolenic Acid

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ABSTRACT

Thirteen-day old rats were given intracranial injections of 1-^{14}C linolenic acid (all *cis* 9,12,15 octadecatrienoic acid) and were sacrificed after 8 hr. Analysis of brain fatty acids showed that 16:0, 18:0, 18:1, 18:3, 20:3, 20:4, 20:5, 22:5, and 22:6 were labeled. The total fatty acid methyl esters were separated into classes according to degree of unsaturation on a $AgNO_3$:SiO_2 impregnated plate. The bands were scraped off and the eluted fatty acids were first analyzed by radio–gas liquid chromatography and then subjected to reductive ozonolysis to determine double bond position. The saturated acids, 16:0 and 18:0, as well as the monounsaturated 18:1, must have been formed from radioactive acetate produced by β oxidation of the injected linolenate. Among the polyunsaturated fatty acids, the triene fraction was characterized and identified as 18:3 ω3 ($\Delta^{9,12,15}$), the starting material, and 20:3 ω3 ($\Delta^{11,14,17}$); the tetraene fraction was identified as 20:4 ω3 ($\Delta^{8,11,14,17}$); the pentaene fraction was identified as 20:5 ω3 ($\Delta^{5,8,11,14,17}$) and 22:5 ω3 ($\Delta^{7,10,13,16,19}$); and, finally, the hexaene fraction was shown to be 22:6 ω3 ($\Delta^{4,7,10,13,16,19}$). The biosynthesis of these ω3 family fatty acids in the brain in situ is discussed.

INTRODUCTION

Many investigators have established that certain brain lipids are rich in polyunsaturated fatty acids (1,2). The ω3 family fatty acids have received much attention because of a rather large amount of 22:6 ω3 occurring in the brain ethanolamine phosphoglycerides, and attempts have been made to postulate a role for these ω3 fatty acids in disease (3) and biochemical reactions (4). In experiments involving oral administration (5), it appears likely that 22:6 is rapidly synthesized in the liver from linolenate and the preformed hexaenoic acid then transported to the brain, rather than being synthesized in the brain itself. In vivo studies by Miyamoto et al. (6) have shown that 22:6 is readily formed from 18:3 in the chick embryonic brain, and recent studies by Yavin et al. (7) have shown that this synthetic reaction also occurs in neuroblastoma cell cultures. The original pathways of biosynthesis of the linolenate family (8) included a direct 18:3 ($\Delta^{9,12,15}$) → 18:4 ($\Delta^{6,9,12,15}$) → 20:4 ($\Delta^{8,11,14,17}$) → 20:5 ($\Delta^{5,8,11,14,17}$) → 22:5 ($\Delta^{7,10,13,16,19}$) → 22:6 ($\Delta^{4,7,10,13,16,19}$) and the optional pathway in which the 18:3 ($\Delta^{9,12,15}$) was first elongated to 20:3 ($\Delta^{11,14,17}$) followed by similar reactions. This scheme has been revised by Sprecher and Lee (9) who have shown that the optional pathway in which 20:3 ($\Delta^{11,14,17}$) would have to be desaturated in the Δ^8 position to form 20:4 ($\Delta^{8,11,14,17}$) does not occur in the liver because of the lack of Δ^8 desaturase. Thus, 20:3 ($\Delta^{11,14,17}$) would not be a true intermediate in the biosynthetic pathway. The present work was undertaken to study the transformations of the linolenate family fatty acids in the brain by isolating and characterizing the intermediates and by choosing intracranial injection to restrict the role of the liver in these reactions.

MATERIALS AND METHODS

Animals

Seven 13-day old Wistar albino rats, male and female, weighing 26.5 ± 1.2 g, were used for intracranial injection.

Tracer

1-^{14}C Linolenic acid (all *cis* 9,12,15 octadecatrienoic acid, 52.5 mc/mM; DHOM Products, Hollywood, CA) was injected intracranially, as an albumin complex, using a Hamilton syringe with a plastic sleeve on the needle 4 mm from the tip. Each rat was given 15 μl containing 3.85 μc of labeled linolenate. The baby rats were returned to the mother, and at the end of 8 hr they were sacrificed.

TABLE I

Percent Distribution of Radioactivity in Brain Total Lipids
8 hr Following Intracranial Injection of 1-^{14}C Linolenic Acid

Lipids	Percent of total radioactivity (thin layer chromatography)
Total lipids	
Total polar lipids	84.9
Total cholesterol	7.8
Total acylglycerides	3.8
Total polar lipids	
Choline phosphoglycerides	49.7
Ethanolamine phosphoglycerides	28.3
Serine + inositol phosphoglycerides	11.1
Cerebroside + ceramide	8.6
Sphingomyelin	1.6
Total ethanolamine phosphoglycerides	
Diacylethanolamine phosphoglycerides	73.1
Alkyl, acyl ethanolamine phosphoglycerides	26.9

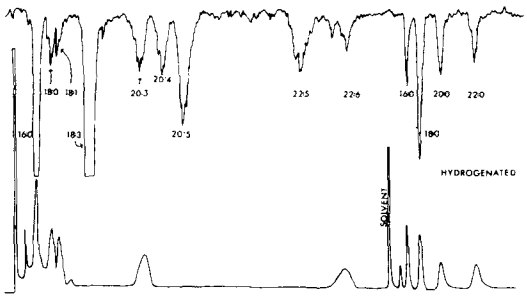


FIG. 1. Gas liquid chromatography of brain total fatty acid methyl esters before and after hydrogenation. Top curve represents radioactivity. 6 ft x 4 mm Apolar 10 C at 180 C.

Lipid Extraction and Fractionation

The brains were excised and washed in distilled water to remove any adhering blood, excess water was blotted out, and the tissue was immersed in cold chloroform:methanol (2:1). The pooled tissue was used for lipid extraction by the method of Folch et al. (10). Fractionation of lipids was performed on thin layer chromatography (TLC) plates to obtain percent distribution of radioactivity in various lipid components (11). Fatty acid methyl esters were obtained by methanolysis using 1% H_2SO_4 in methanol (12). The total methyl esters were separated into classes according to degree of unsaturation using argentation TLC, developed in 5% acetone in toluene as mobile solvent. The various bands were visualized by spraying dichloroflurescein on a portion of the plate spotted with a known mixture of standard fatty acid methyl esters. Corresponding areas were scraped into centrifuge tubes and extracted several times with chloroform: methanol (2:1) to obtain pure fractions. Areas from other TLC plates were scraped directly into scintillation vials to determine radioactivity distribution. Decarboxylation of pure fatty acid, obtained by saponification of methyl esters, has been described before (12).

Radio–Gas Liquid Chromatography

Radio-gas liquid chromatography (GLC) was performed using a Packard GLC Model 824 with a 6 ft x 4 mm glass column packed with Apolar 10 C (Applied Science Laboratory, State College, PA) and operated at 180 C. This instrument gives simultaneous curves for mass and radioactivity. Samples were analyzed before and after hydrogenation over platinum oxide. Identification of fatty acid methyl ester peaks was based on comparison of retention times with known standards. Chain length was ascertained by hydrogenation and degree of unsaturation by position on $AgNO_3$-SiO_2 TLC plate.

Reductive Ozonolysis

Fatty acid methyl esters separated into trienes, tetraenes, pentaenes, and hexaenes were subjected to reductive ozonolysis according to the method described by Stein and Nicholaides (13). As before, aldehyde esters were identified by comparison with known standards.

RESULTS

Table I shows the distribution of radioactivity in total lipids of the brain. Polar lipids comprising pohspho- and glycolipids contained most of the radioactivity. Of the polar lipids, choline phosphoglycerides had higher radioactivity than ethanolamine phosphoglycerides, and the diacyl ethanolamine phosphoglycerides were almost three times more radioactive than

TABLE II

Percent Distribution of Radioactivity in Brain Fatty Acid Methyl Esters 8 hr Following Intracranial Injection of 1-^{14}C Linolenic Acid

Fatty acid (GLC)[a]	Percent of total radioactivity Before Hydrogenation	After	% RCA[b]
16:0	18.7	18.0	11.6
18:0	4.5	34.6	14.3
18:1	5.5	-	19.4
18:2ω6	-	-	-
18:3ω3	24.5	-	-
20:0	-	22.7	-
20:3ω3	8.1	-	-
20:4ω3	5.5	-	21.1
20:5ω3	11.2	-	-
22:0	-	24.7	-
22:5ω3	12.7	-	-
22:6ω3	9.3	-	2.0

[a]GLC = Gas liquid chromatography.

[b]% RCA = Relative carboxyl activity = radioactivity in -COOH/radioactivity of total fatty acid x 100.

the plasmalogen counterpart. These results agree with those obtained when the same tracer was injected intraperitoneally (11).

When the total fatty acid methyl esters were analyzed by radio-GLC (Fig. 1), 16:0, 18:0, 18:1, 18:3, 20:3, 20:4, 20:5, 22:5, and 22:6 were found to be radioactive and were tentatively identified by comparison of their retention times with those of standards. Hydrogenation and reexamination confirmed the chain lengths (Table II, Fig. 1). The relative carboxyl activity (% RCA [radioactivity in -COOH/radioactivity of total fatty acid/x 100]) of 20:4 and 22:6 was found to be relatively low, and this was to be expected inasmuch as 18:3, the immediate precursor, being the starting material, was highly radioactive. Thus, C_3 of the eicosa-ω3-polyunsaturated fatty acid series and C_5 of the docosa-ω3-polyunsaturated fatty acid series would have very much more activity because they are the original C_1 of 18:3, and the carboxyl carbon of the eicosa- and docosa-ω3-polyunsaturated fatty acids would have lower radioactivity (14).

Further confirmation of the identify of these fatty acids was obtained from their movement on the $AgNO_3$:SiO_2 TLC plate. These fractions, separated according to degree of unsaturation (Fig. 2, inset A), were rechromatographed by radio-GLC. Figure 2 shows the purity of the $AgNO_3$:SiO_2 separated fractions as seen by radio-GLC. Ozonolysis of these individual fractions established the position of the first double bond from the carboxyl end. Thus, the polyunsaturated fatty acid peaks were identified as follows: triene fraction, 18:3 ($\Delta^{9,12,15}$) and 20:3 ($\Delta^{11,14,17}$); tetraene

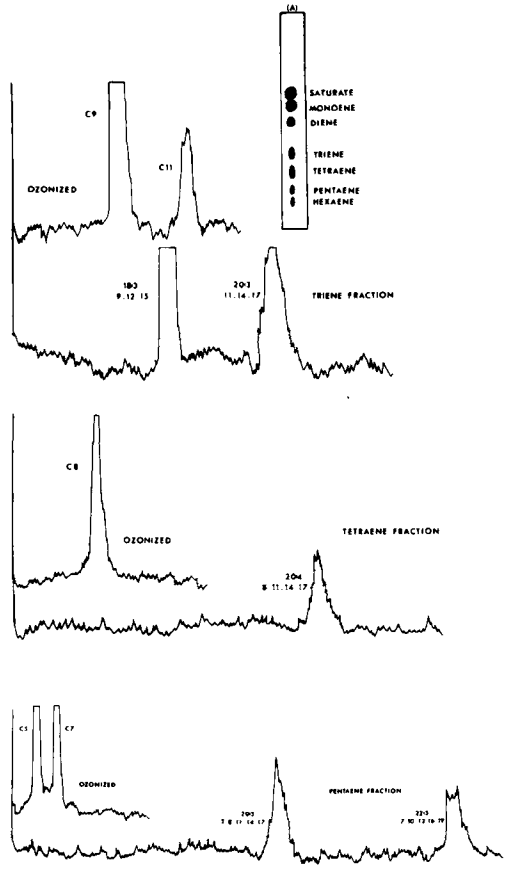


FIG. 2. (Inset A) Thin layer chromatography separation of total fatty acid methyl esters on $AgNO_3$:SiO_2 plate; solvent 5% acetone in toluene. Radio-gas liquid chromatography of triene, tetraene, and pentaene fractions before and after ozonolysis.

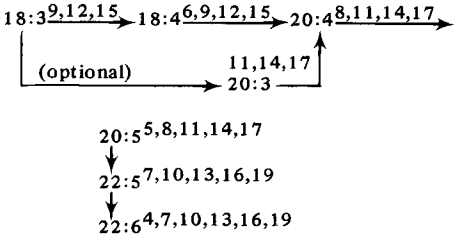
fraction, 20:4 ($\Delta^{8,11,14,17}$); pentaene fraction, 20:5 ($\Delta^{5,8,11,14,17}$) and 22:5 ($\Delta^{7,10,13,16,19}$); and hexaene fraction, 22:6 ($\Delta^{4,7,10,13,16,19}$).

DISCUSSION

In our previous work on the metabolism of 1-^{14}C linolenate (15), we had observed that palmitate isolated from the brain was highly radioactive, but because the tracer was administered by intraperitoneal injection, the liver palmitate was also highly radioactive. Thus, although we knew that it was synthesized de novo from acetate (% RCA ca. theoretical value of 12.5) produced by β oxidation of the injected tracer linolenate, it was difficult to say with certainty whether the palmitate was synthesized in the brain itself or was formed in the liver and then transported directly to the brain via circulating blood. In the present work, the tracer linolenate was injected directly into the brain, and it was noted that radioactivity in the liver was very low. Thus, it is fair to conclude that the major portion of β oxidation and resynthesis occurred in the brain itself. Brain cholesterol was also radioactive (2250 cpm/mg cholesterol, compared to 11101 cpm/mg palmitate), showing synthesis in the brain in situ. The brain stearate was probably a mixture of that synthesized de novo (16,17) and a smaller amount synthesized by chain elongation of the palmitate because the % RCA was slightly higher than the theoretical value for de novo synthesis.

The polyunsaturated fatty acids formed from the 18:3 ($\Delta^{9,12,15}$) all seemed to have radioactivity. Almost one-fourth of the total activity still remained as the original tracer (18:3). Of the total activity, 11-12% was associated with the 20:5 and 22:5 fatty acids. These values are different from those reported by Sprecher and Lee (9), but those authors were dealing with the liver lipids of animals raised on a fat free diet, whereas the present study deals with animals raised on a balanced diet and is limited to the brain. 20:3 ω3 ($\Delta^{11,14,17}$) has a retention time very close to 20:4 ω6 ($\Delta^{5,8,11,14}$). However, $AgNO_3$:SiO_2 TLC separates the triene and tetraene. The separated triene, on ozonolysis, gave a radioactive peak corresponding to C_{11} aldehyde ester, indicating the first double bond position to be 11 and thus confirming the identity of 20:3 as $\Delta^{11,14,17}$. Similarly, the identities of the remaining radioactive peaks were established as 20:4 ($\Delta^{8,11,14,17}$), 20:5 ($\Delta^{5,8,11,14,17}$), 22:5 ($\Delta^{7,10,13,16,19}$), and finally 22:6 ($\Delta^{4,7,10,13,16,19}$).

The metabolic pathway for the biosynthesis of the ω3 family fatty acids was initially proposed by Klenk and Mohrhauer (8):



However, recently Sprecher and Lee (9) have revised this scheme by showing that the optional pathway in which 20:3 ($\Delta^{11,14,17}$) is desaturated to 20:4 ($\Delta^{8,11,14,17}$) does not occur in mammalian liver. This conclusion was reached from the observation that 20:3 ($\Delta^{11,14,17}$) was not a suitable substrate for the Δ^8 desaturase to form 20:4 ($\Delta^{8,11,14,17}$); instead, it formed 20:4 ($\Delta^{5,11,14,17}$) which, not having a methylene interrupted double bond system, acts as a dead end. Thus, 20:3 ($\Delta^{11,14,17}$) is not a true intermediate in the biosynthetic pathway. In the present study, both 20:3 ($\Delta^{11,14,17}$) and 20:4 ($\Delta^{8,11,14,17}$) components were radioactive and contained 8.1% and 5.5%, respectively, of the total radioactivity. The component 20:4 ($\Delta^{8,11,14,17}$) was predicted, but not characterized, by Sprecher and Lee (9). Also in the present study, when the tetraene fraction was isolated in purified form (single radio-GLC peak) and ozonized, no C_5 aldehyde ester peak was obtained, eliminating both 20:4 ω3 ($\Delta^{5,11,14,17}$) and 20:4 ω6 ($\Delta^{5,8,11,14}$). Because 20:3 ($\Delta^{11,14,17}$) was shown to be an unsuitable substrate for Δ^8 desaturase (9), the observed peak of 20:4 ($\Delta^{8,11,14,17}$) must be a product of chain elongation of 18:4 ω3 ($\Delta^{6,9,12,15}$), which has been shown to be an intermediate of the ω3 family fatty acids (8). The trienoic acid 20:3 ω3 ($\Delta^{11,14,17}$), a possible product of direct chain elongation of 18:3 ($\Delta^{9,12,15}$), was not obtained either by Sprecher and Lee (9) in the livers of animals raised on a fat free diet or by Yavin et al. (7) in the neuroblastoma cell culture studies, and both have proposed that 20:3 ω3 may not be a true intermediate. Studies are now in progress to determine whether the brain system is capable of desaturating 20:3 ω3 ($\Delta^{11,14,17}$) to the 20:4 ($\Delta^{8,11,14,17}$) or, like the liver system, lacks the Δ^8 desaturase.

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Monocarboxylic Acids from Oxidation of Acyclic Isoprenoid Alkanes by *Mycobacterium fortuitum*

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ABSTRACT

Mycobacterium fortuitum utilizes certain stereoisomeric mixtures of individual multimethyl branched alkanes as sole carbon source, including 2,6(R), 10(S), 14(RS)-tetramethylhexadecane; 2,6(R),10(S),14(RS)-tetramethylheptadecane; 2,6(RS),10(RS)-trimethyltetradecane, and 2,6(R),10(S)-trimethylpentadecane. Products of oxidation isolated from the bacterial lipids were acids derived predominantly from oxidation of the isopropyl terminus of each alkane, except in the case of 2,6(RS),10(RS)-trimethyltetradecane. With the latter, acids from oxidation at either terminus were detected in comparable proportions.

INTRODUCTION

In earlier work (1), *Mycobacterium fortuitum* was grown on 2,6(R),10(S),14-tetramethylpehtadecane (Fig. 1, I) as sole carbon source, and mono-alcohol and mono-carboxylic acid products were isolated and their respective stereochemical compositions defined by high resolution gas liquid chromatographic analysis. These and related results suggested that, for these products of monoterminal oxygenation, there was specific oxygenation at only one terminus of the alkane. The present study describes products of monoterminal oxygenation derived from metabolism by the same organism of certain other stereochemically defined acyclic isoprenoid type alkanes, i.e., 2,6(R),10(S),14(RS)-tetramethylhexadecane (II, $R=R^1=CH_3$) (configurations at the 6- and 10-positions are as indicated for 2,6(R), 10(S),14-tetramethylpentadecane [1]); 2,6(R), 10(S),14(RS)-tetramethylheptadecane (II, $R=CH_3, R^1=C_2H_5$); 2,6(RS),10(RS)-trimethyltetradecane (III, $R=R^1=CH_3$), and 2,6(R),10(S)-trimethylpentadecane (III, $R=CH_3, R^1=C_2H_5$). These substrates have an isopropyl group as one terminus, and the other terminus comprising ethyl, *n*-propyl, *n*-butyl, and *n*-pentyl moities, respectively. Thus, oxidation at a particular terminus in each would lead to acidic products quite different from those produced by oxidation at the opposite terminus and readily distinguished by a technique such as mass spectrometry.

MATERIALS AND METHODS

Substrates

2,6(R),10(S),14(RS)-Tetramethylhexadecane (II, $R=R^1=CH_3$) was prepared from natural phytol (*trans*-3,7(R),11(R),15-tetramethylhexadec-2-en-1-ol, (British Drug Houses Ltd., Poole, England) by hydrogenolysis with 10% palladium:charcoal in absolute ethanol. 2,6(R),10(S),14(RS)-Tetramethylheptadecane (II, $R=CH_3$ $R^1=C_2H_5$), and 2,6(RS),10(RS)-trimethyltetradecane (III, $R=R^1=CH_3$) were obtained from P. Albrecht, University of Strasbourg (Strasbourg, France) and had been synthesized from commercial natural phytol and farnesol (3,7,11-trimethyldodeca-2,6,10–trien-1-ol), respectively, via chain extension of the aldehydes of these two alcohols (2). 2,6(R), 10(S)-Trimethylpentadecane (III, $R=CH_3, R^1=C_2H_5$) was prepared by zinc amalgam-hydrogen chloride reduction of 6(R),10(R),14-trimethylpentadecan-2-one prepared by ozonolysis of natural phytol. The configuration in 6(R),10(R),14-trimethylpentadecan-2-one at the 6-position remains unchanged in the sequence but is described as S in the alkane product.

The alkane substrates were purified by preparative silver ion thin layer chromatography (TLC) (10% $AgNO_3$ on Silica Gel G by wt with hexane as developer). Substrate purity, as measured by gas liquid chromatography (GLC) on 3m x 0.16cm stainless steel packed with 3% OV-17 on Chromosorb W (Supelco, Bellefonte, PA) was >95%, except for 2,6,10-trimethylpentadecane, which had a purity of ca. 85% and showed three minor impurities.

Organism

Mycobacterium fortuitum (ATCC strain 19709) was grown in shake flasks on the appropriate alkane dispersed in buffered mineral salts medium as described previously (1). Clumping of the bacteria and disappearance of substrate from the medium was observed after

3-4 days growth for the C_{20} (II, $R=R^1=CH_3$) and C_{21} (II, $R=CH_3$ $R^1=C_2H_5$) alkanes and after 6-7 days growth for the C_{17} (III, $R=R^1=CH_3$) and C_{18} (III, $R=CH_3R^1=C_2H_5$) alkanes. In the subsequent isolation of the carboxylic acid products residual alkane was always observed on TLC plates, although for these substrates the extent of utilization was not measured. Under similar conditions, 50-80% utilization of 2,6,10,14-tetramethylpentadecane was observed (1).

Isolation of Acids

Toluene/methanol extracts of the bacteria were hydrolyzed with potassium hydroxide in methanol and the carboxylic acids converted to methyl esters by treatment with boron trifluoride:methanol (1). Purification of the methyl esters was achieved by preparative TLC on Silica Gel G with hexane:ether (95:5) as developer and separation of the products into linear and multimethyl branched ester fractions by urea adduction (1). Yields of total methyl esters were typically 3-4 mg from 50-100 mg amounts of substrates. Multimethyl branched esters constituted 50-80% of the total ester fractions.

GLC and Combined Gas Chromatography-Mass Spectrometry (GC-MS)

Components of multimethyl branched ester fractions were analyzed by GLC and GC-MS using stainless steel columns (3m x 0.16cm) packed with 3% OV-17 on Chromosorb W as described previously (3). Where available, standard esters were compared with the products by coinjection and by comparison of their mass spectra. Other esters for which authentic standards were not available were identified from characteristic mass spectral fragmentations. Relative proportions of identified components were estimated by measurement of peak areas. The gas chromatographic conditions used did not resolve stereoisomers of the substrate alkanes or of the methyl esters of the acidic products.

Authentic Acids

The preparation of certain acids potentially derivable from the substrates by oxidation at C_ω has been described previously (3).

4,8-Dimethyldodecanoic acid (V,$R=CH_3$) was prepared as follows: (a) 2-Methylhexanoic acid (bp 98-101 C ca. 10mm, ν O-H, ca. 3000 cm^{-1}; ν C=O,1705cm^{-1}) was prepared by condensation of diethylmethylmalonate and *n*-butyl bromide, hydrolysis of the resulting diester and decarboxylation. GLC of the methyl ester showed virtually one component (>95%);

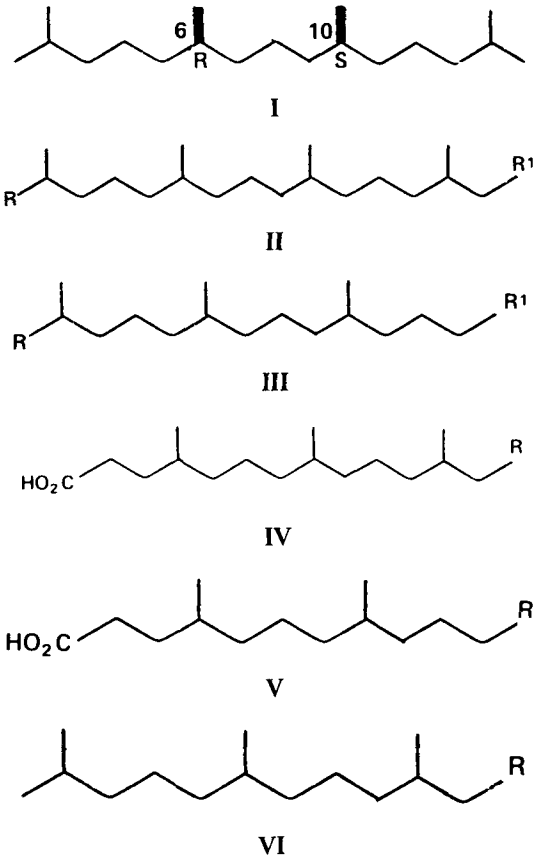

FIG. 1. Numerical key and structures of alkane substrates utilized by *Mycobacterium fortuitum* and of monocarboxylic acid products.

the mass spectrum showed ions at m/e 144 (M) and 88 (rearrangement ion of a 2-methyl substituted methyl ester. (b) 1-Morpholino-4-methylcyclohex-1-ene (4g, 55%; bp 121-123 C ca. 10mm; ν C=C, 1645 cm^{-1}; ν C-O, 1120 cm^{-1}, γ C-H of C=C double bond 790 cm^{-1}) was prepared from 4-methylcyclohexanone (4.2 g) and morpholine (4 g) by the general method of Hünig, et al., (4). (c) 4,8-Dimethyldodecanoic acid (V, $R=CH_3$) was prepared by the general method of Hünig, et al., (5). Crude 2-(2-methylhexanoyl)-4-methylcyclohexanone was obtained via the crude keto-enamine, from 1-morpholino-4-methylcyclohex-1-ene (1.2 g) and 2-methylhexanoyl chloride (0.81 g). Heating under reflux with sodium hydroxide afforded crude 4,8-dimethyl-7-oxo-dodecanoic acid (νO-H ca. 3000 cm^{-1}; ν C=O ca. 1705 cm^{-1}), which was reduced to 4,8-dimethyldodecanoic acid (0.7 g). The crude product was methylated and passed through a short column of alumina (hexane:ether, 50:50). Preparative

TABLE I

Multibranched Acids from Oxidation of Acyclic Isoprenoid Alkanes by *Mycobacterium fortuitum*

Substrate	Point of oxygenation	Possible products of C_1/Cω oxygenation and subsequent oxidation (%)[a]
2,6(R),10(S),14(RS)-Tetramethylhexadecane (II, R=R^1=CH_3)	C_1	2,6,10,14-tetramethylhexadecanoic (55); 4,8,12-trimethyltetradecanoic (45)
	Cω	3,7,11,15-tetramethylhexadecanoic (ND)[b]
2,6(R),10(S),14(RS)-Tetramethylheptadecane (II, R=CH_3,R^1=C_2H_5)	C_1	2,6,10,14-tetramethylheptadecanoic (75); 4,8,12-trimethylpentadecanoic (25)
	Cω	4,8,12,16-tetramethylheptadecanoic (ND); 2,6,10,14-tetramethylpentadecanoic (ND)
2,6(RS),10(RS)-Trimethyltetradecane (III, R=R^1=CH_3)	C_1	2,6,10-trimethyltetradecanoic (28); 4,8-dimethyldodecanoic (20)
	Cω	5,9,13-trimethyltetradecanoic (45); 3,7,11-trimethyldodecanoic (7)
2,6(R),10(S)-Trimethylpentadecane (III, R=$CH_3$$R^1$=$C_2H_5$)	C_1	2,6,10-trimethylpentadecanoic (45); 4,8-dimethyltridecanoic (47)
	Cω	6,10,14-trimethylpentadecanoic (3); 4,8,12-trimethyltridecanoic (5)

[a]Proportion of multimethyl branched acids detected by gas liquid chromatography analysis after removal by urea of straight chain acids.
[b]ND = Not detected at appropriate elution time.

TLC (hexane:ether, 95:5) afforded a component (50 mg, R_f=methyl palmitate) shown by GLC to consist of one major product (ca. 85%; ν C=O, 1740 cm^{-1}). The mass spectrum (GC-MS) had ions at m/e 242 (M), 185 (M-57), 157, 115, 87 (100%), and 74, consistent with the expected structure.

RESULTS

Bacteria grown on the respective substrates as sole carbon source were hydrolyzed with alkali and methyl esters prepared and purified by chromatography and urea adduction, the latter removing linear and most monomethyl branched esters. The principal components of the multimethyl branched ester fraction were analyzed in each experiment by GLC and GC-MS. In all cases, the major components were attributable to degradation via C_1 or Cω in the substrates; no significant peaks could be attributed to products of oxygenation of internal methyl groups. The products identified are listed in Table I.

Identification of Acid Products

Authentic standards corresponding to all of the possible products of Cω oxidation listed in Table I were prepared previously (3), and their methyl esters were used for GLC coinjection and for comparison of mass spectra with those of the products of oxidation. Among the expected products of C_1 oxidation, the only available reference sample was 4,8-dimethyldodecanoic acid (V, R=CH_3). The other C_1 oxidation products were identified from the mass spectral features shown by the methyl esters of acids with methyl branches at 2,6 and 4,8. Esters of this type are readily distinguished from each other and from those with methyl branches at 3,7 and 5,9 by, for example, the m/e value of the most abundant ion which occurs at m/e 88, 87, 101, and 74, respectively, in the spectrum of each of the four types. These and other characteristic fragmentations, adapted from earlier reports (6,7) are summarized as follows for methyl esters of acids derived from C_1 oxidation.

Acids with Branches at Positions 2,6-

The esters of acids (II, R-CO_2H, R^1=CH_3 and R=CO_2H, R^1=C_2H_5, and III, R=CO_2H, R^1=CH_3, and R=CO_2H, R^1=C_2H_5) showed, apart from the appropriate molecular ions, the following characteristic ions: (a) base peak at m/e 88: $[(CH_3)CH{=}C(OH)(OCH_3)]^+$ (McLafferty rearrangement); (b) abundant ion at m/e 101: $[CH_2CH(CH_3)CO_2CH_3]^+$; (c)

TABLE II

Principal Mass Spectral Fragmentations of Some Multibranched Acids from Monoterminal Oxidation of Isoprenoid Alkanes by *Mycobacterium fortuitum*

Acid	Structure	Ions at m/e (relative intensity %)
2,6,10,14-Tetramethylhexadecanoic	$R=CO_2H, R^1=CH_3$ (II)	326(M^+), 88(101), 101(101), 236(18), 129 (20),157(45), 199(4), 227(5)
4,8,12-Trimethyltetradecanoic	$R=CH_3$(IV)	284(M^+), 87(100), 74(40), 211(2), 227(8), 115(6), 157(13), 185(2)
2,6,10,14-Tetramethylheptadecanoic	$R=CO_2H, R^1=C_2H_5$ (II)	340(M^+), 88(100), 101(58), 250(4), 129(8), 157(12), 199(1), 227(1)
4,8,12-Trimethylpentadecanoic	$R=C_2H_5$ (IV)	298(M^+), 87(100), 74(37), 225(4), 241(5), 115(5), 157(9)
2,6,10-Trimethyltetradecanoic	$R=CO_2H, R^1=CH_3$ (III)	284(M^+), 88(100), 101(50), 194(3), 129(6), 157(10)
4,8-Dimethyldodecanoic[a]	$R=CH_3$ (V)	242(M^+), 87(100), 74(47), 169(8), 185(9), 115(9), 157(13)
2,6,10-Trimethylpentadecanoic	$R=CO_2H, R^1=C_2H_5$ (III)	298(M^+), 88(100), 101(42), 208(4), 129(6), 157(10), 199(2)
4,8-Dimethyltridecanoic	$R=C_2H_5$ (V)	256(M^+), 87(100), 74(36), 183(6), 199(7), 115(6), 157(11)

[a]Synthetic standard.

abundant ions M-90; and (d) fragmentations at branch points, e.g., m/e 129, 157, 199, etc.

Acids with Branches at Positions 4,8-

The esters of acids (IV, $R=CH_3$ and $R=C_2H_5$, and V, $R=C_2H_5$) all showed, apart from the appropriate molecular ions, the following characteristic ions: (a) base peak at m/e 87: $[(CH_2)_2CO_2CH_3]^+$; (b) abundant ion at m/e 74: $[CH_2=C(OH)(OCH_3)]^+$ (McLafferty rearrangement); (c) abundant ions M-57, M-73; and (d) fragmentations at branch points, e.g., m/e 115, 157, etc.

Products of Oxidation

The gas chromatogram of the products of oxidation of 2,6(R),10(S),14(RS)-tetramethylhexadecane (II, $R=R^1=CH_3$) showed essentially two peaks. That of longer elution time was coincident with the methyl ester of 3,7,11,15-tetramethylhexadecanoic acid (II, $R=CH_3$, $R^1=CO_2H$) by coinjection, but the mass spectrum (Table II) clearly identified it as the ester of 2,6,10,14-tetramethylhexadecanoic acid (II, $R=CO_2H$, $R^1=CH_3$). Thus, the spectrum showed a base peak at m/e 88, an abundant ion at m/e 236 (M-90) and branch point fragmentations (m/e 129, 157, 199, and 227). Similarly, the spectrum of the component of shorter retention time identified it as the ester of 4,8,12-trimethyltetradecanoic acid (IV, $R=CH_3$). This acid is presumably produced from the C_{20} acid by β-oxidation. Oxidation of 2,6(R),10(S),14(RS)-tetramethylheptadecane (II, $R=CH_3, R^1=C_2H_5$) also gave rise to two products of C_1 oxidation which were identified from their mass spectra (Table II) as the methyl esters of 2,6,10,14-tetramethylheptadecanoic (II, $R=CO_2H$, $R^1=C_2H_5$) and 4,8,12-trimethylpentadecanoic (IV, $R=C_2H_5$) acids. In this case, the methyl esters of the expected products of C_ω oxidation, i.e., 4,8,12-16-tetramethylheptadecanoic (II, $R=CH_3, R^1=CH_2\cdot CO_2H$) and 2,6,10,14-tetramethylpentadecanoic (III, $R=CH_3, R^1=CH(CH_3)\cdot CO_2H$) acids, had different retention times from the two products of C_1 oxidation and were not detected on the chromatograms. With the substrate containing the four possible isomers, i.e., 2,6(RS),10(RS)-trimethyltetradecane, products of oxygenation at both C_1 and C_ω, 2,6,10-trimethyltetradecanoic (III, $R=CO_2H$, $R^1=CH_3$) and 5,9,13-trimethyltetradecanoic (III, $R=CH_3$, $R^1=CO_2H$) acids, and their respective β-oxidized products, 4,8-dimethyldodecanoic (V, $R=CH_3$) and 3,7,11-trimethyldodecanoic (VI, $R=CO_2H$) acids, were readily separated on the chromatogram and were identified from their mass spectra (Table II for the C_1 oxidation products). Acids derived by oxidation at either terminus of this substrate were detected in comparable proportions, e.g., 2,6,10-trimethyltetradecanoic (28%) and 5,9,13-trimethyltetradecanoic (45%). $C\omega$ oxidation of 2,6(R),10(S)-trimethylpentadecane (III, $R=CH_3$ $R^1=C_2H_5$) gave rise to minor proportions of 6,10,14-trimethylpentadecanoic (III, $R=CH_3$, $R^1=CH_2\cdot CO_2H$) and 4,8,12-trimethyltridecanoic (VI, $R=CH_2\cdot CO_2H$) acids which (Table I) were identified from their retention times and by comparison of their mass spectra

with those of authentic standards. The major products (>90%) of oxidation, however, were derived from C_1 oxidation, and were identified from their mass spectra (Table II) as 2,6,10-trimethylpentadecanoic (III, $R=CO_2H$, $R^1=C_2H_5$) acid and its β-oxidation product, 4,8-dimethyltridecanoic (V, $R=C_2H_5$) acid.

DISCUSSION

The isolation of monocarboxylic acids from the various cultures does not necessarily indicate that the predominant pathway of oxidation is via monoterminal oxygenation. Diterminal pathways have also been recorded for the oxidation of 2,6,10,14-tetramethylpentadecane (I) by a *Corynebacterium sp.* (8) and by *Brevibacterium erythrogenes* (9), and of 2,6,10,14-tetramethylhexadecane (II, $R=R^1=CH_3$) by the yeast *Torulopsis gropengiesseri* (10). Monoterminal oxidation of 2-methylhexane by *Pseudomonas aeruginosa* has been observed and, although products of oxygenation at either terminus were detected, C_6 oxidation with β-oxidation from the same terminus was apparently preferred (11).

In the present study, a high degree of selectivity was observed (Table I) in the isolation of products from C_1 oxidation of the alkanes (II, $R=R^1=CH_3$ and $R=CH_3$, $R^1=C_2H_5$ and III, $R=CH_3$, $R^1=C_2H_5$), which each had the 6(R),10(S) configuration. For the substrate, 2,6,10-trimethyltetradecane (III, $R=R^1=CH_3$), containing all four stereoisomers in equal proportions, products of both C_1 and C_ω oxidation and the corresponding β-oxidation products were detected in more comparable proportions.

For the alkanes 2,6,10,14-tetramethylhexadecane (II, $R=R^1=CH_3$), 2,6,10-14-tetramethylheptadecane (II, $R=CH_3$, $R^1=C_2H_5$), and 2,6,10-trimethylpentadecane (III, $R=CH_3$, $R^1=C_2H_5$), it is possible that monoterminal oxidation occurs at both C_1 and C_ω, but preferential adaptation to the C_ω oxidized products occurs, leaving the observed C_1 oxidation products to accumulate. It is also possible, however, that there is preferential oxidation at C_1 leading to the observed products (Table I). In this case there may be a parallelism with the oxidation of 2,6(R),10(S),14-tetramethylpentadecane (I) by the same organism, for which it was suggested that the initial oxidation is directed by the configuration of carbon atoms 6 and 10 (1). The observed C_1 and C_ω oxidation for alkane in III ($R=R^1=CH_3$), the only substrate with mixed configurations at carbon atoms 6 and 10, may also be explained by this hypothesis. However, at present there is no simple method of deciding if one or more isomers of this substrate is preferentially utilized, or of determining the stereochemistry of all of the four acid products isolated.

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SHORT COMMUNICATIONS

Cuticular Lipids of Insects: VIII. Alkanes of the Mormon Cricket *Anabrus simplex*[1]

ABSTRACT

The cuticular hydrocarbons of the Mormon cricket, *Anabrus simplex*, are all saturated and consist of n-alkanes (29+ %), 3-methylalkanes (12%), internally branched monomethylalkanes (26+ %), and dimethylalkanes (28+ %). The principal n-alkane is the C_{29} component, with a range from C_{23} to C_{33}. The 3-methylalkanes range from C_{28} to C_{32}, and the internally branched monomethyl- and dimethylalkanes range from C_{29} to C_{39}. When the branched alkanes of *A. simplex* are compared to those from other insects in the order Orthoptera, interesting patterns of methyl branching are observed.

INTRODUCTION

One of the earliest chemical studies of insect cuticular lipids was that of the Mormon cricket, *Anabrus simplex* (1). Baker et al. (1) reported that hydrocarbons comprised the majority of the cuticular lipids of this insect and suggested that n-alkanes predominated. They were, however, unable to assign definitive structures. The hydrocarbons of *A. simplex* have been re-examined by gas liquid chromatographic and mass spectral analyses, and the structures of all the major components are reported in this communication.

EXPERIMENTAL PROCEDURES

Adult *A. simplex* were collected from wild populations in southwestern Montana. The insects were sacrificed and stored at -20 C until used. The cuticular lipids were extracted by immersing the insects in hexane for 10 min.

The hydrocarbons were isolated by silicic acid column chromatography, checked for unsaturation by argentation thin layer chromatography (TLC), and quantitated by gas liquid chromatography (GLC) as described elsewhere (2). The branched alkanes were isolated by inclusion of the n-alkanes in 5 Å molecular sieve, and separated by preparative GLC. Mass spectra of the branched alkanes were obtained as described earlier (2).

RESULTS

The hydrocarbons amounted to 0.5 mg per g insect. Argentation TLC showed all the hydrocarbons to be saturated. The alkanes range in carbon chain length from 23 to 39+ carbons and consist of more than 27 components, as determined by GLC analysis.

Four major types of alkanes are present. n-Alkanes account for 29+ % of the hydrocarbon fraction; 3-methylalkanes comprise 12%; and internally branched monomethyl- and dimethylalkanes make up 26 and 28%, respectively (Table I).

The n-alkanes range from 23 to 33 carbons and have an odd number of carbons atoms. They were identified by GLC retention times compared to standards and by disappearance after inclusion in molecular sieve. The C_{29} homologue predominates, followed by the C_{27} and C_{31} compounds.

The 3-methylalkanes, which have an even number of carbons, range from 28 to 32 carbons (Table I). They elute 0.3 carbon units in front of n-alkanes of the same total carbon number and were identified by their characteristic M-29 and smaller M-57 peaks in the mass spectra of each homologue. The major alkane in this series is 3-methylheptacosane, followed by 3-methylnonacosane and 3-methylhentriacontane.

The internally branched monomethylalkanes were identified from their mass spectra by the characteristic fragmentation on each side of the methyl branch, with retention of the positive charge on the fragment containing the branching methyl group as described elsewhere (2). These alkanes elute 0.7 carbon units in front of n-alkanes of the same total carbons (2-4). The internally branched monomethylalkanes range from 28 to 38 total carbons, and each homologue contains an even number of carbons, with 3 to 6 isomers present in each homologue (Table II). From the C_{30} monomethylalkanes to the C_{32}, C_{34}, C_{36}, and C_{38} homologues,

[1]Scientific Journal Series 631, Agricultural Experiment Station, University of Montana, Bozeman, Montana 59715.

TABLE I

Percentage of Total Alkanes of the Mormon Cricket, *Anabrus simplex*

Number of carbons	Normal	3-Methyl	Internal methyl	Dimethyl
23	Tr[a]			
25	1			
27	8			
28		6	4	
29	16			2
30		4	4	
31	4			3
32		2	2	
33	Tr			Tr
34			3	
35				3
36			12	
37				13
38			1	
39				7
Total[b]	29+	12	26+	28+

[a]Trace = >0.1% but <1.0%.

[b]In addition to the compounds listed, larger than trace quantities were observed of unidentified branched alkanes with retention times on gas liquid chromatography equivalent to C_{36} (2%) and $C_{36.3}$ (1%) and greater than C_{39} (1%).

TABLE II

Internally Branched Mono- and Dimethylalkanes of *Anabrus simplex* According to Positions of the Methyl Group(s) in Order of Decreasing Quantities

Total carbons	Position of the methyl group(s)	Alkane
	Monomethylalkanes	
28	11-,13-,5-,7-	methylheptacosane
30	5-,13-,15-,11-,7-,9-	methylnonacosane
32	7-,5-,9-,11-,13-,15-	methylhentriacontane
34	9-,11-,13-,7-	methyltritriacontane
36	11-,13-,15-	methylpentatriacontane
38	13-,15-,17-,11-	methylheptatriacontane
	Dimethylalkanes	
35	13,17-,11,15-	dimethyltritriacontane
37	15,19-,13,17-,11,15-	dimethylpentatriacontane
39	15,19-,13,17-,17,21-,11,15-	dimethylheptatriacontane

the position of the methyl group of the major isomer increases from positions 5 to 7, 9, 11, and 13. In all the homologues present, the methyl group is located on an odd numbered carbon.

The dimethylalkanes are of 29 to 39 total carbons. Mass spectra were interpreted as described elsewhere (2-4). These alkanes elute about 1.3 to 1.4 carbon units in front of n-alkanes of the same total number of carbons (2-4). The branched methyl groups are located on odd numbered carbon atoms near the center of the carbon chain and are separated by three methylene groups (Table II).

DISCUSSION

Interesting patterns of methyl branched alkanes are observed in insects of the order Orthoptera, which includes the grasshoppers, crickets, and cockroaches, among others (5). The seven species of cockroaches from which cuticular hydrocarbons have been examined possess 3-methyl- and internally branched monomethylalkanes, with the majority of the alkanes containing 31 carbons or less (6-9). The three species of grasshoppers studied have internally branched monomethyl- and dimethylalkanes, with 3-methylalkanes being absent or present in trace amounts. The three species of crickets studied appear unique among insects in that they possess 2-methylalkanes and no 3-methylalkanes (10,11).

Although given the common name Mormon cricket, *A. simplex* is not a true cricket but is

instead in the family Tettigonidae, which includes the longhorned grasshoppers and katydids. Results presented in this paper show that 2-methylalkanes characteristic of crickets are absent, whereas considerable amounts of 3-methyl, internally branched monomethyl-, and dimethylalkanes are present. The alkanes of *A. simplex* are similar to those of the grasshoppers *Melanoplus sanguinipes* and *Melanoplus packardii* in that the n-alkanes contain the shortest chain length components (C_{23} to C_{33}), the 3-methylalkanes are from C_{28} to C_{32}, and the internally branched monomethylalkanes and dimethylalkanes contain the longest chain components.

Not enough insects have been studied to date to propose chemotaxonomic rules, yet the interesting patterns of methyl branched hydrocarbons noted above in four families of Orthoptera suggest that such a possibility may exist.

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[Received September 29, 1975]

In Vitro Activity of the Fatty Acyl Desaturases of Human Cancerous and Noncancerous Tissues

ABSTRACT

The microsomal desaturase activity of human cancerous and noncancerous tissues was measured in vitro using 1-^{14}C,-11,14-eicosadienoic and 1-^{14}C-oleic acids as substrates. Tissues used were a case of ovarian cancer, a urinary bladder cancer, a rectal cancer, and a nonspecific colonic ulcer with appropriately normal tissues. When 11,14-20:2 was used as substrate, radioactive tetraene and triene were produced. The tetraene was identified by radio gas chromatography as arachidonic acid (5,8,11,14-20:4), and the triene had a retention time of 5,11,14-20:3. Thus, the possibility arises that a Δ8 desaturase was involved. In the Δ6 desaturase, with the urinary bladder cancerous tissue, the desaturase activity appeared to be decreased in comparison to neighboring tissue, whereas with the colonic cancer tissue, the desaturase appeared to be relatively increased, though the number of samples was inadequate for confidence.

INTRODUCTION

The attack on cancer can be carried out only with thorough knowledge of its metabolic characteristics. One of the properties of rapidly dividing cells is the need for unsaturated fatty acids as components of the phospholipids necessary for the formation of cell membranes. The source of these fatty acids and their metabolism within the malignant cell thus becomes a matter of considerable importance.

instead in the family Tettigonidae, which includes the longhorned grasshoppers and katydids. Results presented in this paper show that 2-methylalkanes characteristic of crickets are absent, whereas considerable amounts of 3-methyl, internally branched monomethyl-, and dimethylalkanes are present. The alkanes of *A. simplex* are similar to those of the grasshoppers *Melanoplus sanguinipes* and *Melanoplus packardii* in that the n-alkanes contain the shortest chain length components (C_{23} to C_{33}), the 3-methylalkanes are from C_{28} to C_{32}, and the internally branched monomethylalkanes and dimethylalkanes contain the longest chain components.

Not enough insects have been studied to date to propose chemotaxonomic rules, yet the interesting patterns of methyl branched hydrocarbons noted above in four families of Orthoptera suggest that such a possibility may exist.

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In Vitro Activity of the Fatty Acyl Desaturases of Human Cancerous and Noncancerous Tissues

ABSTRACT

The microsomal desaturase activity of human cancerous and noncancerous tissues was measured in vitro using 1-^{14}C,-11,14-eicosadienoic and 1-^{14}C-oleic acids as substrates. Tissues used were a case of ovarian cancer, a urinary bladder cancer, a rectal cancer, and a nonspecific colonic ulcer with appropriately normal tissues. When 11,14-20:2 was used as substrate, radioactive tetraene and triene were produced. The tetraene was identified by radio gas chromatography as arachidonic acid (5,8,11,14-20:4), and the triene had a retention time of 5,11,14-20:3. Thus, the possibility arises that a Δ8 desaturase was involved. In the Δ6 desaturase, with the urinary bladder cancerous tissue, the desaturase activity appeared to be decreased in comparison to neighboring tissue, whereas with the colonic cancer tissue, the desaturase appeared to be relatively increased, though the number of samples was inadequate for confidence.

INTRODUCTION

The attack on cancer can be carried out only with thorough knowledge of its metabolic characteristics. One of the properties of rapidly dividing cells is the need for unsaturated fatty acids as components of the phospholipids necessary for the formation of cell membranes. The source of these fatty acids and their metabolism within the malignant cell thus becomes a matter of considerable importance.

In particular, the various abilities of the malignant cells to transform the precursor fatty acids – oleic, linoleic, and linolenic – into the ω9, ω6, and ω3 polyunsaturated fatty acids apparently required by membrane phospholipids may represent a difference from normal cells. Chiappe et al. (1) have reported that both the Δ9 and Δ6 desaturases are depressed in two rat hepatomas as compared with normal liver tissue. Because the Δ5 desaturase may be under different regulation and because different tumors in different species may have distinct characteristics, the various desaturases were investigated in several human cancers.

MATERIALS AND METHODS

1-^{14}C-11,14-eicosadienoic acid was purchased from DHOM Products, Ltd. (North Hollywood, CA) and 1-^{14}C-oleic acid from New England Nuclear Corp. (Boston, MA). Both substances were over 99% pure by thin layer chromatography (TLC) and radio gas liquid chromatography (GLC) and did not contain any of the metabolic products concerned.

Tissues studied in this research were a case of chronic nonspecific ulcer (21 year old male), a case of ovarian cancer (51 year old female), a case of urinary bladder cancer (61 year old female), and a case of rectal cancer (75 year old male).

Tissues were collected during surgical procedures under sterile conditions at the City of Hope Medical Center (Duarte, CA) and were placed immediately in ice cold Hank's solution. A portion of each specimen was subjected to histological examination to confirm the malignant or normal condition of the tissues. All procedures were carried out at 4 C unless stated otherwise.

Each tissue was homogenized with 4 parts (v/w) of homogenizing buffer (2), and the homogenate was centrifuged at 600 x g for 30 min. The supernatant was centrifuged at 9,000 x g for 30 min, and the resulting supernatant was centrifuged at 105,000 g for 90 min. The pellet from the last centrifugation, the microsomal fraction, was suspended in homogenizing buffer (2:1, v/v) with sonication, and the protein content was measured by the method of Lowry et al. (3). Including the buffer solution, each vial contained 3-10 mg of microsomal protein and the following additions, in μmoles: adenosine triphosphate, 8; coenzyme A, 0.4; reduced nicotinamide adenine dinucleotide, 5; KF, 6; nicotinamide, 2; $MgCl_2$, 7.5; glutathione, 2.2: phosphate buffer, pH 7.3, 62; and 1 μc (1 μm) of substrate fatty acid (in solution in 0.05 ml 0.1 *N* NH_4OH and 0.15 ml 1% Triton) in a total volume of 4 ml 0.15 *M* KCl and 0.25 *M* sucrose. The final pH of the solution was 7.1. The vials were shaken for 30 min at 37 C, and the reaction was stopped by addition of 0.1 ml of 9*N* H_2SO_4. The total lipid was extracted by the method of Bligh and Dyer (4). After evaporation of the chloroform at 40 C under N_2, the lipids were subjected to methanolysis with 2 ml of 5% methanolic HCl (for up to 50 mg) for 30 min at 85 C. The methyl esters were extracted with pentane, washed with water, and freed from solvent. One portion of the methyl ester mixture was separated on 10% $AgNO_3$-impregnated silica gel plates (5) using 5% acetone in toluene as developing solution. Areas containing fatty acids with different degrees of unsaturation were located by spraying with an ethanolic solution of 2′,7′-dichlorofluorescein and by comparison with authentic standards (Nu-Chek Prep, Inc., Elysian, MN). Each fraction was scraped off the plate, and the radioactivity in each area was measured in the liquid scintillation spectrometer (Packard, Model 3003).

A second portion of the methyl ester mixture was separated by TLC as described above, and each fraction was further analyzed by radio gas chromatography using the Packard gas chromatograph with gas proportional counter model 894, fitted with a 6 ft x 4 mm glass column packed with Apolar 10C (Applied Science Laboratory, State College, PA) and operated at 185 C column temperature. Peaks were identified, where possible, by comparison with authentic standards.

RESULTS

With 20:2ω6 as substrate, radioactivity was found in the areas of the TLC plate corresponding to diene, triene, and tetraene (Table I). The tetraenoic acid was identified as arachidonic (5,8,11,14-20:4) by comparison of its GLC retention time with that of an authentic standard. The retention time of the single component of the triene zone was the same as that of 5,11,14-20:3. Ozonolysis was carried out with both fractions (6) but, because of the small amounts involved, was not definitive. Throughout the experiment, the total radioactivity of tetraene was higher than that of triene.

In Table II can be seen the results of experiments designed to test the activity of the Δ6 desaturase. In the urinary bladder cancer, enzyme activity, as measured by diene production per mg microsomal protein, appeared to be decreased as compared with that of normal

TABLE I

Products Formed from 1-^{14}C-11,14-Eicosadienoic Acid on Incubation with Microsomal Fractions from Human Cancerous and Normal Tissues[a]

Tissue	Radioactive Fatty Acids Isolated (%)					
	11,14-20:2	20:3		5,8,11,14-20:4		
Normal colonic	83	7	1.9[b]	10	2.7[b]	
Ovarian cancer	92	3	0.7[b]	5	1.1[b]	
Urinary bladder cancer	90	4	0.2[b]	6	0.4[b]	
Urinary bladder normal	92	3	1.1[b]	5	1.8[b]	
Colonic cancer	83	6	1.8[b]	11	3.1[b]	
Normal colonic	87	6	0.7[b]	7	0.7[b]	

[a]Each vial contained, in 4 ml, 3-10 mg microsomal protein and the following additions, in μmoles: adenosine triphosphate, 8; coenzyme A, 0.4; reduced nicotinamide adenine dinucleotide, 5; potassium fluoride, 6; nicotinamide, 2; $MgCl_2$, 7.5; glutathione, 2.2; phosphate buffer, pH 7.3, 62; and 1 μc (1 μm) of substrate fatty acid (in solution in 0.05 ml 0.1 *N* NH_4OH and 0.15 ml 1% triton). Vials were incubated 30 min at 37C, and fatty acid methyl esters were prepared and quantitated using radio gas chromatography.

[b]Expressed as percent of radioactive fatty acids produced per mg of microsomal protein.

surrounding tissue. In the colonic cancer, however, the Δ6 desaturase activity appeared to be increased.

DISCUSSION

Following incubation of 11,14-eicosadienoic acid with the microsomal fraction from human cancerous and noncancerous tissues, radioactivity was found in diene, triene, and tetraene fatty acids. The methyl eicosatetraenoate had the same GLC retention time as methyl arachidonate and was probably identical with that acid. The methyl ester of the trienoic fatty acid had a retention time corresponding to that of 5,11,14-20:3, the expected product from a liver system (7), but could not be further identified because of the small amount of product available. The diene zone yielded only the starting substrate, 20:2ω6, and the 1-^{14}C-11,14-eicosadienoic acid used in the experiment was shown to be pure by radio GLC and not to contain any radioactive 18:2.

Thus, there is a need to explain the origin of the arachidonate. In the incubation system, KF was used to inhibit chain elongation, and retroconversion of the 11,14-20:2 to 9,12-18:2 followed by elongation and desaturation to arachidonate (8) was shown to be unlikely because a trial experiment using 1-^{14}C-18:2 did not yield arachidonate under these conditions.

TABLE II

Products Formed from 1-^{14}C-Oleic Acid on Incubation with Microsomal Fractions from Human Cancerous and Normal Tissues[a]

Tissue	Radioactive Fatty Acids Isolated (%)		
	9-18:1	6,9-18:2	
Urinary bladder cancer	97.5	2.5	0.14[b]
Urinary bladder normal	99	1.0	0.38[b]
Colonic cancer	98.8	1.2	0.34[b]
Normal colonic	98.8	1.2	0.14[b]

[a]Each vial contained, in 4 ml, 3-10 mg microsomal protein and the following additions, in μmoles: adenosine triphosphate, 8; coenzyme A, 0.4; reduced nicotinamide adenine dinucleotide, 5; potassium fluoride, 6; nicotinamide, 2; $MgCl_2$, 7.5; glutathione, 2.2; phosphate buffer, pH 7.3, 62; and 1 μc (1 μm) of substrate fatty acid (in solution in 0.05 ml 0.1 *N* NH_4OH and 0.15 ml 1% triton). Vials were incubated 30 min at 37C, and fatty acid methyl esters were prepared and quantitated using radio gas chromatography.

[b]Expressed as percent of radioactive fatty acids produced per mg of microsomal protein.

The alternative pathways are (a) conversion of 11,14-20:2 to 8,11,14-20:3, which has been shown to proceed readily to arachidonate, and

(b) conversion of 11,14-20:2 to 5,11,14-20:3 and conversion of this acid to arachidonate, as has been suggested by Takagi (9) from in vivo experiments with rats but denied by Schlenk et al. (10).

Recently, Sprecher and Lee (7) have carried out experiments with rat liver microsomes that appear to deny the existence of a Δ8 desaturase and their results have been confirmed in this laboratory. In our experiments with human tissues, however, more than 10% of substrate 11,14-20:2 was converted to arachidonate in some cases, and either of the possible routes would require desaturation at the 8 position.

As Nakazawa et al. have reported previously (11), the phospholipid and triglyceride fatty acid composition of biopsied human liver tissues were distinctly different from those of corresponding rat tissues, and it is possible to consider the existence of a Δ8 desaturase in human tissue. Further study will be necessary to clarify the route from 20:2ω6 to arachidonate and the ω6 pathway in human cancerous and normal tissues.

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LETTER TO THE EDITOR

Calculation of the Rate of Utilization of Albumin-bound Free Fatty Acids from Specific Radioactivity Data

Sir: The rate of uptake of free fatty acid (FFA) by the isolated perfused rat liver, when calculated from differences in portal and hepatic venous concentrations of mass of FFA (μmoles/ml), was observed previously to be less than that calculated from simultaneous measurements of radioactivity of FFA (dpm/ml) (1). The difference in rate of uptake of FFA between that estimated from chemical determinations and that from radioactive measurements was explained by the existence of two distinct and poorly miscible pools of albumin-bound FFA, one of which turns over rapidly and one slowly (2).

Since there appears to be poor equilibration between the radioactive tracer and the total mass of FFA due to the existence of these distinct pools of serum albumin-bound FFA, it may be expected that the rate of utilization of FFA calculated from specific radioactivity of the perfusate FFA in vitro, or plasma FFA in vivo, following a single injection or constant infusion of a radioactive fatty acid may be incorrect in comparison to that calculated from the chemically measured uptake of FFA. (The uptake of the total exchangeable pool of plasma FFAs by the liver can be measured accurately with either palmitic or oleic acid as a tracer, although differences are observed in the fractional uptake of individual fatty acids from a mixture of FFA [3]).

This expectation was confirmed in recent studies which evaluated the conversion of [1-^{14}C] oleic acid to various metabolic products by the isolated liver. Livers isolated from fed female rats were perfused with different amounts of oleic acid under steady state conditions. The basic perfusate consisted of defibrinated rat blood and Krebs-Henseleit bicarbonate buffer (2:1, v/v), pH 7.4. A complex of [1-^{14}C] oleic acid and bovine serum albumin containing 15.0±1.3, 54.5±0.7, or 111.3±3.7 μmoles was added to the medium at 0 hr, and 34.8±3.0, 126.2±1.6, or 258.1±8.7 μmoles were infused per hr for groups I, II, and III, respectively. Aliquots of

TABLE I

Rates of Uptake and Incorporation of Free Fatty Acid by Perfused Rat Liver[a]

		Incorporation of exogenous FFA into total metabolites[c] calculated from specific activities of:			
	[A]	[B]		[C]	
	Uptake of FFA[b]	FFA taken up by the liver[d]		FFA in perfusate plasma[e]	
Group	(μmoles/g liver/hr)	(μmoles/g liver/hr)	(B/A) (100)	(μmoles/g liver/hr)	(C/A) (100)
I	3.4 ± 0.4	3.3 ± 0.3	96.6 ± 3.0	12.7 ± 0.9	377.6 ± 33.2
II	13.2 ± 0.7	13.3 ± 0.7	101.3 ± 3.0	19.8 ± 2.6	149.5 ± 14.2
III	28.3 ± 1.1	26.6 ± 1.3	94.5 ± 5.8	30.2 ± 2.3	107.8 ± 11.4

[a]Liver from female rats was perfused with increasing amounts of [1-^{14}C] oleic acid (groups I, II, and III) under steady state conditions. Data in table are mean values ± standard error of the mean (n=4).

[b]Uptake of FFA (μmoles/g liver/hr) between T_1 (1 hr after start of infusion of FFA) and T_4 (3 hr later) was calculated as follows: uptake = ($[FFA]_{T1}$ + [FFA] infused - $[FFA]_{T4}$)/(3) (liver wt). Concentrations of FFA were determined chemically. [FFA] = (μmole/ml) x (total volume).

[c]μMoles of oleic acid incorporated into total products esterification and oxidation = total radioactivity recovered in metabolites ÷ by specific activity of the FFA taken up by the liver (d) or specific activity of FFA in the perfusate plasma (e).

[d]Specific activity of the FFA was calculated from the radioactivity (dpm/g liver/hr) and mass (μmoles/g liver/hr) of the FFA actually taken up by the liver. Uptake of radioactivity was calculated as in b, except that radioactivity in FFA, separated by thin layer chromatography from total lipids, was substituted for chemical mass.

[e]The specific activity of the total FFA in the perfusate plasma was calculated from the radioactivity (dpm/ml) and concentration (μmoles/ml) of FFA after separation by thin layer chromatography from lipid extracts of aliquots from the perfusate plasma taken at T_1 and T_4.

perfusate were taken for analysis 1 hr and 4 hr after addition of the fatty acid to the medium. The average concentrations of FFA in the cell-free perfusate were 0.27±0.01, 0.43±0.03, and 0.89±0.07 μmoles/ml, for groups, I, II, and III, respectively. The percent of FFA taken up by the liver recovered in total metabolic products of esterification and oxidation, in liver plus perfusate, was about 95-100% in all groups when the specific activity of FFA actually taken up by the liver was used for this calculation (Table I). In contrast, the values calculated from the specific activity (dpm/μmole) of the FFA in the perfusate plasma were significantly ($P<0.05$) higher, particularly in group I; these data are incompatible with actual mass values.

Clearly, the metabolic disposition of albumin-bound FFA cannot be estimated precisely from its specific radioactivity in the perfusate in these in vitro experiments, particularly when the molar ratio FFA:albumin is low and the proportion of the slow turnover pool of FFA relative to that of the fast turnover pool becomes larger. These sources of error may apply also to in vivo studies and should be considered in calculation of the data.

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[Received September 29, 1975]

ERRATUM

In the title and abstract of the paper "Occurrence of 7-Methyl-7-Hexadecenoic Acid, the Corresponding Alcohol, 7-Methyl-6-Hexadecenoic Acid, and 5-Methyl-4-Hexadecenoic Acid in Sperm Whale Oils" by Pascal, J.C., and Ackman, R.G., Lipids 10(8) 478 (1975), "5-methyl-4-hexadecenoic acid" should read "5-methyl-4-tetradecenoic."

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Contribution towards the Glyceride Structure of Kamala Seed Oil

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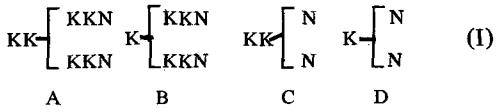
ABSTRACT

Kamala (*Mallotus philippinensis*) oil was crystallized using acetone at temperatures progressively lowered from ambient to -15 C. The five fractions thus obtained were subjected to intensive analysis using a variety of methods. These included thin layer chromatography before and after hydrogenation, cryoscopic mean molecular wt determination, fatty acid analysis by ultraviolet absorption and by gas liquid chromatography of the hydrogenated silylated esters, determination of total unsaturation using the Rosenmund-Kuhnhenn reagent, hydroxyl value determination, nuclear magnetic resonance, lipase hydrolysis, ester number determination both as ferric hydroxamate complexes and infrared absorption, and glycerol determination by silylation followed by gas liquid chromatography. The evidence is consistent with the presence in kamala seed oil of polyacid glycerides carrying from 3 to 8 fatty acids.

INTRODUCTION

In 1962, Achaya and Aggarwal (1) reexamined the analytical data of Kapadia and Aggarwal (2) on fractions of kamala (*Mallotus philippinensis*) seed oil (KSO) crystallized using acetone at progressively lower temperatures ranging from ambient to -60 C. They noted that all the fractions contained glycerol, that a high mol wt was associated with a low glycerol content and a low hydroxyl value, and that the molar ratios of kamlolenic (K) acid to other normal fatty acids in these fractions were 6:2, 5:2, 2:2 and 1:2. Achaya and Aggarwal (1) suggested that KSO consisted of the following four glyceride types. In these the hydroxyl group of 18-hydroxy-9,11,13-octadecatrienoic acid (K acid) was esterified with the carboxyl group of another K acid or of a normal fatty acid (N).



(I)

The estimated molar proportions of these glycerides were 40, 18, 17, and 25%, respectively. Each glyceride type had one free hydroxyl group at the end of the KK or K moiety in the 2-position of the glyceride. Symmetrical glyceride types were preferred to asymmetrical types on the basis of glyceride esterification concepts. This also satisfied the analytical requirements.

The present work was designed to test the presence of these four glyceride types in KSO. After acetone crystallization of KSO into five fractions (2), each of these fractions was examined by a number of analytical procedures. Use was also made of special techniques, such as ester group determination by a ferric hydroxamate colorimetric procedure, ester group estimation by IR, glycerol determination by a new GLC procedure, lipase hydrolysis, and NMR. Part of this work was briefly reported in 1965 (3). This and earlier evidence is reported in a recent review of glyceride structure (4).

EXPERIMENTAL PROCEDURES

Extraction of Oil

When small lots of fresh KSO were needed, the kernels of kamala seed were inspected, discolored seeds rejected, and the white kernels ground with anhydrous sodium sulphate and Soxhlet extracted with diethyl ether. To avoid polymerization during isolation, KSO and its fractions were brought to volume in ether or benzene, and the stock solution preserved under refrigeration. Aliquots were used for individual analytical estimations.

Crystallization of Oil

KSO (200 g) was refluxed with acetone (2 liters) for 30 min, and the solution was cooled and kept at ambient temperature (27 ± 2 C) for 2 days. The lower viscous mass (Fraction I, 80.5 g, 40.3%) was maintained in a refrigerator with solvent benzene added as a stabilizer. The upper acetone layer was decanted, partly evaporated to 1200 ml, and kept at 0 C for 24 hr to yield a precipitate (Fraction II, 43.3 g, 21.6%). The filtrate was crystallized from 1200 ml acetone at -15 C for 24 hr to yield another precipitate (Fraction III, 19.9 g, 10.0%). The

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filtrate constituted Fraction IV. Later, this filtrate was resolved from 420 ml acetone at -15 C for 24 hr to give a precipitate (Fraction IV-N, 36.3 g, 18.1%) and solubles (Fraction V, 20.0 g, 10.0%).

Fraction I (40.3%), separated in our investigation, is less in wt than the corresponding fraction (53.2%) obtained by Kapadia and Aggarwal (2). The present Fraction III corresponds analytically to their Fraction II, while the present Fraction V (10.0%) resembles their Fractions IV + V + VI (11.3%).

TLC of KSO and Hydrogenated KSO

Separation of KSO on silica gel TLC plates using numerous solvent systems, followed by visualization using charring or dichlorofluorescein spraying, usually resulted in streaks, suggesting polymerization on the plate. As KSO could not be separated on silica gel TLC plates, it was hydrogenated in ethyl acetate using 10% palladium on carbon at 25 psi hydrogen pressure for 48 hr. After shaking at room temperature for 24 hr, fresh catalyst was added, and shaking resumed for 24 hr. Hydrogenated KSO samples obtained in this way were sticky, semisolid materials, and had iodine values (IV) ranging from 50 to 100. They were poorly soluble in solvents except chloroform. Hydrogenation reduced polymerization tendencies without altering the glyceride linkages in KSO. Separation of hydrogenated products by TLC or crystallization was unsuccessful.

Separation of KSO by silicic acid chromatography as described by Evans, et al., (5) was also attempted, but the resolution claimed by them could not be achieved.

Mean Mol Wt Determination by Cryoscopy

Using a Beckman freezing point apparatus and benzene as solvent (molar freezing pt depression = 5.12), mean mol wt determinations were made on peanut oil as a control (mean mol wt = 877), on KSO, and on the 5 KSO fractions.

Fatty Acid Composition Determined by UV Absorption

Pure kamlolenic acid (mp 86.7 C), prepared by extraction of KSO acids with light petrol followed by repeated crystallization of the product using ethyl acetate, showed in cyclohexane a specific extinction coefficient ($E^{1\%}_{1\,cm}$) at 270 nm of 1813 which is in agreement with that reported by Gupta, et al. (6). Aliquots of the mixed acids of KSO and the other crystallized fractions were examined in cyclohexane at 270 nm, and the wt-% of K in each determined. Percent N acids were given as 100-K.

Analysis by GLC

Two materials, KSO and Fraction V, were analyzed by GLC. An F&M Model 1609 with flame ionization detector was used. Mixed methyl esters of KSO prepared via the acids, were analyzed at 200 C on an 8 ft x 3/16 in column of 20% DEGS on Chromosorb W (40-60 mesh). They yielded a broad, tailed peak of methyl kamlolenate. Triangulation of peaks for known esters, taking areas as proportional to wt-% and ignoring kamlolenate, showed 16:0 (13.0%); 18:0 (8.7%); 18:1 (26.1%); and 18:2 (52.2%). Total hydrogenation of KSO acids, followed by esterification and GLC separation on a polyester column, gave a better defined hydroxy ester peak, but the area of 55% was lower than expected for KSO.

Silylation (7,8) of the hydrogenated methyl ester using hexamethyl disilazane and trimethylchlorosilane, followed by isothermal GLC resolution (200 C) on a 2 ft column of SE-30 (2% on 40-60 mesh Chromosorb W), gave clear peaks for 16:0, 18:0, and 18-hydroxy-stearate (retention times = 2.24, 18:0 = 1.00).

It has been shown earlier (9), using ricinoleate mixtures, that silylation of a hydroxyl group necessitates a downward peak area correction of 18/21 or 6/7 to allow for the 3 extra carbons introduced. The composition of KSO was calculated on the basis of combined GLC data. Fraction V was analyzed similarly.

Determination of Total Unsaturation

The Rosenmund-Kuhnhenn reagent (pyridine-bromine sulphuric acid-mercuric acetate) as employed by Planck, et. al., (10) was used to determine the total unsaturation of KSO and its fractions containing conjugated systems. Triplicate IV by this method for pure kamlolenic acid averaged 250.3 (calc. 258.9).

Hydroxyl Values

The cold acetylation procedure of Pathak and Aggarwal (11) using acetyl chloride in anhydrous ether, followed by saponification value determination, was employed on KSO and its fractions.

Analysis by NMR

Solutions of KSO and its crystallized glycerides in carbon tetrachloride (ca. 15% concentration) were scanned in a Varian A60-A instrument (Riker Laboratories, Northridge, CA). Integrals of the peak at 0.9-1.0 ppm for terminal methyl and 3.9-4.3 ppm for ester methylene protons were compared with values for peanut oil used as a reference material. Tetramethylsilane was used as the reference signal.

Lipase Hydrolysis

Short term (2 min) lipolysis using pancreatic lipase (steapsin, Nutritional Biochemical Corp., Cleveland, OH) according to the semi-micro (50 mg glycerides) procedure of Luddy, et. al., (12) was applied to hydrogenated Fraction V. This was followed by silica gel TLC using a solvent system of chloroform:acetone:ammonium hydroxide (80:20:1). Fatty acids (at the origin) and monoglycerides (Rf ca. 0.3) were isolated, esterified, silylated, and analyzed by GLC. For hydrogenated KSO, the lipolysis time was increased to 5 min.

Ester Group Determination by Chemical Method

Esters react with hydroxylamine to form hydroxamic acids, which, in turn, can react with ferric ion to form a red complex. The procedure used was that of Snyder and Stephens (13) employing alkaline hydroxylamine and ferric perchlorate. The precautions stressed by Skidmore and Entenman (14) regarding water content during the reaction of ester groups with hydroxylamine were observed. Scrupulous cleanliness was maintained in the glassware. KSO and its fractions were measured as aliquots in benzene solution, alkaline hydroxylamine added, and the mass boiled down to yield the hydroxamic derivative. This was dissolved in 10 ml of a 1:1 benzene:ethanol mixture. Addition of acidic ferric perchlorate gave a clear, wine red solution which was immediately examined at 530 nm. Use of alcohol instead of a benzene:alcohol mixture led to hazy solutions with KSO glycerides. Good linearity was obtained with peanut oil and methyl oleate in various concentrations with the plots also passing through the origin. Averages of duplicate values for 4 concentrations of each fatty material were used. The number of ester groups was calculated on the basis of absorbance, concentration, and mean mw. The mean mw was determined by cryoscopy, assuming a value of three ester groups for peanut oil.

Ester Group Determination by IR Absorption

Acyloxy groups of esters gave rise to two strong characteristic frequencies: A) the C=O stretching vibration at ca. 1730 cm^{-1}; and B) the C-O stretching vibration at ca. 1155 cm^{-1}. Because the former peak was stronger and comparatively isolated, it was employed for the present purpose. KSO and its fractions were scanned in 15% carbon tetrachloride solution in 0.1 mm cells on a Perkin-Elmer Model 221 spectrophotometer. Peanut oil was scanned at two concentrations (3 and 6%). The actual absorbance was calculated by subtracting the baseline absorbance connecting the intersections at 1670 and 1780 cm^{-1} from the absorbance at the tip of the absorption peak. The number of ester groups in KSO and its glycerides was calculated on the basis of absorbance, concentrations, and mean mw, again assuming a value of three ester groups for peanut oil.

TABLE I

Comparison of Two Methods of Analysis of Kamala Seed Oil

Component acids	Present study (wt-%)	Kapadia and Aggarwal (2) (wt-%)
16:0	3.2	10.7[a]
18:0	2.2	---
18:1	6.9	9.3
18:2	13.6	11.2
Kamlolenic acid	72.0	68.8
Unknown ester[b]	2.1	---

[a]Total wt-% of 16:0 + 18:0.

[b]Polyester equivalent chain length (ECL) 20.7; silicone ECL 22.6.

Glycerol Determination

Rajiah, et. al., (15), have described the GLC estimation of glycerol extracted from saponified glycerides with pyridine. It was resolved as the trimethylsilyl ether on a silicone column using hexadecane as an inert internal standard. The procedure was standardized using pure glycerol solutions, and was applied to glycerol determination in a number of fats, including KSO and its fractions.

RESULTS AND DISCUSSION

The results of certain analytical methods will be discussed separately before the quantitative tabulated data are reviewed.

The present GLC analysis of KSO is probably more accurate than the analysis by Kapadia and Aggarwal (2) using classical methods. A comparison of the data from the two studies is given in Table I. UV analysis in the present study showed 70.0% of K acid in KSO. For Fraction V, GLC analysis of the hydrogenated esters showed 16:0, 9.4 wt-%; 18:0, 57.6 wt-%; 18-hydroxystearate, 33.0 wt-%. (K acid was 33.7 wt-% by UV analysis.)

Integration of the NMR peaks at 0.9-1.0 ppm for terminal methyl protons and at 3.9-4.3 ppm for ester methylene protons as recorded for peanut oil, KSO, and fractions of KSO are presented in Table II.

The ratios of terminal methyl protons to ester methylene protons are lower than calculated for peanut oil and KSO, and higher than calculated for Fractions II, III, and IV. The

TABLE II

Integration of NMR Peaks of Peanut Oil, Kamala Seed Oil (KSO), and Fractions of KSO

Glyceride sample	NMR peaks 0.9-1.0 ppm (relative area)	NMR peaks 3.9-4.3 ppm (relative area)	Area ratio
Peanut oil	2.7	1.3	2.08
KSO	1.2	2.0	0.60
Fraction I[a]	--	--	--
Fraction II	1.35	2.5	0.54
Fraction III	1.3	2.4	0.54
Fraction IV	2.5	1.55	1.61

[a]Fraction I was lost during collection.

TABLE III

Composition of Hydrogenated Fraction V and 2-Monoglyceride Derivative of Fraction V of Kamala Seed Oil

Glyceride sample	Mole-% normal acid	Mole-% hydroxystearic acid
Fraction V	68	32
2-Monoglyceride	18	82

TABLE IV

GLC Analysis of Total Hydrogenated Kamala Seed Oil and Acids Cleaved by Lipase

Glyceride sample	Mole-% normal acid	Mole-% hydroxystearic acid
Total hydrogenated KSO	29	71
Acids cleaved by lipase	93	7

calculated ratios of terminal methyl protons to ester methylene protons in peanut oil (9/4 or 2.25) and in glyceride type B (6/12 or 0.50) have a 4.5:1 relationship to each other. The NMR ratios determined in this study (2.08; 0.54) are related in approximately the same order.

For short term lipase hydrolysis, hydrogenated Fraction V and hydrogenated KSO were employed as substrates. The composition of hydrogenated Fraction V, and of the 2-monoglyceride obtained from it, are shown in Table III. This fraction is believed to consist almost entirely of the glyceride type N-K-N, and the lipase data are in good accord.

With regard to total KSO, assuming that the postulated glyceride types do exist, and that lipase cleaves the 1- and 3-glyceride ester linkages in normal fashion, the 2-monoglyceride moiety derived from KSO should consist of -KK- and -K-. The TLC migration characteristics of -K- are fairly normal (Rf 0.3). However, TLC migration of -KK-, which would comprise the greater proportion of the total, monoglyceride, is not known. Accordingly, only the fatty acids cleaved from hydrogenated KSO by lipase were collected as the ammonium soaps at the origin, subjected to GLC analysis as silylated methyl esters. The data are presented in Table IV.

If the postulated structures are correct, and

TABLE V

Experimental Data on Kamala Seed Oil (KSO) and KSO Fractions

Characteristics	Whole KSO	Fr. I	Fr. II	Fr. III	Fr. IV	Fr. IV-N	Fr. V
Wt-%	100.0	40.3	21.6	10.0	28.1	18.1	10.1
Mole-%	100.0	29.8	17.5	8.7	44.0	24.9	19.1
Mean mw	1702	2260	2054	1932	1068	1227	890
Glycerol (wt-%)	5.3	4.0	4.3	4.6	8.7	7.3	10.2
Hydroxyl value	26.2	26.9	28.3	30.7	50.2	49.2	64.3
Total iodine value	214.3	222.1	217.6	216.4	182.2	192.7	149.4
No. of ester groups							
Chemical determination	6.1	10.9	10.2	9.1	3.5	--	--
IR	5.2	11.5	10.0	8.0	3.3	3.5	2.8
Component acids (UV analysis)							
Kamlolenic acid (mole-%)	68.8	73.3	71.3	68.5	46.6	49.0	32.5
Normal fatty acids (mole-%)	31.2	26.7	28.7	31.5	53.4	51.0	67.5

TABLE VI

Calculated Characteristics of Postulated Kamalas Seed Oil Glyceride Types[a]

Characteristics	Type A	Type B	Typce	Type D
Mol wt	2270.2	1993.8	1164.6	888.3
Glycerol (wt-%)	4.1	4.6	7.9	10.4
Hydroxy value	24.7	28.1	48.2	63.2
Total iodine value	230.2	223.8	186.9	159.6
No. ester groups	8	7	4	3
Mole-% K[b] acid in acids	75.0	71.4	50.0	33.3
No. terminal methyl protons/ No. ester methylene protons (ratio)	6/14 (0.43)	6/12 (0.50)	6/6 (1.00)	6/4 (1.50)

[a]Using mol wt of 294.4 for kamlolenic acid and mol wt and iodine value of 277.9 and 118.0, respectively, for normal acids.

[b]K = kamlolenic acid.

if lipase cleaves 1- and 3-position ester linkages attached to glycerol, the cleaved acids should contain both K and N acids, whereas, the results show almost exclusively N acids. This could mean A) that only the terminal primary ester link in glycerides like KKN-KK-KKN and KKN-K-KKN is being attacked by lipase to yield N acids, or B) lipase is able to act only on the simpler triglycerides present, such as N-KK-N and N-K-N, particularly the latter. The cleaved lipase data on KSO and Fraction V are not definitive, but are not inconsistent with the glyceride types postulated to occur in KSO.

Total Analytical Evidence

Table V shows the experimental data obtained on KSO and its fractions. The calculated analytical data for the 4 glyceride types postulated to be present in KSO are shown in Table VI.

The mean mw of the fractions are in line with their expected compositions. Fraction I closely corresponds to glyceride type A, Fraction III to type B, Fraction IV-N to type C and Fraction V to type D. Fraction II is a mixture of types A and B. Thus, as in the earlier work of Kapadia and Aggarwal (2), crystallization has resulted in nearly perfect resolution into glyceride types, perhaps because their characteristics and properties differ so widely from each other.

The glycerol contents determined by GLC are consistent with the expected compositions, and with those determined by Kapadia and Aggarwal (2) on their own crystallized KSO fractions. The hydroxyl values are also consistent with composition. The total iodine value was not determined in the earlier crystallization work (2), but the present values are consistent with the expected compositions.

Determination of the number of ester groups by the ferric hydroxamate colorimetric procedure shows values for the fractions which are higher than expected. Thus, Fraction I, which should carry eight ester groups if it were mainly composed of glyceride type A, shows an ester number of 10.9. On the other hand, KSO itself shows an ester value of 6.1 as compared to a calculated figure from its glyceride type composition of 6.3. This could indicate that esters of even greater complexity than octaacid glyceride are present. Alternatively, it is possible that the analytical method is itself unsatisfactory. This is the first application of it to products of such complexity. It does show that in mono-, di-, and triglycerides, the slopes of the calibration curves are not the same per ester group (16). Possibly, a correction factor related to mol wt, not apparent in materials like methyl oleate or ordinary triglycerides, may be involved.

IR estimation of ester groups also gave lower values than expected for KSO (5.2) and for Fraction V (expected value = 3.0; obtained value = 2.8), but higher values for all the other KSO fractions. Two concentrations of peanut oil gave values for absorbance/concentration which differed by ca. 5%, showing that concentration played a part in the ester material. Because 1 meq of an ester group does not have an absolute value as obtained from its absorbance, but rather, is related to other structural features, the present ester group determinations using IR are only qualitatively indicative of the presence of multi-ester groupings. Component acid analyses in terms of K acid and N acids using UV absorption are again consistent with the proposed glyceride types in the various fractions.

Glyceride Types in KSO

The present crystallization operation and analytical evidence fully substantiates the results of Kapadia and Aggarwal (2), but has yielded slightly different proportions (mol-%) of the postulated glyceride types. The similarity

between the molar proportions of Type A + Type B (56%), and of Type C + Type D (44%), in the present analysis and in the earlier one (58,42% respectively), could be fortuitous. Both the present and previous KSO samples had very similar mean mw (1702 vs. 1686). While the present experimental evidence does not provide unequivocal proof of the existence in KSO of polyacid triglycerides, none of it is inconsistent with such structures.

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Bile Acid Pool in Wistar Rats

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ABSTRACT

The bile acid pool was found to be ca. 50 μmoles/100 g body wt in male and female rats maintained on standard laboratory chow and ca. 30 μmoles/100 g body wt in those maintained on a standard semisynthetic diet. The distribution of bile acids within the pool was similar in plasma, liver, and intestinal tract, except for a higher concentration of deoxycholic acid in the intestinal tract. Sex differences in bile acid composition were found to be influenced by the dietary regimen of the animals.

INTRODUCTION

The bile acid pool of the rat has been estimated by analysis of the bile acids of the intestinal contents (1), of the bile produced after creation of a biliary fistula (2,3), and of the portal blood (4). It has also been estimated more indirectly by isotopic means (5,6). The pool size in these studies has ranged from ca. 12 to 25 μmoles bile acid/100 g body wt (7,8). From studies on the feedback regulation of bile acid synthesis in the rat, Shefer et al. (9) have estimated that the pool of cholyl-taurine in male rats is ca. 35-40 μmoles/100 g body wt. These workers suggested that previous estimates of the size of the bile acid pool were too low and that this might explain previous failures to demonstrate a regulatory effect of bile acids on bile acid biosynthesis (10).

Because of the controversial data in the literature and because such information was essential for the interpretation of other investigations to be conducted in our laboratory, we undertook a direct analysis of the size of the bile acid pool in the rat. Because this pool can vary with the sex (11) and nutritional status (12) of the animal and because our experimental program involved the use of a well-defined but semisynthetic diet, we studied both male and female rats on both standard laboratory chow and the semisynthetic diet.

METHODS

Wistar rats obtained from High Oak Ranch (Toronto, Canada) and weighing ca. 200 g were studied. Twenty-four animals, 12 male and 12 female, were kept in a constant temperature environment (22 C) in darkness from 7 p.m. to 7 a.m. and allowed water ad libitum. One week after admission to the animal facilities, six male and six female animals were started on a powdered semisynthetic diet (Teklad Mills, TD-72460 Basal Diet: casein 27%, sucrose 63.9%, corn oil 5%, salt mixture USP XIV 4%, and vitamin mixture 0.1%). Three weeks after admission to the animal facility and after an 18-hr fast, the animals were sacrificed by exsanguination under ether anesthesia.

As much blood as possible was removed via the aorta, the intestinal tract was removed after ligation of the distal esophagus and rectum, and the liver was removed after ligation of the common bile duct. Plasma was obtained by centrifugation of the blood at 3,000 x g for 20 min at 4 C. The intestinal tract and the liver were each homogenized vigorously in normal saline, 2 ml/g tissue, using a close fitting Teflon-glass homogenizer.

To 5 ml of plasma, and to aliquots of the intestinal tract and liver homogenates, were added tracer amounts of cholyl[1-^{14}C]glycine (CA[1-^{14}C]G) (Amersham Searle, Arlington Heights, IL, sp act 49.4 mCi/mmole) and a known amount of 5β-cholanic acid (Applied Science Laboratories, Inc., State College, PA). Portions of the plasma and tissue homogenates were then extracted with 20 volumes of 0.1% ammonium hydroxide in absolute ethanol for 4 hr at 60 C. The supernatant obtained after centrifugation at 3,000 x g for 30 min at 4 C was saved and this extraction procedure repeated twice. The three ethanol extracts were combined and evaporated to dryness under nitrogen after aliquots were taken for radioactivity analysis. Studies of the radioactivity of these extracts showed an average recovery of 90% of the added CA[1-^{14}C]G. The residue was taken up in 5N NaOH and subjected to hydrolysis at 115 C overnight in sealed tubes. The neutral lipids were removed by three extractions of the alkali solution with petroleum ether at 30-60 C and, after acidification on ice with 2.5N HCl to pH 1, the acidic steroids were removed by repeated extractions with diethyl ether. The diethyl ether extracts were pooled, checked for radioactivity, and then evaporated under nitrogen. Previous studies in our laboratory confirmed that these conditions of alkaline

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TABLE I

Distribution of Bile Acid Pool in Wistar Rats[a]

	Purina Chow diet		Semisynthetic diet	
	Male	Female	Male	Female
Intestinal tract (μmole/100 g body wt)	50.40 ± 5.10	51.6 ± 5.40	29.30 ± 0.60	27.8 ± 1.0
Liver[b] (μmole/g liver)	0.19 ± 0.01	0.33 ± 0.01	0.18 ± 0.01	0.19 ± 0.01
Blood (μmole/100 ml plasma)	2.96 ± 0.24	2.97 ± 0.22	1.99 ± 0.11	1.97 ± 0.12

[a]Values: mean ± standard error; six animals/group.

[b]Liver wt (g): male, 9.3 ± 0.5; Female, 8.1 ± 0.4 (no significant difference).

hydrolysis were equally effective for both glycine and taurine conjugated bile acids (11). The completeness of the hydrolysis was indicated by the absence of ^{14}C-radioactivity in the diethyl ether extracts and of CA[1-^{14}C]G in the aqueous layer. The bile acids were methylated, and the trifluoroacetate and trimethyl silyl derivatives of the methylated bile acids were analyzed by gas chromatography as previously described (11,13). The final concentrations of bile acid were calculated on the basis of reference to the internal standard of 5β-cholanic acid (11). Student's t test was used for the statistical analysis, with $P > 0.05$ indicating no significant difference.

RESULTS

The bile acid content of the intestinal tract, blood, and liver of the rats in these studies is presented in Table I. It can be calculated from the data that the total bile acid pool was ca. 100 μmoles in those animals on the Purina Chow diet and 60 μmoles in those on the semisynthetic diet. The reduction in pool size associated with the semisynthetic diet was reflected to an equal degree in the blood, liver, and intestinal tracts. It is apparent that 97% of the bile acid pool is located in the intestinal tract, regardless of sex or diet.

There was no sex difference in the size of the bile acid pool in these animals, nor in the concentration of bile acids in the intestinal tract or blood. However, the female rats did have a significantly higher concentration of bile acids in the liver ($P < 0.02$).

The bile acid composition of the intestinal tract, blood, and liver is presented in Table II. Cholic acid (CA) comprised 55-73% of the bile acid pool. In the intestinal tract, the relative concentrations of CA, lithocholic acid (LCA), and, in those receiving the semisynthetic diet, of chenodeoxycholic acid (CDCA) were significantly higher ($P < 0.02$) in the female rats. The relative concentrations of deoxycholic acid (DOCA), β-muricholic acid (βMCA), and hyodeoxycholic acid (HyoDOCA) were significantly higher ($P < 0.05$) in the male.

Diet did not appear to influence the relative bile acid composition of the intestinal tract of male rats. Although female rats on the laboratory chow diet appeared to have relatively more CA and relatively less CDCA than did female rats on the semisynthetic diet, the differences, in fact, were not significant.

In general, there were fewer sex differences in the bile acid composition of the liver and no significant differences between the two dietary regimens.

The bile acid composition of the plasma was not significantly different from that of the intestinal tract, except that no sex differences in the distribution of CDCA or hyoDOCA were found. As in the case of liver, there were no differences between the two dietary regimens.

DISCUSSION

This study has demonstrated that the size of the bile acid pool in Wistar rats supplied by High Oak Ranch (Toronto), weighing 200 ± 25 g, and maintained on Purina Chow diet is 40-60 μmoles/100 g body wt. This value is at least twice that of published estimates and closely approximates that calculated by Shefer et al. (9) to be required for inhibition of bile acid synthesis under basal conditions. As reported previously, the bulk of the bile acid pool is located in the intestinal tract (1), and its size and composition are affected by diet (12). In the studies reported here, the bile acid pool of animals maintained on a semisynthetic diet was only 60% of that found in animals maintained on standard laboratory chow.

The observation that both the concentration and relative composition of the bile acids in the

TABLE II

Molar Percent Distribution of Bile Acids in Wistar Rats[a]

Bile acid	Intestinal tract			Liver			Blood		
	Male	Female	P	Male	Female	P	Male	Female	P
Purina Chow diet									
Cholic acid	54.8	71.3	<0.001	62.5	69.8	NS[b]	57.8	69.4	<0.05
Deoxycholic acid	15.9	7.8	<0.001	9.1	6.0	<0.05	13.9	9.1	<0.05
Chenodeoxycholic acid	11.9	11.6	NS	11.1	10.6	NS	11.0	11.5	NS
β-Muricholic acid	7.9	2.3	<0.01	8.3	2.4	<0.001	7.4	2.4	<0.02
Lithocholic acid	0.8	1.6	<0.001	1.0	1.8	NS	0.8	1.7	<0.001
Hyodeoxycholic acid	8.7	5.4	<0.001	10.1	6.6	<0.02	9.1	7.7	NS
Semisynthetic diet									
Cholic acid	55.4	63.3	<0.01	63.3	72.9	<0.01	60.8	68.9	<0.02
Deoxycholic acid	15.1	8.9	<0.001	9.6	5.9	<0.001	13.2	8.8	<0.01
Chenodeoxycholic acid	11.1	14.6	<0.05	9.6	9.0	NS	11.1	11.7	NS
β-Muricholic acid	8.1	2.9	<0.001	7.3	2.7	<0.001	7.1	2.4	<0.001
Lithocholic acid	0.8	2.0	<0.02	1.1	1.6	NS	0.8	2.0	<0.001
Hyodeoxycholic acid	8.3	6.4	<0.05	9.0	8.0	NS	7.0	7.9	NS

[a]Six animals per group.

[b]NS = No significant difference, P >0.05.

plasma reflected changes in the bile acid content of the intestinal tract suggests that analysis of plasma bile acids might prove useful in future studies on the bile acid pool in general. There is clearly a need for much more investigation and technological work in this area.

The fact that the bile acid composition of the liver did not compare strictly with that of the intestinal tract presumably reflects differences in bile acid metabolism in these two areas. However, the similarities were more striking than the differences. Although these static quantitative values cannot be expected to reflect much of the drama of bile acid metabolism, it is of interest that the relative composition of the bile acid pool considered in these studies was so similar in the three compartments.

While substantial quantitative influences of diet were recorded in these studies, it may be reassuring that no significant qualitative influences were documented. The relative composition of the bile acid pool of the rats in these studies was essentially the same with the two dietary programs.

The results of these studies can be applied with confidence only to our own experimental program. However, they may serve to emphasize that the bile acid pool of the experimental animal varies with its age, sex, size, and strain and with the diet and other experimental conditions involved.

ACKNOWLEDGMENTS

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Cholesteryl Ester Metabolism in Tissue Culture Cells: II. Source of Accumulated Esterified Cholesterol in Fu5AH Rat Hepatoma Cells

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ABSTRACT

Previous investigations had demonstrated that Fu5AH rat hepatoma cells accumulated large quantities of esterified cholesterol when grown in hyperlipemic rabbit serum. The present investigation has determined the sources of the cellular esterified cholesterol when the cells were grown in hyperlipemic serum. Cellular esterification of endogenous and exogenous free cholesterol contributed 10% and 30%, respectively. The remaining 60% of the accumulated cellular esterified cholesterol was derived from exogenous (serum) cholesteryl esters. Evidence for the hydrolysis of a portion of the incorporated esterified cholesterol is presented. A stimulation of free cholesterol incorporation and cellular esterification is elicited by hyperlipemic serum and serum lipoproteins when compared to normolipemic serum present at equivalent exogenous cholesterol concentrations. The effect of hyperlipemic serum is reduced by Tween-80 and Triton WR-1339. Comparative data on esterified cholesterol accumulation, free cholesterol incorporation, and cellular cholesterol esterification in Fu5-5 rat hepatoma cells, L-cells, and rabbit aortic medial cells are presented.

INTRODUCTION

Accumulation of cholesteryl esters occurs in a number of pathological conditions (1). However, even in atherosclerosis, the most thoroughly investigated situation, no firm conclusions can as yet be drawn regarding the origin of the cholesteryl esters that accumulate in the aorta (2). Investigations using experimental systems ranging from whole animals (3,4) to aortic organ culture (5,6), have demonstrated that a portion of the accumulated cholesteryl ester is derived from the esterification of serum free cholesterol. Accelerated rates of esterification of cholesterol have been observed in the atheromatous vessel, presumably in response to the hyperlipemic state (6-8). Numerous studies have demonstrated that various tissues, including the aorta and liver, have the enzymatic capability of both esterifying cholesterol and hydrolyzing cholesteryl esters (1-12). Inasmuch as the cholesterol moiety of the cholesteryl ester found in tissue can be derived from a number of sources, it has been difficult to obtain firm estimates of the contribution of any one metabolic process to the accumulation of cellular cholesteryl esters.

Tissue culture provides a number of distinct advantages for the study of cholesteryl ester metabolism, among which is utilization of dispersed homogeneous cell populations in easily manipulated closed systems. Although many cells in culture have been shown to have a low cholesteryl ester content when grown in the presence of normocholesteremic sera (13), evidence has accumulated that cells are capable of incorporating exogenous cholesteryl esters (14-18), hydrolyzing cholesteryl esters (14,15,19), and esterifying free cholesterol (16,20,21). In addition, manipulation of the culture medium, particularly of the serum or serum lipoproteins present in the medium, results in either increased esterification of cholesterol and/or accumulation of cholesteryl esters (16,20-23). A previous investigation in this laboratory demonstrated that the Fu5AH rat hepatoma cell will accumulate large quantities of cholesteryl ester when exposed to hyperlipemic rabbit sera (24). This cholesteryl ester accumulation, which is not elicited by any concentration of normal serum (1-50%), is accompanied by the appearance of numerous cytoplasmic inclusions, many of which have anisotropic properties similar to those described for the cholesteryl ester-rich inclusions of atherosclerotic aorta (25). The purpose of the present investigation was to study the cellular metabolism of cholesterol in tissue culture cells exposed to hyperlipemic rabbit sera.

EXPERIMENTAL PROCEDURES

Cells and Growth Conditions

Monolayers of the tissue culture cells used in these experiments were grown in the presence

[1] This work was done during the tenure as Established Investigator of the American Heart Association.

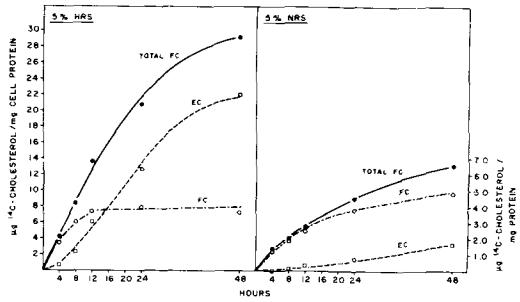

FIG. 1. Incorporation and esterification of free cholesterol by Fu5AH cells grown in 5% normal (NRS) and hyperlipemic (HRS) rabbit serum. ●——● total exogenous free cholesterol (FC) incorporated, □– – –□ exogenous FC recovered as cellular esterified cholesterol (EC), ○– – –○ exogenous FC recovered as cellular FC.

of normo- or hypercholesteremic sera using experimental conditions previously described (24). The Fu5-5 and Fu5AH rat hepatoma cell lines were derived from the Reuber H35 rat hepatoma (26-29). Cultures of rabbit aortic medial cells were initiated from aortic explants (30,31). All cells were grown in Eagle's minimal essential medium (MEM; Auto-Pow, Flow Labs, Rockville, MD) supplemented with 10% fetal calf serum. Cells obtained from a single 60 mm petri dish (Falcon Plastic, Oxnard, CA) provided sufficient material for routine isotopic and gas liquid chromatographic (GLC) analysis.

All cells were harvested when monolayers were ca. three-quarters confluent. Isolated lipoprotein fractions were added to the tissue culture medium in the absence of any other proteins or whole serum.

Serum and Serum Lipoproteins

Normal rabbit serum (NRS) was obtained from animals maintained on rabbit chow. Unless otherwise noted, the cholesterol induced hyperlipemic rabbit sera (HRS) was obtained from rabbits fed with rabbit chow augmented with 2% cholesterol and 6% corn oil (32). All animals were fasted for 24 hr prior to bleeding. Total cholesterol (TC) levels in such sera were in the range of 1,000-3,000 mg/dl, with esterified cholesterol (EC): free cholesterol (FC) ratios between 2.6-3.5. Surfactant induced hyperlipemic serum was obtained from rabbits that had received three intraperitoneal injections (250 mg) of Triton WR-1339 (Ruger Chemical Co., Philadelphia, PA) over a period of 9 days. The serum obtained from these animals had a TC of 800-1,500 mg/dl and EC:FC ratios averaging 0.2. In vitro treatment of sera with surfactant was accomplished by the addition to the sera of appropriate dilutions of Triton WR-1339 or Tween 80 (Atlas Chemical Industries, Wilmington, DE). This material was incubated 18 hr at 37 C prior to addition to cell cultures. Hyperlipemic rabbit serum was also obtained from animals maintained for 6 months on a cholesterol free, semipurified diet formulated to produce hyperlipemia (33). EC:FC ratios ranged from 2.9 to 3.4. Cholesterol values for these sera are presented in the text. Unless otherwise noted, sera were labeled with 4-^{14}C cholesterol (250 mCi/mM, New England Nuclear, Boston, MA) by addition in ethanol (34) at a final concentration of 0.1 μCi/ml of tissue culture medium. Ethanolic solutions containing the labeled FC were added to serum and held 18-24 hr at 4 C before the serum was added to tissue culture medium. Esterification of the added FC ranged from 0 to 0.5%. Ethanol concentrations never exceeded 0.5% in the medium. In all experiments using 4-^{14}C cholesterol labeled lipoproteins, the whole sera were labeled prior to lipoprotein separation using the celite exchange procedure of Avigan (35). Radiolabeled HRS containing both 7α-^{3}H-cholesterol and 7α-^{3}H-cholesteryl esters was obtained from hyperlipemic rabbits 48 hr after intubation of 1 mCi 7α-^{3}H-cholesterol (12.1 Ci/mM, New England Nuclear). Isolated lipoprotein fractions were obtained from normal and hyperlipemic sera by sequential centrifugation (No. 40 rotor, Spinco, Au: city, state, please) in KBr solutions (36,37). The following density classes were obtained: d $<$ 1.006 g/ml (30 min, 26,000 x g), d $<$ 1.019 (16 hr, 110,000 x g), d 1.019-1.063 (20 hr, 110,000 x g), d 1.063-1.21 (48 hr, 110,000 x g). Each fraction was washed by recentrifugation in KBr solutions of appropriate density. In some experiments, noted in the text, the lipoprotein d 1.063-1.21 g/ml was washed by adjusting this lipoprotein fraction to d 1.063 g/ml, recentrifuging (110,000 x g, 48 hr), and collecting the material in the lower one-third of the tube. Purified lipoprotein fractions were dialyzed for 24 hr against 0.15 M NaCl containing 1.0 mM ethylenediaminetetraacetic acid, followed by dialysis for 24 hr in serum-free tissue culture medium.

Analytical Procedures

Specific analytical procedures have been detailed previously (24). Lipid extracts were analyzed for FC and EC content using GLC techniques (24). TC content was determined on material saponified by the method of Ishikawa et al. (38). FC and EC were separated by thin layer chromatography on Silica Gel G strips (Baker-flex, J.T. Baker Chemical Co., Phillipsburg, NJ) developed in petroleum ether:ethyl ether:acetic

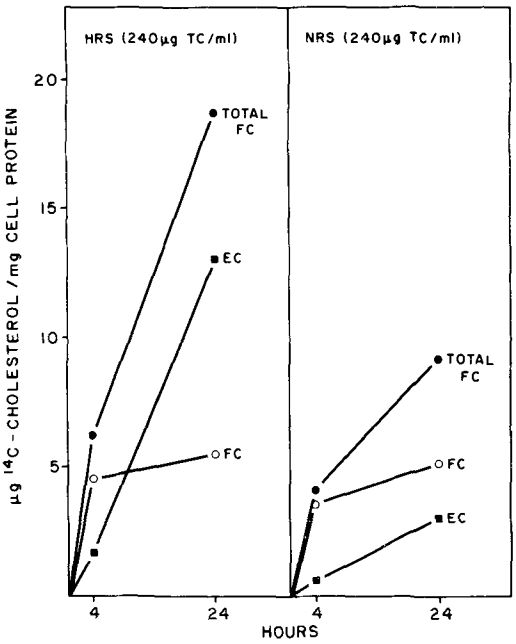

FIG. 2. Incorporation and esterification of free cholesterol (FC) by Fu5AH cells grown in normal (NRS) and hyperlipemic (HRS) rabbit serum adjusted to the same exogenous total cholesterol (TC) concentration. ●——● total exogenous FC incorporated, ■——■ exogenous FC recovered as cellular esterified cholesterol (EC), ○——○ exogenous FC recovered as cellular FC.

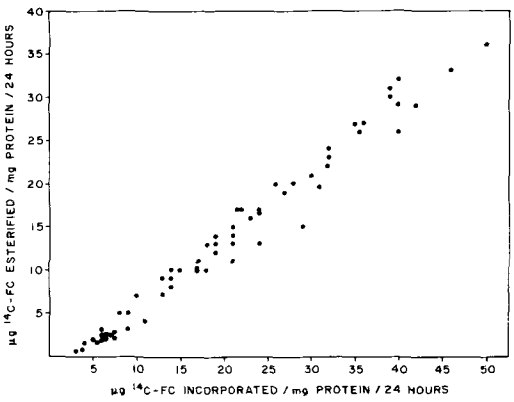

FIG. 3. Relationship between exogenous free cholesterol (FC) incorporated and amount of incorporated FC recovered as cellular esterified cholesterol (EC).

acid (75:24:1). Radioactivity in the separated bands or in lipid extracts was measured in a Packard or Intertechnique spectrometer using 0.6% diphenyloxazole and 0.2% demethyldiphenyloxazole-benzene in toluene. Cellular sterol synthesis experiments were conducted quantitating sodium-2-^{14}C-acetate (24.5 Ci/mM, New England Nuclear,Boston, MA) incorporation into digitonin precipitable sterols as previously described (39,40). All values for the incorporation of exogenous unesterified cholesterol (FC) were calculated from the specific activity of the labeled FC in the medium at the start of the experiment. Throughout the text, the value for percent esterification represents the fraction of the labeled unesterified sterol taken up from the medium and recovered as cellular esterified cholesterol.

RESULTS

Incorporation and Esterification of Free Cholesterol

The incorporation of ^{14}C-cholesterol by Fu5AH cells cultured in normal and hyperlipemic rabbit serum and its distribution between cellular FC and EC are shown in Figure 1. When grown in 5% serum containing labeled FC, these cells show a rapid incorporation of exogenous FC, illustrated in Figure 1 as total FC, with a much greater total incorporation and higher EC levels occurring in HRS grown cells. Although there was rapid incorporation of ^{14}C-FC into the NRS grown cells, there was no accompanying increase in cholesterol content over that seen at 0 time. The increase in incorporation and the concurrent higher levels of labeled EC are directly related to the greater percentage of FC esterified in cells cultured in a HRS supplemented medium. Thus, after 24 hr incubation in HRS, an average of 69% (50-85%) of the incorporated radiolabeled cholesterol is recovered as EC, whereas this value for NRS is only 25% (range 13-34%). A similar pattern of uptake and esterification of FC was observed when NRS and HRS were added to cells at the same level of exogenous cholesterol (240 µg/ml TC, Fig. 2). The presence of HRS again produces a greater incorporation of FC, a higher percent of esterification, and much greater accumulation of EC than does NRS. Thus, a direct correlation appears to exist between the esterification of FC and the ability of the cell to incorporate FC and accumulate EC. Both esterification and accumulation are markedly elevated in the presence of HRS and are not solely dependent on exogenous cholesterol levels. In addition, it was determined that new enzyme synthesis was not required to obtain the high esterification values observed in HRS grown cells because activity was not reduced when cells were simultaneously exposed to cycloheximide (2 µg/ml).

Pooled data obtained from a series of experiments using a number of different samples of HRS and HRS lipoproteins and added over a

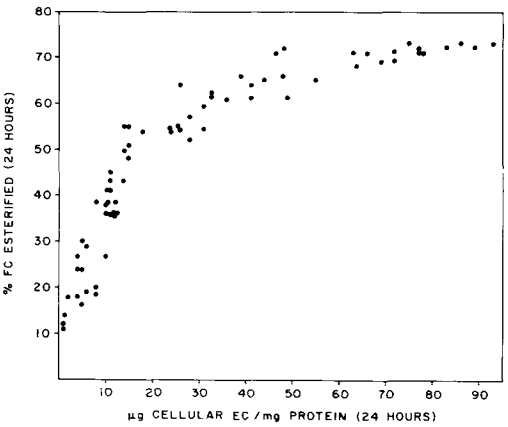

FIG. 4. Relationship between cellular esterified cholesterol (EC) content and free cholesterol (FC) esterification in Fu5AH cells. Percent FC esterified = μg FC esterified/μg FC incorporated.

range of concentrations illustrate a linear relationship between cellular FC incorporation and esterification of the incorporated cholesterol (Fig. 3). Figure 4 illustrates the relationship between percent esterification and the accumulation of cellular EC. A linear relationship can be seen between the percent of FC esterified and cellular EC content below cellular EC levels of ca. 20 μg/mg cell protein. Above levels of 50 μg/mg protein, maximum levels of percent esterification (65-70%) are observed.

The possible influence of lecithin:cholesterol acyltransferase (LCAT) on the cellular incorporation and esterification of FC from HRS and NRS was examined using heated (30 min, 60 C) and unheated sera. Little or no LCAT activity was observed in media containing HRS, possibly due to the low final concentration of HRS (1-5%). LCAT activity was observed in media containing high concentration of NRS (15-50%) and resulted in changes in the FC:EC ratio and esterification of a portion of the added ^{14}C-cholesterol. Although this activity produced some differences in the observed incorporation and esterification of FC from NRS, cellular incorporation, esterification, and EC content were consistently lower in cells grown in NRS than in cells exposed to HRS when heated or unheated sera were adjusted to comparable exogenous cholesterol levels.

Cellular Response to Isolated Lipoproteins

Since previous experiments had demonstrated that the lipoproteins isolated from hyperlipemic sera differ in their ability to promote accumulation of EC (24), experiments were conducted to determine if such differences also existed in the stimulation of esterification. Prior to the isolation of lipoproteins, normal and hyperlipemic sera were labeled by either direct addition of ^{14}C-FC in ethanol or by celite exchange. Similar results were obtained with both labeling procedures. Representative results are shown in Table I for whole serum and lipoprotein density <1.019. All fractions were added to the culture medium at equivalent FC concentrations (75 μg/ml). NRS and NRS lipoprotein produced ca. 30% esterification, consistent with the esterification usually observed with NRS. Neither NRS nor NRS d <1.019 lipoprotein gave esterification or accumulation comparable to that produced by HRS lipoproteins. Although the greatest stimulation of esterification occurred with HRS lipoprotein d < 1.019, all fractions demon-

TABLE I

Effect of Rabbit Serum Lipoprotein on Free Cholesterol Incorporation and Esterification in Fu5AH Cells: Comparison Between Lipoprotein of Normal and Hyperlipemic Sera[a]

Serum or lipoprotein fraction[b,c]	Cell content (μg/mg protein)		Exogenous free cholesterol incorporation (μg/mg protein)		
	FC	EC	FC	EC	% Esterification[d]
NRS					
Whole serum	12.29	9.00	7.02	3.16	31
d<1.019	19.22	20.76	5.50	3.16	37
HRS					
Whole serum	19.41	62.17	8.43	22.38	73
d<1.019	21.72	75.24	8.68	23.17	73

[a]FC = Free cholesterol, EC = esterified cholesterol, NRS = normal rabbit serum, HRS = hyperlipemic rabbit serum.

[b]Sera labeled with ^{14}C-free cholesterol by celite exchange prior to isolation of lipoproteins.

[c]Serum and lipoprotein added to cell cultures at 75 μg/ml FC. Incubation time 24 hr.

[d]% Esterification = $\frac{\mu g \text{ FC esterified}}{\mu g \text{ FC incorporated}}$.

TABLE II

Effect of Hyperlipemic Rabbit Serum Lipoproteins on Free Cholesterol Incorporation and Esterification in Fu5AH Cells

Lipoprotein fraction[a]	FC content of medium[b] (μg/ml)	Cell content (μg/mg protein)		Exogenous free cholesterol incorporation (μg/mg protein)		
		FC[c]	EC[c]	FC	EC	% Esterification[d]
d<1.019	75[e]	21.72	75.24	8.68	23.17	73
	20	14.83	32.90	4.86	7.79	60
	10	13.75	24.05	3.84	4.58	54
d 1.019-1.063	75	19.58	58.06	7.11	16.23	70
	20	14.67	25.74	4.22	5.72	57
	10	12.90	15.47	2.90	2.66	47
d 1.063-1.21	75	17.34	36.18	7.27	13.62	65
	20	13.80	17.65	4.31	5.02	54
	10	14.13	12.97	3.46	2.69	44

[a]Serum labeled with ^{14}C-free cholesterol by celite exchange prior to isolation of lipoproteins.

[b]Incubation time 24 hr.

[c]FC = Free cholesterol, EC = esterified cholesterol.

[d]% Esterification = $\frac{\mu g \text{ FC esterified}}{\mu g \text{ FC incorporated}}$.

[e]Values taken from Table I.

TABLE III

Effect of Medium Supplements on ^{14}C-Acetate[a] Incorporation into Digitonin Precipitable Sterol in Fu5AH Cells

Medium supplemented[b]	μg FC[c] ml medium	% Serum	Synthesis (% DLP[d] control)	% Efflux $\frac{\text{cpm medium}}{\text{Total cpm}}$
DLP (5 mg/ml)	0	0	100[e]	22.6
DLP (5 mg/ml) + lecithin (20 μg/ml) + cholesterol (40 μg/ml)	40	0	38.5	37.9
Normal rabbit serum	8	5	35.5	23.4
Hyperlipemic rabbit serum	50	1.9	7.9	23.4
Hyperlipemic rabbit serum	150	5.6	8.5	27.7

[a]0.8 μCi (1.7 mg) ^{14}C-acetate/ml medium for 24 hr.

[b]Cells grown in supplements for 24 hr.

[c]FC = Free cholesterol.

[d]DLP = Delipidized serum protein.

[e]1068 cpm in digitonin precipitable sterol/mg cell protein.

strated a stimulatory effect (Table II). To assess possible differences between these lipoprotein fractions, three major HRS lipoproteins were added to cultures at a range of concentrations. Table II shows an elevation of esterification and accumulation in the order of lipoprotein d < 1.019 > d 1.019 - 1.063 > d 1.063 - 1.21. However, even at the lowest concentration (10 μg/ml FC), the least stimulatory hyperlipemic lipoprotein (d 1.063 - 1.21) produced a greater esterification than did any lipoprotein fraction of normal rabbit serum when added to the culture medium at 75 μg/ml FC.

Source of Cellular Esterified Cholesterol

A series of experiments have shown that, after 24 hr in HRS, an average 28% (21-40%, n=10) of the accumulated cellular EC was derived from the influx and esterification of FC. The cellular EC not derived from this source could have originated from (a) synthesis of FC and its esterification during the 24 hr incubation; (b) esterification of cellular FC present at 0 time; and/or (c) incorporation of exogenous EC, either by intact uptake or by

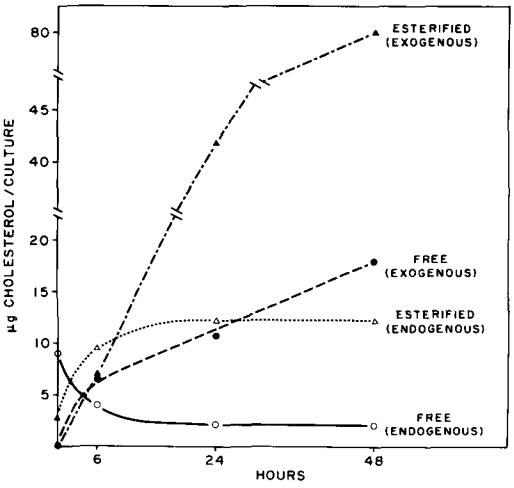

FIG. 5. Esterification of endogenous and exogenous free cholesterol by Fu5AH cells grown in 5% hyperlipemic rabbit serum.

hydrolysis and reesterification to EC. To test the first of these possibilities and assess the ability of cells to synthesize cholesterol during growth in NRS and HRS, the incorporation of ^{14}C-acetate into digitonin precipitable material was assayed. The amount of acetate incorporated into sterol in cells grown in delipidized serum protein supplemented medium (DLP) was assumed to represent maximum cellular synthesis and therefore set equal to 100% (Table III). NRS (5%) produced a 64% decrease which was similar to that produced by supplementing DLP medium with 20 μg/ml lecithin plus 40 μg/ml FC. HRS produced the greatest inhibition of synthesis (>90%) when added to 50 or 150 μg/ml FC. Thus, de novo cholesterol synthesis is markedly reduced in the presence of HRS and, hence, could not contribute importantly to the accumulation of cellular cholesteryl esters.

To determine the contribution of endogenous sterol to the accumulation of cellular EC, endogenous sterol was prelabeled by growth of cells for 5 days (1 subculture) in DLP medium containing 7α-^{3}H-cholesterol (40 μg/ml FC, 20 μg/ml lecithin, 5 mg/ml DLP). These cells were then shifted to medium containing ^{14}C-cholesterol labeled HRS. Figure 5 shows the time course of esterification of both endogenous ^{3}H- and exogenous ^{14}C-cholesterol. At 0 time, 83% of the endogenous ^{3}H-sterol was present as FC. Upon addition of HRS, rapid esterification occurred so that after 6 hr incubation, 60% of the ^{3}H was recovered as EC. After 24 hr, 85% of the endogenous (^{3}H) and 70% of the incorporated exogenous FC were recovered as EC. Based on 0 time specific activities, the amount of endogenous cholesterol present in the cells prior to exposure to HRS was shown to account for < 10% of the cellular sterol after 24 hr growth in HRS (Table IV). The contribution of exogenous FC to the cellular sterol pool is also shown in Table IV. As much as 40-50% of the cellular FC and 60-70% of the cellular EC are derived from a source other than endogenous or exogenous FC.

The above experiments indicate that a sizable portion of the cellular EC could be derived from exogenous esterified sterol. To ascertain the contribution of exogenous EC to cellular cholesterol, serum cholesterol was labeled in vivo by feeding ^{3}H-cholesterol to a hyperlipemic rabbit. The serum obtained was then labeled with ^{14}C-FC by in vitro celite exchange (35) and fractionated as described above. The isolated lipoproteins thus contained ^{14}C-FC, ^{3}H-FC, and ^{3}H-EC. The ^{3}H:^{14}C ratio of FC and the specific activities were determined to assess the contribution of exogenous FC to cellular sterol. The contribution of exogenous EC was taken as the difference between total ^{3}H-sterol in the cell and the ^{3}H-sterol which was calculated to be derived from exogenous FC. This latter figure was based on the incorporation of ^{14}C-FC.

Cells were incubated for 24 hr with each lipoprotein fraction adjusted to the same exogenous FC concentration (50 μg/ml). The contribution of exogenous FC and EC to the accumulated cellular sterol is shown on Table V. Total uptake of exogenous cholesterol from the lipoproteins was greatest from d < 1.019. The contribution of exogenous EC to the total cholesterol incorporated followed the order of lipoprotein density, being greatest in d < 1.006 (82%) and least in d 1.063-1.21 (38%). In all cases, it was observed that a fraction of the incorporated EC underwent hydrolysis and could be recovered as FC in the cell, the value being 23% hydrolysis in whole serum and ranging from 26 to 41% with the isolated lipoproteins. These values are based on the amount of labeled EC incorporated from the medium and recovered in the cells as FC.

Cellular Response to HRS Produced with Cholesterol-Free Diet

Previous experiments have shown that EC accumulation in Fu5AH cells is stimulated by hyperlipemic rabbit, pigeon, and monkey sera (24), while the present investigations have demonstrated an accompanying stimulation of

TABLE IV

Source of Accumulated Cholesterol in Fu5AH Cells Incubated in Hyperlipemic Rabbit Serum

	μg Cholesterol/culture						
		μ Source of cholesterol					
	Cell content (24 hr incubation)	Endogenous FC[a]	% of total[b]	Exogenous FC[c]	% of total[b]	Difference[d]	% of total
Experiment 1							
Free	30.0	2.0	7	16.8	55	11.5	38
Ester	123.1	6.0	5	32.8	27	90.3	68
Total	153.1	8.0	5	49.6	32	95.8	63
Experiment 2							
Free	27.5	2.2	8	10.8	39	14.5	53
Ester	141.0	12.3	9	41.8	30	86.9	61
Total	168.5	14.5	9	52.6	31	32.9	60

[a]Cholesterol in cells prior to exposure to 5% hyperlipemic rabbit serum

[b]% of cellular content.

[c]Represents ^{14}C-free cholesterol incorporated from hyperlipemic rabbit serum.

[d]Difference represents cellular cholesterol which could be derived from exogenous cholesteryl ester.

TABLE V

Incorporation of Exogenous Free and Esterified Cholesterol by Fu5AH Cells

Lipoprotein fractions[a]	Cell sterol	Cell content[b] (μg/mg protein)	Exogenous source of incorporated cholesterol (μg/mg protein) FC	EC	% Esterification of FC	% Hydrolysis of EC	% of total incorporated cholesterol derived from exogenous EC
Hyperlipemic rabbit serum	FC	18.3	8.4	8.4	68	23	58
	EC	54.1	18.0	28.4			
d<1.006 (30 min)	FC	17.3	3.4	10.2	60	26	82
	EC	43.3	5.1	29.1			
d<1.019	FC	19.9	9.4	9.7	64	26	60
	EC	50.6	16.5	27.5			
d 1.019-1.063	FC	19.3	9.2	9.2	62	29	57
	EC	39.0	15.0	22.6			
d 1.063-1.21[c]	FC	14.5	6.4	4.6	59	52	36
	EC	22.4	9.3	4.3			

[a]Whole serum and lipoprotein fraction added to culture medium to yield 50 μg/ml free cholesterol (FC). Incubation time, 24 hr. % Esterified cholesterol (EC) in HRS and lipoprotein fractions: HRS, 72%; d <1.006 = 80%; d <1.019 = 71%; d 1.019-1.063 = 67%; d 1.063-1.21 = 73%.

[b]0 time cell content: FC = 16.9 μg/mg protein; EC = 6.7 μg/mg protein.

[c]Lipoprotein fraction washed by recentifugation from d 1.063 gm/ml (see Experimental Procedures).

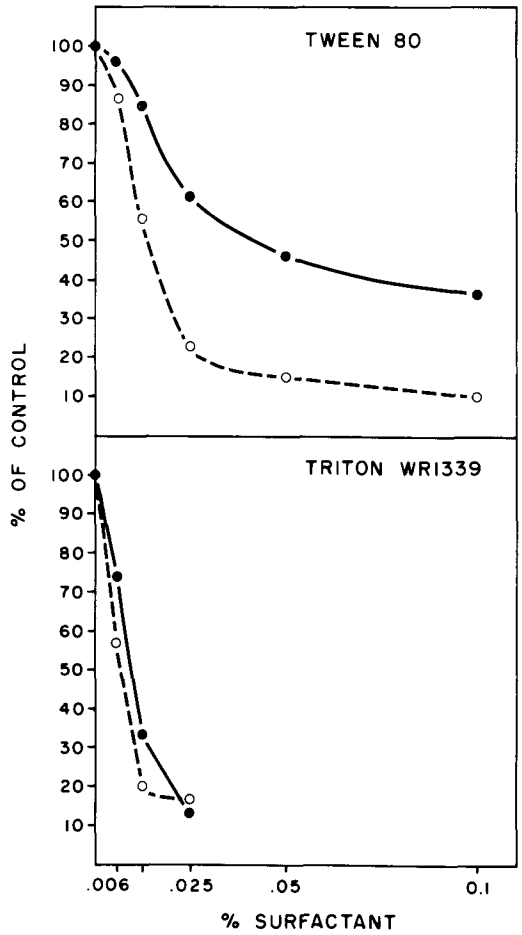

FIG. 6. Effect of Tween 80 and Triton WR-1339 on esterified cholesterol accumulation and exogenous free cholesterol esterification by Fu5AH cells grown for 24 hr in hyperlipemic rabbit serum preincubated with surfactant. o——o esterified cholesterol content, •——• percent exogenous free cholesterol esterified. Control values (100%) were obtained from untreated hyperlipemic rabbit serum (5%).

esterification with HRS lipoproteins. In all of the above studies, sera were obtained from cholesterol fed animals; thus, the cellular response may be due to new or modified lipoproteins produced by cholesterol feeding. To test the correlation between cholesterol feeding and cellular response, sera were obtained from rabbits made hyperlipemic with a cholesterol free, semipurified diet (33). A number of sera having cholesterol values ranging from 60 to 800 mg/dl were obtained from animals on this dietary regime. Sera obtained from the semipurified diet elicited cellular accumulation of EC and stimulation of FC esterification with the magnitude of percent esterification ranging from that seen with NRS to values approaching those produced with HRS obtained by cholesterol feeding.

Serum lipoproteins were isolated from a rabbit fed the semipurified diet (serum TC, 800 mg/dl) and tested for their ability to produce a cellular response. The results obtained were similar to those shown in Tables I and II, with the highest response (63% esterification) being obtained using lipoproteins d<1.019; however, lipoprotein d 1.063-1.21 elicited a lower response (35%) for this density class than had been previously observed with HRS lipoproteins.

Effect of Surfactants

To obtain HRS by means other than dietary manipulation, a rabbit was injected with Triton WR-1339, a treatment known to induce severe hyperlipemia (41-43). When added to culture medium, this serum failed to produce the cellular response of increased esterification and accumulation seen with HRS obtained from cholesterol fed animals. However, since Triton treatment results in detectable levels of Triton in the circulation (44,45), experiments were conducted to determine if surfactants, added in vitro to HRS, could influence EC accumulation and/or FC esterification. The esterification and accumulation of EC produced by HRS was drastically reduced in a dose dependent manner when the HRS was preincubated with either Triton WR-1339 or Tween 80. Figure 6 shows the effect on esterification and EC content produced by increasing concentrations of each surfactant. Both were present at levels lower than those which produced marked cellular toxicity. This inhibitory effect was not seen when the Triton WR-1339 was added to cell cultures for 18 hr and then removed before exposure to untreated HRS for 5 hr.

Response of L-Cells and Aortic Medial Cells to HRS

The stimulation of esterification of FC and accumulation of cellular EC in response to HRS seems to be common to several cell types and not an exclusive phenomenon of Fu5AH cells. L-cells, rabbit aortic medial cells, and Fu5-5 hepatoma cells, as well as Fu5AH cells, were cultured in the presence of 5% HRS for 48 hr and compared for cholesterol esterification and accumulation (Table VI). In NRS, L, aortic medial, and Fu5-5 cells showed <1% esterification and have low levels of EC in contrast to 31% esterification and somewhat higher EC levels of Fu5AH cells. In response to HRS,

TABLE VI

Comparative Cholesterol Metabolism of Fu5AH, Fu5-5, L, and Aortic Medial Cells

Serum[a] / Cells	Cell content μg/mg protein FC[b]	Cell content μg/mg protein EC[b]	Exogenous free cholesterol[c] μg/mg protein FC	Exogenous free cholesterol[c] μg/mg protein EC	% Esterified
Hyperlipemic rabbit serum					
Fu5AH	18.72 ± 0.64[d]	86.39 ± 7.18	9.35 ± 0.75	30.36 ± 3.36	76
Fu5-5	26.24 ± 0.95	20.41 ± 1.66	11.68 ± 1.44	4.92 ± 1.11	30
L	24.78 ± 1.70	8.76 ± 1.45	17.16 ± 1.32	6.90 ± 1.80	29
Aortic medial[e]	34.99 ± 3.69	8.01 ± 1.63	17.08 ± 1.72	0.90 ± 0.18	5
Normal rabbit serum					
Fu5AH	14.77 ± 0.59	7.55 ± 1.39	3.72 ± 0.20	1.70 ± 0.19	31
Fu5-5	12.72 ± 0.46	0.43 ± 0.11	1.15 ± 0.12	<0.01	<1
L	14.31 ± 1.10	1.05 ± 0.47	7.35 ± 0.59	0.04 ± 0.01	<1
Aortic medial	23.38 ± 1.53	3.31 ± 1.00	7.41 ± 0.95	0.05 ± 0.01	<1

[a]Normal and hyperlipemic sera at 5%. Incubation time 48 hr.

[b]FC = Free cholesterol, EC = esterified cholesterol.

[c]Represents ^{14}C-cholesterol incorporated from medium. μg calculated from exogenous cholesterol specific activity. Average of at least six determinations.

[d]mean ± standard deviation.

[e]Rabbit aortic medial cells, second to eighth cell doubling.

however, all cells showed a stimulation of esterification and an increase in FC and EC content, with Fu5AH attaining the highest level of cellular EC and the greatest percent esterification.

DISCUSSION

The influx of FC from serum and the accumulation of EC has been observed in lesions in atherosclerotic aorta (2,4,6). Intact segments of atherosclerotic arterial tissue, as well as cell-free homogenates of aortic tissue, have been shown to have enhanced ability to esterify cholesterol with fatty acids over similar nondiseased tissue (7,8,46,47). This elevated esterification has been further shown to be due primarily to increased fatty acyl-CoA:cholesterol acyltransferase activity in atherosclerotic aortic microsomes (8,47). However, firm estimates of the origin of the cholesterol which accumulates in the aorta as cholesteryl ester are difficult to obtain (2,5).

A previous report from this laboratory described the general parameters which resulted in EC accumulation when Fu5AH rat hepatoma cells were exposed to hyperlipemic rabbit sera (24). In earlier investigations, results from balance studies quantitating changes in cholesterol levels in growth media and cells suggested that the EC which accumulated in the cells could be derived from both exogenous FC and EC (24). The present investigation has established the origin of the cellular EC and further investigated the interaction of Fu5AH cells with hyperlipemic serum lipoproteins.

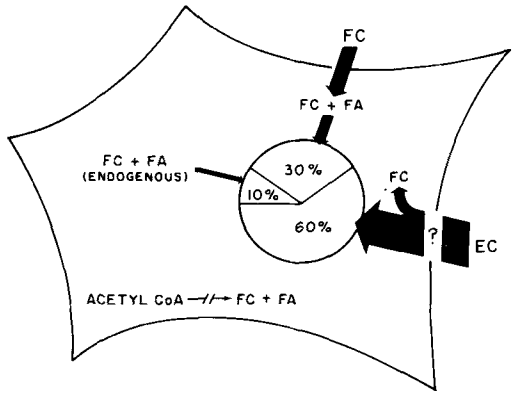

FIG. 7. Schematic representation of the sources of esterified cholesterol which accumulates in Fu5AH cells exposed to hyperlipemic rabbit serum. FC = free cholesterol, EC = esterified cholesterol, FA = fatty acid.

Four different pools of cholesterol could contribute to the esterified sterol present in tissue culture cells exposed to hyperlipemic serum: (a) exogenous FC incorporated from the growth medium and subsequently esterified by the cell, (b) cholesterol synthesized and esterified by the cells during the time of exposure to hyperlipemic sera, (c) endogenous FC present in the cell prior to exposure to HRS that is esterified during the experimental period, and (d) exogenous EC that is incorporated from the hyperlipemic sera. The last could occur by incorporation of the entire molecule or with hydrolysis followed by reesterification. The

contribution of cholesterol from each of these sources has been quantitated in the Fu5AH cell system, and the results are illustrated in Figure 7. Serum cholesterol contributes ca. 90% of the sterol which accumulates in these cells during a 24 hr exposure to HRS.

In the presence of both ^{14}C-labeled NRS and HRS, there is a rapid incorporation of ^{14}C-FC into the cellular FC pool (Figs. 1 and 2). In the presence of normal serum, there is no consequential change in the cellular FC content while, in the presence of HRS, there can be up to a twofold increase in cellular FC content (24). Thus, the incorporation of ^{14}C-FC from NRS probably reflects an exchange with cellular FC. In the presence of HRS, the cellular FC content (24) and the incorporation of exogenous ^{14}C-FC into the cellular FC pool reached a plateau after 8-12 hr incubation. Labeled EC continued to increase, however, indicating that even when the cellular FC pool has become saturated, additional exogenous FC is incorporated and esterified. The magnitude of both incorporation and esterification of exogenous FC is closely correlated with the net amount of EC which accumulates in these cells (Fig. 4). A linear relationship has been demonstrated between total FC incorporated and the amount of FC esterified (Fig. 3), with both processes being decidedly stimulated in the presence of hyperlipemic sera. It is unclear whether this increased esterification is dependent on increased FC influx, resulting in increased substrate availability, or whether the increased ability of the cell to esterify FC permits additional incorporation of FC by shifting FC to a large intracellular pool of EC, stored as lipid inclusions. Regardless of mechanisms, the incorporation and esterification of FC contributes ca. 30% of the accumulated EC.

Although the results illustrated in Figure 5 indicate that, upon exposure to HRS, endogenous (0 time) FC undergoes rapid and extensive esterification, this pool, because of its limited size, contributed only ca. 10% of the accumulated EC (Table (IV). The fraction of this material that undergoes esterification closely parallels the esterification of exogenous FC, suggesting that the fate of endogenous FC could serve as a reliable measure of relative esterification in experimental systems in which exogenous FC esterification cannot be easily determined. The presence of hyperlipemic serum in the medium has been shown to block effectively de novo synthesis of cholesterol in these cells (Table III), thereby eliminating any important contribution to cellular EC by newly synthesized FC. A total of 40% of the cellular EC present in the cells at 24 hr can therefore be accounted for by esterification of exogenous incorporated FC (30%) and endogenous FC (10%), with no consequential contribution by de novo synthesis.

Exogenous cholesteryl esters comprise the single largest source of the Fu5AH esterified cholesterol, contributing the remaining 60%. Incorporation of exogenous cholesteryl esters has been previously demonstrated in a number of different cell systems (14-18), sometimes accompanied by evidence of cellular cholesteryl hydrolysis (14,15,19). The present experiments do not indicate whether all of the incorporated EC undergoes hydrolysis followed by reesterification or whether some of the exogenous ester is incorporated intact. It is evident from the results in Table V that the cell is capable of hydrolyzing EC, inasmuch a fraction of the exogenous EC incorporated is recovered as cellular FC.

Comparison of the cholesterol metabolism of Fu5AH cells exposed to normo- and hyperlipemic rabbit serum and serum lipoproteins indicates that the hyperlipemic sera produce increased EC influx, increased FC influx and esterification, resulting in cellular accumulation of EC. This response is not due simply to high levels of exogenous cholesterol, but is specifically linked to the hypercholesteremic state. Recent investigations of lipoproteins in diet induced hypercholesteremic animal sera have demonstrated marked shifts in lipoprotein content and composition (48-51). A major change occurs in rabbit lipoproteins, $d < 1.019$, resulting in new or modified very low density lipoproteins or "intermediate" lipoproteins that are enriched in EC and may exhibit modified apoprotein patterns (48-50). It is this lipoprotein density class that elicits the greatest response in Fu5AH cells. The other lipoprotein fractions isolated from hyperlipemic rabbit sera also stimulate, to various degrees, the esterification of FC and cellular EC accumulation. This lack of specificity may be due to incomplete separation of hyperlipemic lipoproteins; the presence of new or modified lipoproteins which separate by density techniques as a continuum among the different fractions, or a change, to various degrees, in all serum lipoproteins in the hypercholesteremic rabbit. Cholesterol feeding, however, is not necessary for the production of cell recognizable lipoprotein changes, because Fu5AH cells exposed to lipoproteins obtained from animals made hyperlipemic by feeding a cholesterol free, semipurified diet also demonstrated increased esterification and cellular EC content.

The data presented here are not sufficient to reveal the precise mechanism by which hyper-

lipemic lipoproteins stimulate cholesterol uptake and esterification. The extent to which FC and EC are incorporated by physical exchange or by lipoprotein uptake is not known. The observation that the cellular response is inhibited by surfactants does not, at this time, provide data on possible mechanisms. Triton WR-1339 has been shown to modify lysosomes (52), to interfere with LCAT (44,45) and lipoprotein lipase (53), and to modify lipoprotein composition and density (42,54). The observation that pretreatment of cells with WR-1339 did not inhibit EC suggests that the surfactants act by modifying the serum lipoproteins and not by directly affecting cellular metabolism. It is interesting to note that the inhibition of EC accumulation produced by surfactants in this cell system parallels the effect of surfactants in vivo. A number of studies have shown reduced aortic lipid deposition in cholesterol fed animals injected with selected surfactants (55-57). It is thus possible that the Fu5AH cells could serve as a model for the development of tissue culture systems that could be used to screen physiologically useful compounds that modify cholesterol flux and/or accumulation.

The data presented in Table VI on the comparative response to HRS of L-cells, rabbit aortic medial cells, and Fu5-5 and Fu5AH rat hepatoma cells demonstrate that all of these cells exhibited elevated percent esterification values and increased cholesterol content when compared to cells grown in normocholesteremic sera. These results are similar to those of Chen et al. (22), who demonstrated both increased esterification and EC levels in L-cells, rabbit aortic medial cells, and rabbit skin fibroblasts grown in HRS. Although these cells demonstrated a similar response, the magnitude of response differed considerably. The data presently available are not sufficient to indicate whether the mechanisms of accumulation and sources of the cellular EC are similar to those of the Fu5AH cells. The observation that Fu5-5 rat hepatoma cells have a lower percent esterification value and EC content than do the closely related Fu5AH is not a characteristic expressed by all rat hepatoma cells. The difference in the magnitude of EC accumulation and esterification between these two related hepatomas suggests that similar differences could exist between clones of other cells. Such clonal variation in cellular response to hyperlipemic sera, particularly if observed in aortic medial cells, could, in part, explain the focal development of lesions in atherosclerosis.

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Lung Lamellar Bodies Lack Certain Key Enzymes of Phospholipid Metabolism

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ABSTRACT

Palmitoyl CoA-glycerol-3-phosphate acyltransferase, phosphatidate phosphohydrolase, and phospholipase A were assayed in subcellular fractions of rat lung, including lamellar bodies, the putative site of storage and secretion of lung surfactant. The specific activity of each of these enzymes in lamellar bodies was relatively low and could be entirely accounted for by a small contamination of the lamellar bodies fraction by microsomes, as quantitated by the presence of the microsomal marker reduced triphosphopyridine nucleotide cytochrome *c* reductase. These data indicate that lamellar bodies are not the site of synthesis of the lipid component of pulmonary surfactant by pathways involving these enzymes.

INTRODUCTION

Mammalian lung alveoli are lined with a highly surface active material known as pulmonary surfactant, which prevents alveolar collapse at end-expiration by reducing surface tension at the air-tissue interface (1). Pulmonary surfactant consists of both protein and lipid, the major component being dipalmitoyl lecithin, a highly surface active phospholipid (2). Alveolar type II cells appear to be the site of surfactant synthesis (3). These cells contain unique subcellular structures, the lamellar bodies, which store the surfactant material in concentric osmiophilic lamellae prior to its secretion onto the alveolar surface (1). These unique subcellular structures have proven amenable to isolation due to their high lipid content and resulting low density relative to other subcellular organelles. A number of studies have appeared in which the structure and composition of isolated lamellar bodies have been investigated and shown to contain certain hydrolytic enzymes characteristic of lysosomes (4-8). Cytochemical evidence has been reported which is consistent with the presence of phosphatidate phosphohydrolase in lamellar bodies (9). With the exception of two preliminary reports (10,11), however, no direct evidence regarding the presence of the enzymes of phospholipid metabolism in lamellar bodies has been reported, and their role in surfactant lipid synthesis remains largely a matter of speculation. We have examined this possible role by assaying three key enzymes of phospholipid metabolism in lamellar bodies, microsomes, and mitochondria of rat lung. We report that the low activities of these enzymes in lamellar bodies parallel the activity of a microsomal marker enzyme and therefore may be attributed to a small microsomal contamination of the lamellar body fraction.

EXPERIMENTAL PROCEDURES

Lamellar bodies were isolated by a modification of the procedure of Page-Roberts (4). Male Sprague-Dawley rats (250-275 g) were killed by concussion followed by decapitation. The lungs were rapidly removed and chilled in 0.33 M sucrose, 0.01 M tris(hydroxymethyl)aminomethane) HCl, pH 7.4 (sucrose-tris). The parenchyma was scraped from the tracheobronchial tree and all visible remnant vessels and bronchi removed and discarded. The tissue was homogenized in a Potter-Elvehjem type homogenizer, mechanically driven at 250 rpm. A loose fitting teflon pestle (0.017 in. clearance) was used for 4-6 strokes, followed by 4-6 strokes with a tighter fitting teflon pestle (0.007 in. clearance). The homogenate was strained through 6 layers of cheesecloth and centrifuged 10 min at 600 x g to remove nuclei and cellular debris. The supernatant was centrifuged 10 min at 1,600 x g. The resulting pellet was discarded and the supernatant was centrifuged 10 min at 16,000 x g. The pellet was resuspended in 0.33 M sucrose and used as starting material for preparation of lamellar bodies and motochondria according to Page-Roberts (4). An intermediate fraction, obtained by centrifuging the 16,000 x g supernatant at 20,000 x g for 20 min, was discarded and the microsomal fraction isolated from the resulting supernatant by centrifugation at 100,000 x g for 45 min. Protein concentration of the resulting fractions was determined according to Lowry et al. (12), using bovine serum albumin as a standard. Lipid extractable phosphate was determined by the method of Ames (13).

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TABLE I

Specific Activities of Phospholipid Metabolizing Enzymes and Marker Enzymes in Subcellular Fractions of Rat Lung

Fraction	TPNH cytochrome *c* reductase	Acyl CoA-glycerol-3-phosphate acyltransferase (nmoles/min/mg protein)	Phospholipase A	Phosphatidate phosphohydrolase	Monoamine oxidase
Homogenate	13.3	1.7	2.4	10.0	0.4
Mitochondria	17.3	4.5	13.5	10.6	7.1
Microsomes	69.4	9.2	13.0	16.0	0.5
Lamellar bodies	9.4	0.8	1.3	1.5	ND[b]
Lamellar bodies/Microsomes x 100	13.5	8.7	10.0	9.4	

[a]TPNH = Reduced triphosphopyridine nucleotide.
[b]Not detectable.

Reduced triphosphopyridine nucleotide (TPNH) cytochrome *c* reductase (14) and monoamine oxidase (15) were assayed according to published procedures. Palmitoyl CoA glycerol-3-phosphate acyltransferase activity was determined in an assay mixture containing 0.1 M tris-HCl pH 8.0, 1 mM dithiothreitol, 7 mg/ml bovine serum albumin, 0.2 mM palmitoyl CoA, and 3.6 mM [^{14}C] glycerol-3-phosphate (4250 cmp/nmole) in a total volume of 0.07 ml. The mixture was preincubated 5 min at 37 C and the reaction started by addition of 5-50 μg of the listed subcellular fraction protein. The reaction was incubated 10 min at 37 C and terminated by addition of 3.8 ml of methanol: chloroform: 5% trichloroacetic acid (2:1:0.8). Lipids were extracted according to Bligh and Dyer (16) and counted in 10 ml of scintillation solution. Specific activity is expressed as nmoles of glycerol-3-phosphate incorporated into lipid per min per mg of protein. Phosphlipase A was assayed as described elsewhere (17), using 120 μM 1-acyl-2[^{14}C]-linoleoyl phosphatidylethanolamine (155 cpm per nmole) as substrate and 5-50 μg of subcellular fraction protein. Activity is expressed as nmoles radio-labeled fatty acid released per min per mg of protein. Phosphatidate phosphohydrolase activity was determined in an assay mixture containing 50 mM tris-maleate pH 7.0, 3.5 mg/ml bovine serum albumin, 0.7 mM dipalmitoyl glycerol phosphate, and 48-66 μg of protein from the listed subcellular fraction in a total volume of 0.2 ml. The reaction mixture was incubated at 37 for 20 min and the reaction terminated by adding 0.1 ml of 5% trichloroacetic acid. Phosphate released was determined in the supernatant after removal of precipitate by centrifugation. Specific activity is expressed as nmoles of phosphate released per min per mg of protein.

All enzyme assays were linearly proportional to time and added protein over the ranges used. Assays were performed in duplicate, and the averages, which were within ± 5-10% of the individual assays, were reported. Values in Table I were obtained by assaying all of the enzymes listed in subcellular fractions from a single preparation. The results are representative of those obtained from two or more preparations for each of the enzymes listed.

RESULTS AND DISCUSSION

The lamellar bodies preparation obtained by this scheme contained 3.0 μmoles of lipid extractable phosphate per mg of protein, compared to a corresponding ratio of 0.73 for the microsomal fraction.

The activities of the phospholipid metabolizing enzymes and appropriate marker enzymes for microsomes (TPNH cytochrome *c* reductase) and mitochondria (monoamine oxidase) in the various subcellular fractions of rat lung are shown in Table I. Palmitoyl CoA glycerol-3-phosphate acyltransferase catalyzes the first step in the de novo pathway of lipid synthesis. Phosphatidate phosphohydrolase hydrolyzes phosphatidic acid to diglyceride, which can then react with the appropriate activated polar head groups to form phospholipid. The hydrolysis of phosphatidic acid has been suggested as the rate limiting step in phospholipid synthesis in liver (18). This enzyme has been recently shown to increase in the lungs at birth (19). Phospholipase A has been shown in lung microsomes to catalyze specifically the hydrolysis of fatty acyl residues at the two position of phospholipid (17), which normally is esterified by unsaturated fatty acids. This specificity is consistent with a role for this enzyme in the synthesis of disaturated surfactant lipid by a remodeling pathway (20-22). TPNH cytochrome *c* reductase in lung was shown in a recent study to be similar to the enzyme in liver, where its utility as a microsomal marker has been established (23). Cytochromes b_5 and P-450, which were used as microsomal markers by Page-Roberts (4) in assessing the contamination of a lung lamellar body preparation, have very low activity in lung and are difficult to quantitate (24). The results of the enzyme assays are expressed in terms of specific activity rather than total activity because the lamellar bodies are derived from a small portion of the total cell population and are, therefore, recovered in small amounts relative to other subcellular fractions. As seen in Table I, the palmitoyl CoA glycerol-3-phosphate acyltransferase and phosphohydrolase specific activities are highest in microsomes, while phospholipase A is equally active in mitochondria and microsomes. The specific activities of these enzymes in lamellar bodies are in all cases 10% or less of the specific activity in the microsomal fraction, consistent with 13.5% contamination of lamellar bodies by the microsomal marker enzyme TPNH cytochrome *c* reductase. Monoamine oxidase, a mitochondrial marker enzyme, was not detectable in the lamellar body fraction.

In experiments using the procedure of Gil and Reiss (6) or the unmodified method of Page-Roberts (4) to isolate lamellar bodies, we found higher contamination (20-30%) of the lamellar bodies with microsomes as calculated from the specific activity of TPNH cytochrome *c* reductase. The specific activity of palmitoyl CoA glycerol-3-phosphate acyltransferase was proportionately higher in these more contaminated preparations.

Additional studies, comparing the microsomal palmitoyl CoA glycerol-3-phosphate acyltransferase with the activity of this enzyme in lamellar bodies, showed a pH optimum of 8 and a K_m for glycerol-3-phosphate of 2.2 mM, common to both fractions. This further suggests the microsomal origin of the acyltransferase activity detectable in lamellar bodies.

Regardless of the origin of the enzyme activities detected here in the lamellar bodies fraction, the low specific activities relative to lung microsomes (presumably the site of "normal" phospholipid biosynthesis) make it seem unlikely that lamellar bodies are responsible for the relatively rapid synthesis of pulmonary surfactant lipid (25) by pathways involving the enzymes studied here, namely the de novo pathway involving acylation of glycerol-3-phosphate or the deacylation-reacylation cycle. Rooney et al. (10) have reported, in abstract form, very low levels of activity of choline phosphotransferase and glycerolphosphate phosphatidyl-transferase in lamellar bodies, indicating that metabolism of the polar head groups of the major lipid components of lamellar bodies may occur elsewhere.

The possibility of a direct acyl exchange between acyl CoA and phosphatidylcholine, without an intermediate deacylation by phospholipase A, is suggested by the lamellar body-catalyzed transacylation reported by Engle and Longmore (11) and the lack of phospholipase A in lamellar bodies reported here. This possibility is an interesting one inasmuch as such an enzyme has not previously been documented in mammalian tissues.

ACKNOWLEDGMENT

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Preparation and Purification of Lipid Hydroperoxides from Arachidonic and γ-Linolenic Acids

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ABSTRACT

Commercial soybean lipoxygenase may be used under carefully controlled reaction conditions to give high yields of lipid hydroperoxides. Lipid hydroperoxides so derived from γ-linolenic or arachidonic acid may be purified by high pressure liquid chromatography. Thus, commercial lipoxygenase serves as a viable source for 100 mg quantities of lipid hydroperoxides.

INTRODUCTION

We have recently developed a method for the study of peroxy radical cyclization reactions based on the radical induced decomposition of unsaturated hydroperoxides. Our objective has been to utilize this method in an investigation of a model reaction for the biosynthesis of prostaglandins (1-2). Toward this end, it was necessary to obtain isomerically pure lipid hydroperoxides on a preparatively useful scale.

Autoxidation of fatty acids yields hydroperoxides which are not only mixtures of positional isomers but are racemic as well (3). Lipoxygenase catalyzed oxidation, on the other hand, has a demonstrated positional specificity and gives hydroperoxides which are enantiomerically pure (3). The enzymatic reaction was therefore the method of choice for our purposes.

The lipoxygenase reaction has been the subject of numerous investigations (4-14). A variety of factors have been shown to be important in determining the product specificity in the oxygenation of fatty acids by lipoxygenase. These factors include pH, temperature, presence or absence of Ca^{2+}, method of substrate preparation, and source and homogeneity of the enzyme. The factors which affect the isolated yield of the hydroperoxides have been studied to a lesser extent. In general, the absorbance increase at 234 nm is the only estimation of hydroperoxide yield. In fact, using commercial lipoxygenase and conditions reported in the literature, we could prepare lipid hydroperoxides from γ-linolenic acid in no more than 5% isolated yield.

We report here a study of the soybean lipoxygenase oxidation of γ-linolenic and arachidonic acids. The purpose of this work was to maximize the isolated yields of various lipid hydroperoxides and, in so doing, make the lipoxygenase system conveniently useful in a preparative sense. The cornerstone of this study was high performance liquid chromatography (HPLC). By the use of this technique, both the hydroperoxy fatty acids and the corresponding methyl esters, I-III, could be obtained in pure form (Fig. 1). I, an ω-10 hydroperoxide from γ-linolenic acid, is particularly interesting because it serves as a starting material for model studies on prostaglandin biosynthesis. The corresponding ω-10 hydroperoxide from arachidonic acid could not be prepared in useful yields with our reaction conditions.

MATERIALS AND METHODS

γ-Linolenic acid, arachidonic acid, and linoleic acid (99%+) were obtained from Nu-chek Prep (Elysion, MN) and used without further purification.

Soybean lipoxygenase was obtained from Sigma Biochemical Co. (St. Louis, MO) and was assayed with linoleic acid: pH 9, 90,000 u/mg; pH 7, 45,500 u/mg. The method used was that

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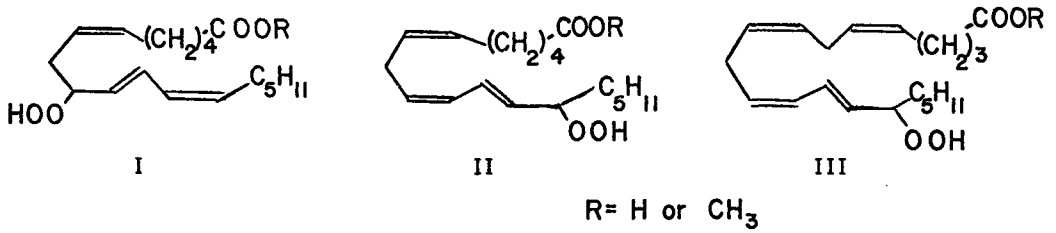

FIG. 1. Hydroperoxides formed from soybean lipoxygenase and γ-linolenic and arachidonic acid.

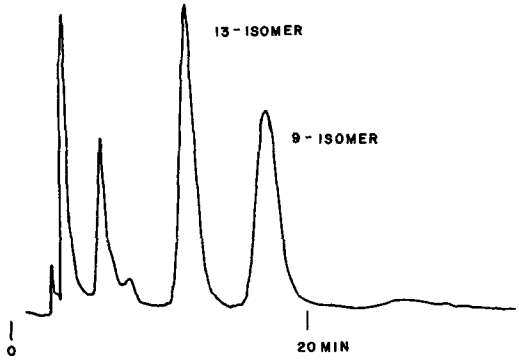

FIG. 2. Analytical high performance liquid chromatography trace for separation of γ-linolenic hydroperoxide isomers. Methyl ester, ultraviolet detection.

prescribed by Sigma Biochemical Co.

Substrate Solutions

Ethanol dispersion: A stock solution of the substrate (0.088M) was prepared in 95% ethanol. Aliquots were diluted to the appropriate concentration with borate (0.047M, pH 9.0) or phosphate (0.047M, pH 7.0) buffer.

Ammonium salt: The substrate was treated with 2M NH_4OH (1 ml/50 μl substrate) and was diluted with water (ca. 50 ml/50 μl substrate). The pH of the solution was adjusted to 7.0 or 9.0 using 0.1N hydrochloric acid. The final concentration was obtained by dilution with borate or phosphate buffer.

Incubation

The substrate solution was equilibrated at 0 or 25 C and was combined with the enzyme solution (1 mg/ml). The reaction was maintained at the correct temperature with a steady stream of O_2 gassing.

Isolation

The reaction was terminated by acidifying to pH 4 and extracting with ether (3x, 100 ml). The extracts were washed with water (2x, 50 ml), dried ($MgSO_4$), and evaporated. If the methyl ester was to be prepared, the residue was treated with diazomethane (excess, 5 ml ether, 0 C, 30 min) and the solvent then removed.

Chromatography

Analytical liquid chromatography was performed on a Waters ALC 202 (Waters Associates, Framingham, MA). 6 ft x 1/8 in. of Corasil II was used. 8 ft x 3/8 in. of Porasil A was used for preparative separations.

Silica Gel H: HF-254 (4:1), 0.25mm, was used for thin layer chromatography (TLC). The spots were made visable under ultraviolet light, and peroxides were detected by $Fe(SCN)_2$, and charring with cerium sulphate.

Solvent systems: Analytical HPLC: acids, acetic acid: 2-propanol: hexane (1:5:994); methyl esters, 2-propanol:hexane (1:249). TLC:acids, acetic acid: 2-propanol:hexane (1:20:229); methyl esters, ether:hexane (1:1). Preparative HPLC:acids, acetic acid:2-propanol: hexane (1:10:989); methyl esters, 1-propanol: hexane (1:249).

Product Identification

Hydroperoxide products were identified by $NaBH_4$ reduction and hydrogenation to the hydroxystearates, followed by oxidation (CrO_3) to the ketostearate and mass spectral analysis (15).

RESULTS

A general method for the estimation of the conversion of fatty acids to hydroperoxides in a lipoxygenase catalyzed reaction has been developed. Two factors influenced the yield of a particular hydroperoxide isomer: the overall conversion of substrate to hydroperoxides and the ratio of the isomers obtained.

The conversion factor for a given set of reaction conditions was estimated by TLC of the ethereal extracts. The materials generally present in the extracts were grouped into three rough categories on the basis of R_f. The hydroperoxides were clearly identifiable on the plates both by their quenching of flourescence at 254 nm and by a strong positive response to the ferrous thiocyanate reagent. Many of the reactions contained residual amounts of the starting materials, which had a distinctly higher R_f than the product hydroperoxides. Other by-products of reaction were all more polar than the hydroperoxides by TLC. Three types of compounds were therefore generally present in a given reaction mixture: substrate fatty acid, hydroperoxides, and polar by-products.

The relative amounts of the hydroperoxide isomers formed were determined by analytical HPLC. The product mixtures were injected without prior purification. A typical analytical separation for the 9 and 13 hydroperoxide-γ-linolenate methyl esters is presented in Figure 2. R_s values were calculated according to the relationship $Rs = 2(t_{r2} - t_{r1})/(w_1+w_2)$ where t_r=retention time and w=peak width. R_s values in the range 2.3-2.5 for the separation of the 9 and 13 hydroperoxide isomers of γ-linolenic acid methyl ester were typical. Free fatty acid hydroperoxide separations were as good as

TABLE I

Product Analysis of Soybean Lipoxidase and Fatty Acid Reactions[a]

Experiment number	Substrate (S) form	Concentration S (10^3M)	Concentration E (10^{-3} units/ml)	pH	Temperature (C)	Time (min)	TLC S	TLC Hydroperoxides	TLC By-products	HPLC Hydroperoxide (isomers (%) ω10	HPLC Hydroperoxide (isomers (%) ω6
1	salt, C 18	3.29	18	9	0	30	0	++	0	0	100
2	EtOH, C18	.78	2.1	9	0	5	0	++	0	0	100
3	EtOH, C18	.78	2.1	7	0	5	+	++	0	11	89(34)
4	EtOH, C18	.94	4.5	7	0	45	0	+	++	80	20
5	salt, C18	3.29	11.3	7	25	90	0	+	++	50	50
6	salt, C18	3.29	22.5	9	25	90	0	+	++	56	44
7	EtOH, C18	1.61	4.5	7	0	45	0	+	++	70	30
8	salt, C18	1.20	2.8	7	25	5	+	+	++	43	57
9	salt, C18	.63	1.8	7	25	5	0	++	++	68	32
10	salt, C18	.26	.72	7	25	5	0	++	+	42	58
11	salt, C18	.26	.72	7	25	2	0	++	0	48	52
12	salt, C18	.32	.72	7	25	2	0	++	0	47(25)	53(32)
13	EtOH, C18	.77	2.1	7	25	5	+	++	++	55	45
14	EtOH, C20	.80	3.0	9	20	5	0	++	0	0	100
15	salt, C20	.32	.72	7	25	2	0	+	++	0	100

[a]0 = not detectable, + = minor component, ++ = major component, numbers in ( ) represent isolated yields. S = fatty acid, E = lipoxygenase, TLC = thin layer chromatography, HPLC = high performance liquid chromatography, EtOH = ethanol , C20 = arachidonic acid, C18 = γ-linolenic acid.

those of the methyl esters. Linoleic fatty acid hydroperoxides could also be separated by these techniques.

TLC and HPLC data from a series of reactions run on γ-linolenic and arachidonic acid are presented in Table I. As noted, very little difficulty was encountered in the preparation of II. The enzyme demonstrated a high degree of specificity for the 13 position of γ-linolenic acid at pH 9 and 0 C regardless of the substrate form employed (Table I, Expt. 1-2). The crude esterification reaction mixture was virtually all hydroperoxide by TLC, and only the 13 isomer could be detected by analytical HPLC. II was also the predominant product at pH 7 and 0C when the reaction was terminated after 5 min (Expt. 3). Preparative HPLC of this mixture resulted in a 34% isolated yield based on the fatty acid. Longer reaction times at pH 7 and 0 C led to the formation of polar by-products (Expt. 4) at the expense of the 13 isomer.

Obtaining the reported ratio of the 9 to 13 isomers at a high level of conversion was less readily accomplished. With reaction conditions qualitatively similar to those described by Roza and Francke (10)(Expt. 5-6) and Christopher et al. (11) (Expt. 7), but with commercial lipoxygenase rather than purified enzyme, poor yields of I were obtained. Polar by-products were identified by TLC as the major components of the oxidation mixtures. The formation of these by-products was, however, suppressed by increased dilutions and reduced reaction times (Expt. 8-12) for the substrate solubilized as the ammonium salt and oxidized at room temperature and pH 7. Preparative HPLC resulted in a 25% isolated yield of I. A similar change in reaction conditions for the substrate in an ethanol dispersion (Expt. 13) was only moderately successful in improving the yield of I.

The ω6 hydroperoxide from arachidonic acid, III, was prepared in high yield with the pH 9 ethanol dispersion method (Expt. 14). Analytical HPLC showed only minute amounts of other hydroperoxides present, and TLC showed the hydroperoxide to be the major product. With conditions that maximize the ω10 hydroperoxide in γ-linolenic acid (Expt. 15), no significant change in hydroperoxide ratio for arachidonic acid was noted. Several more polar by-products were formed with these reaction conditions, however.

DISCUSSION

The conversion of unsaturated lipids to hydroperoxides by lipoxygenase enzymes appears to be an important chemical event in plants, e.g., potato (7), wheat (8), and soybean (5), and a lipoxygenase system in mamallian blood has recently been investigated (9).

The chemical reactivity of the lipid hydroperoxides formed in the lipoxygenase systems has not been studied in any detail. A primary difficulty in studies of lipid hydroperoxide has been the preparation and isolation of pure compounds from readily available enzymes. Methods described here overcome this difficulty, making quantities of pure hydroperoxide on the 100 mg scale available from commercial lipoxygenase.

The recent report that HPLC is useful in the analytical separation of the methyl ester hydroperoxides of linoleic acid (16) has prompted us to report our studies based on this technique. We find that HPLC is a useful tool in separating hydroperoxides derived from γ-linolenic and arachidonic acids. Both the methyl ester and the free fatty acid hydroperoxides may be isolated on a preparative scale. Separations of (50-100 mg) the 9 and 13 hydroperoxy methyl esters I and II have been achieved routinely, and separations of larger samples would appear to be straightforward.

We find that commercial lipoxygenase is a useful synthetic reagent only under carefully controlled conditions. Reaction time is of extreme importance. Long reaction times result in the formation of polar nonperoxide by-products. Contaminants in the commercial material are probably responsible for those side reactions, inasmuch as experiments reported with purified enzyme preparations apparently do not result in significantly reduced peroxide product yield in long time-scale reactions (10,11). Dilution, pH, and reaction media also affect product yield.

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Configuration at C-24 of 24-Methyl and 24-Ethylcholesterol in Tracheophytes[1]

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ABSTRACT

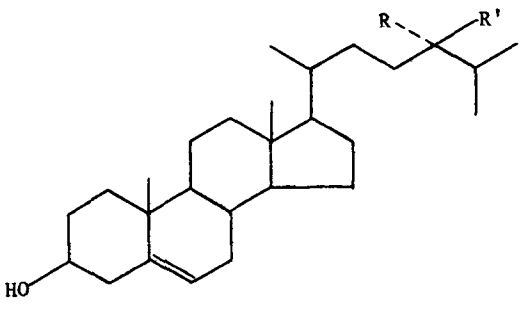
24-Ethylcholesterol was shown by proton magnetic resonance spectroscopy to have only the α-configuration in a series of tracheophytes ranging through the evolutionary hierarchy from ferns through gymnosperms and primitive angiosperms to climax angiosperms. 24-Methylcholesterol, however, was consistently an epimeric mixture, with the 24α-epimer present in about twice the concentration of the 24β-epimer. 24-Methylcholesterol was always present in smaller amount than the 24-ethylcholesterol.

INTRODUCTION

The configuration at C-24 of sterols is believed to be predominantly of the β-series in algae (1) and fungi (2) and the α-series in tracheophytes (3), where the α-configuration (R when the side chain is saturated and S when a Δ^{22}-bond is present) is taken to mean the substituent points toward the observer when the molecule is viewed in the usual way with the side chain arranged to the right (C-22 away from C-13) in the staggered conformation (Scheme 1). In the β-configuration, the substituent points away from the observer. However, 24α-alkylsterols have been reported to occur in one algal species (4) and 24β-alkylsterols in two tracheophyte families (5-8). To shed more light on what rules govern the stereochemistry, we have determined the configurations of 24-methyl and 24-ethylcholesterol which occur in a series of vascular plants arranged in an evolutionary hierarchy. Proton magnetic resonance (PMR) spectroscopy was used as a tool; it has recently been shown (9-12) to distinguish the α- and β-epimers.

The plants chosen were as follows: The New York fern, *Dryopteris novaboracensis* (L.) Gray, was selected as a representative of a lower vascular plant. The stone pine, *Pinus pinea*, was selected as representative of a gymnosperm because the maternal haploid endosperm and the diploid embryo could be examined separately, which allowed any generational and developmental phenomena to be manifest if they existed. For higher plants, we chose first the tulip tree, *Liriodendron tulipifera* L. because it is a representative of the order Magnoliales, family Magnoliaceae, which, due to morphological and geographical factors, is believed to comprise the most ancient living angiosperms (13-15). Time has limited the *Liriodendron* genus to only two species, *L. tulipifera* and the Chinese *L. Chinese* Sargent. Plants of the order Magnoliales are thought to be either on or close to the line which led to the remainder of the woody dicotyledons. In a parallel line, the order Ranales is believed to occupy an analogous place in the evolution of monocotyledons. We therefore obtained specimens of the May apple, *Podophyllum peltatum* L., which is in one of seven families in the order Ranales. Analogous to the *Liriodendron* genus, the May apple occurs only in eastern North America and eastern Asia. Finally, we examined three representatives of the higher dicotyledons, a species in the family Cruciferae (cabbage, *Brassica oleracea* L.) because it is herbaceous and two in the Leguminosae (soybeans, *Glycine max* [L.] Merr., and peas, *Pisum sativum* L.) because they are woody.

[1]Presented at AOCS Sterol Symposium, 48th Annual Meeting, Philadelphia, September, 1974.



SCHEME 1. 24-Alkylcholesterol. 24α-Methyl, R = H, R' = CH_3; 24β-methyl, R = CH_3, R' = H; 24α-ethyl, R = H, R' = C_2H_5; 24β-ethyl, R = C_2H_5, R' = H.

EXPERIMENTAL PROCEDURE

Stigmasterol was obtained from common commercial sources. Brassicasterol was the gift of H. Kircher and was derived from rapeseed. Pure sitosterol was prepared from stigmasterol, and the acetate was found to crystallize in hexagonal prisms, mp 120.0-120.5 C. The

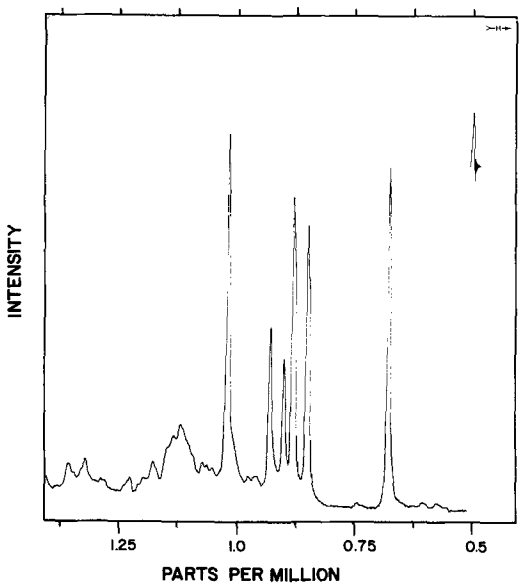


FIG. 1. Nuclear magnetic resonance spectrum of authentic cholesteryl acetate of commercial origin.

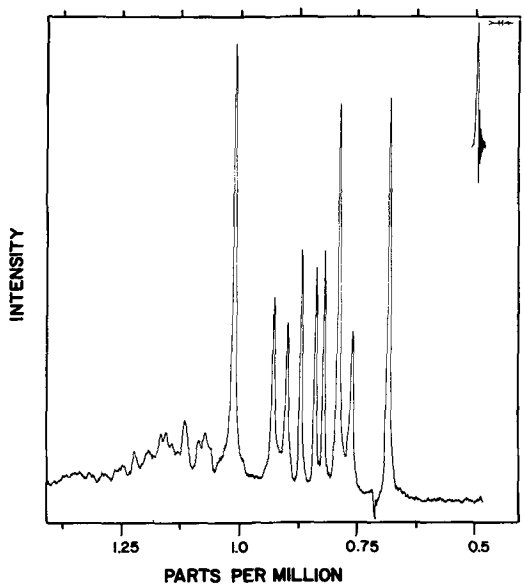


FIG. 2. Nuclear magnetic resonance spectrum of authentic campesterol from soybeans.

epimer of stigmasterol (poriferasterol) was isolated from *Chlorella ellipsoidea* and was kindly sent to us by G. W. Patterson. The epimer of sitosterol (clionasterol) is a much rarer compound. Insufficient poriferasterol was available for chemical conversion to it. One sample was obtained as the benzoate from the marine invertebrate collection of the late W. Bergmann through the courtesy of W. M. Stokes. It is presumably of algal origin through ingestion by the invertebrates. Gas liquid chromatographic analysis showed it to be a mixture with at least four other sterols in substantial quantities. After hydrolysis to the alcohol, the component possessing the retention time of 24-ethylcholesterol was obtained as a single chromatographic entity by repeated and sequential preparative gas liquid chromatography (GLC) and was found to have mp 137.5-138.5 and $[\alpha]_D^{24}$ -33.6. However, these constants, as well as the GLC retention time, were indistinguishable from those of the pure sitosterol (mp 137.0-137.5, $[\alpha]_D^{24}$ - 33.6) which was prepared from stigmasterol. No consequential depression of the melting points occurred on admixture. We therefore do not regard the marine invertebrate "clionasterol" as being necessarily authentic. The bergmann sample was alternatively purified by preparative reversed phase thin layer chromatography (TLC). Gas liquid chromatographic analysis showed it to be 83% 24-ethylcholesterol, 16% of a component with the retention time near that of its

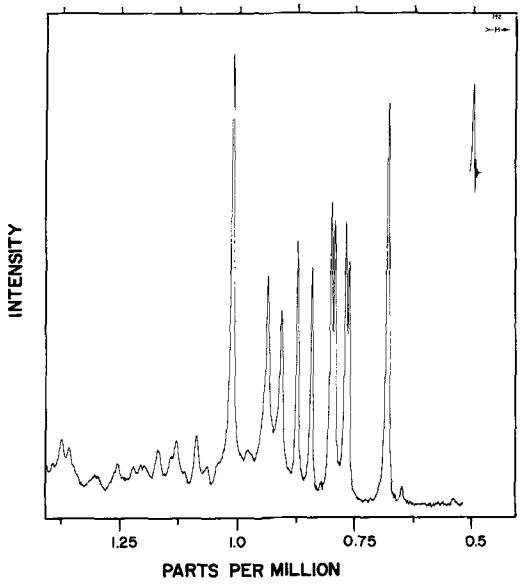


FIG. 3. Nuclear magnetic resonance spectrum of dihydrobrassicasterol synthesized from ergosterol.

Δ^{22}-derivative, and 1% of a component with the retention time of 24-methylcholesterol. A final sample was obtained from *Chlorella ellipsoidea* through the courtesy of G.W. Patterson. The acetate was recrystallized (giving leaflets) and found to melt at 137.5-138.5 in close agreement with expectation. It moved as a single entity in GLC and had the same retention

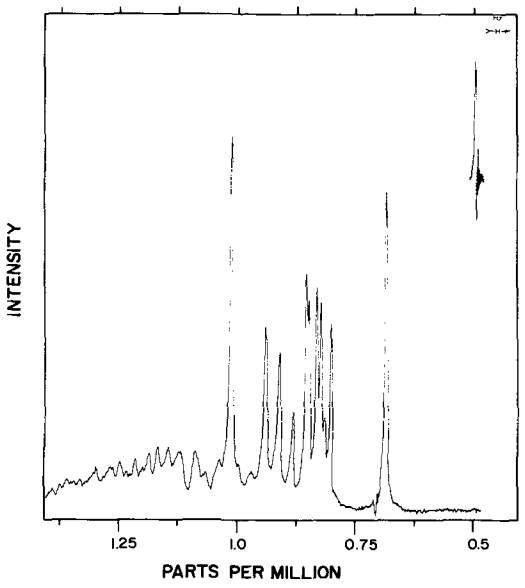


FIG. 4. Nuclear magnetic resonance spectrum of authentic sitosterol synthesized from stigmasterol.

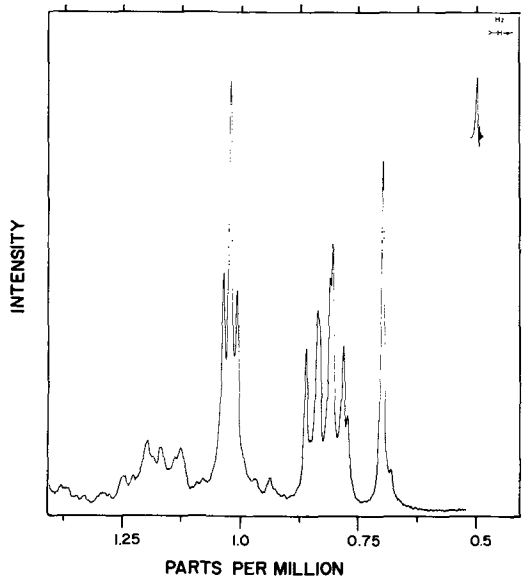


FIG. 5. Nuclear magnetic resonance spectrum of authentic stigmasteryl acetate of commercial origin, presumably from soybeans.

as sitosteryl acetate.

Campesterol (24α-methylcholesterol) was obtained from Applied Science Laboratories (State College, PA), who isolated it from soybeans by equilibrium crystallization on a very large scale. After crystallization, it melted at 159-160; acetate 138-139. Although, as shown in the present work, the fact that soybeans contain both epimers of 24-methylcholesterol would appear to cast doubt on the authenticity of this sample of campesterol, we proved, as will be seen, that both it and synthetic dihydrobrassicasterol were epimeric because of their very different PMR spectra. Comparison of the two spectra also revealed that the two samples of these epimeric materials were configurationally quite pure. Ergosterol was used to obtain authentic 24β-methylcholesterol (dihydrobrassicasterol) by a series of reactions developed in Barton's laboratory (16). The 24β-methyl compound melted at 157-158.

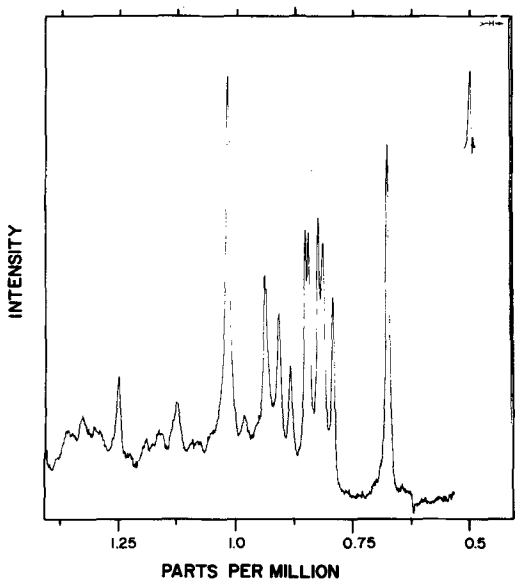


FIG. 6. Nuclear magnetic resonance spectrum of authentic clionasteryl acetate from *Chlorella ellipsiodea*.

In all cases, the 4,4,-desmethylsterols isolated from leaves or seeds were obtained as a multifraction band in alumina chromatography. In one of these, the fern, gas liquid chromatographic monitoring of the individual fractions showed that the less polar half of the band was homogenous from fraction to fraction but the more polar half was inhomogenous and contained three additional compounds in small amounts, which will be described in a subsequent communication. In the other cases, the entire 4,4-desmethylsterol band was homogenous. The homogenous fractions were combined for a given plant, nonfractionally crystallized to remove traces of contaminants, and used in this form for the spectroscopic studies or for further chromatographic separation of the homologs. Inconsequential changes occurred in the steroidal composition before and after recrystallization, as shown by gas liquid chro-

matographic analysis. The mass spectrum of all the samples showed them to have the assigned structures. The constant predominance of 24-ethylcholesterol was especially pronounced in the lower plants. Except for the compounds mentioned in the fern case, no evidence for any sterols was found other than cholesterol (trace amounts on occasion), 24-methylcholesterol, 24-ethylcholesterol, and occasional small amounts of a Δ^{22}-derivative. Consequently, the sterols studied were typical of dominant sterols (17).

Nuclear magnetic resonance (NMR) spectroscopy was performed on 2.5-6.0 mg of sterol in deuterated chloroform at room temperature in 1-2% solutions at 220 MHz by the Ontario Research Foundation (Ontario, Canada), under contract with Morgan-Schaffer (Montreal 252, Quebec, Canada). Mass spectroscopy was performed by the latter organization. Optical rotations were determined on a Rudolf (O.C. Rudolf and Son, Inc., Caldwell, NJ) Model 80 high precision instrument in $CHCl_3$. GLC was performed at 235 with 1% of nitrile silicone gum (XE-60) deposited on silanized Chromosorb W equipped with a stream splitter to allow collection of fractions. Interconversion of acetates and free sterols was accomplished by hydrolysis in refluxing 10% methanolic KOH for 15 min or by acetylation for 16 hr at room temperature with acetic anhydride in pyridine. Melting points were taken on a Kofler apparatus, and crystallizations were from methanol.

Sterols were isolated from plant specimens by continuous extraction with acetone in a Soxhlet extractor ca. 24 hr or until no chlorophyll appeared in the extract. The extracted material was saponified in 10% methanolic KOH at reflux for 1 hr, and the neutral lipid was chromatographed on Al_2O_3 containing 3% by wt of water in a system of ether graded into hexane. This yielded, inter alia, a 4,4-dimethyl- and a 4-desmethylsterol fraction. Separation of the C-24 homologs in the latter fraction was achieved either by reversed phase chromatography on a thin layer of paraffin impregnated kieselguhr with the system paraffin oil:acetone-water, 4:1 (18), followed by removal of the paraffin oil from the eluted fractions by chromatography on alumina, or by column chromatography on a lipophilic-hydrophobic Sephadex (19). The latter material (Lipidex-5000) was purchased from Packard Instrument Co., Inc. (Downers Grove, IL). The solvent system was 5% heptane in methanol. At a flow rate of 10 ml per hr, 50 mg of a sterol mixture was resolved on 400 cm^3 (ca. 200 g) of the Sephadex

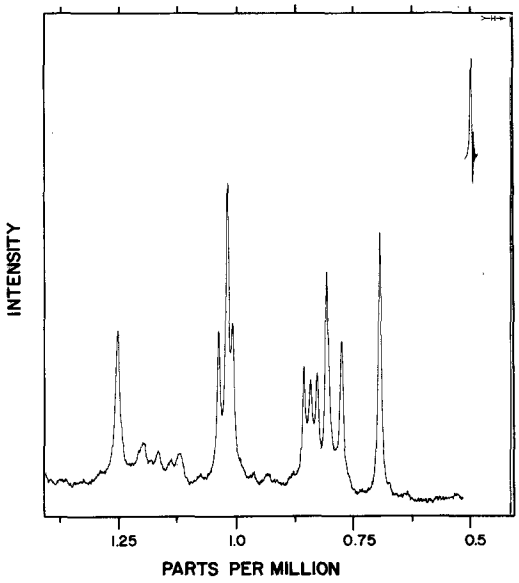


FIG. 7. Nuclear magnetic resonance spectrum of authentic poriferasteryl acetate from *Chlorella ellipsoidea*.

TABLE I

Positions of Nuclear Magnetic Resonance Absorption of Authentic Sterols at 220 MHz

Sterol	C-18	C-19	C-21	C-28	C-29	C-26,27
Cholesterol	149s	222s	200d	-	-	190d, 189d
Campesterol	150s	222s	200d	177d	-	187d, 170d
Dihydro-brassicasterol	150s	222s	203d	172d	-	188d, 171d
Sitosterol	150s	222s	203d	-	186t	184d, 179d
Clionasterol	150s	222s	204d	-	188t	183d, 178d
Stigmasterol	154s	222s	225d	-	177t	186d, 175d
Poriferasterol	153s	222s	225d	-	178t	185d, 174d
Brassicasterol	154s	223s	224d	201d	-	184d, 181d

[a]Values are rounded to the next higher number and given as displacements in Hz from the signal for tetramethylsilane. s = Singlet, d = doublet, and t = triplet. Only the center is given. For the doublets J = 6, and for the triplets J = 7. The values can be converted to ppm for comparison with the figures by dividing by 220.

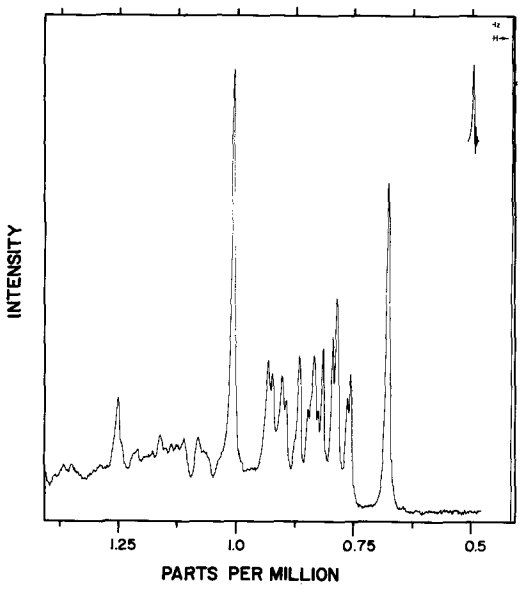


FIG. 8. Nuclear magnetic resonance spectrum of an authentic mixture of 25% sitosterol, 30% dihydrobrassicasterol, and 45% campesterol.

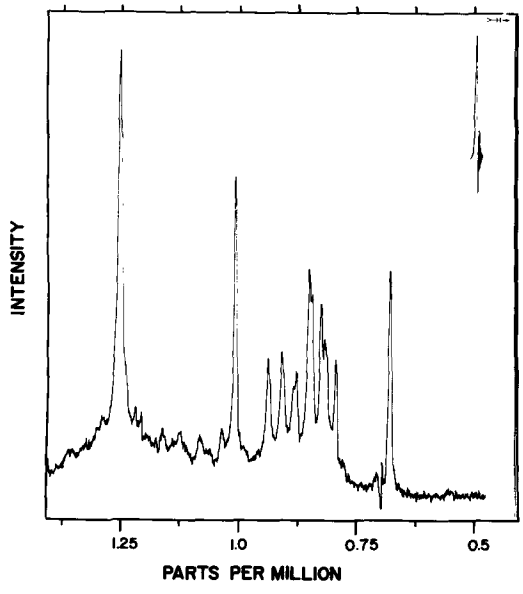


FIG. 9. Nuclear magnetic resonance spectrum of a "clionasterol" sample derived from the marine invertebrate collection of the late W. Bergmann.

in a column of 2.5 cm inside diameter. The Sephadex column was found to allow separation of larger amounts in less time and with less effort than did reversed phase chromatography.

The fronds of the fern (*Dryopteris novaboracensis* [L.] Gray), the leaves of the tulip tree (*Liriodendron tulipifera* L.), May apple (*Podophyllum peltatum* L.), and cabbage (*Brassica oleraceae*), and the ungerminated seeds of the pea (*Pisum sativum* L.) were used. In the case of soybeans (*Glycine max* [L.] Merr.), the commercially available sterols ("sitosterol") (Sigma Chemical Company, St. Louis, MO) that remain after removal of stigmasterol were directly separated by chromatography on Sephadex. The gymnosperm studied was the stone pine (*Pinus pinea*). Seeds lacking their shells were incubated on moist filter paper for 7-10 days at room temperature until a root appeared, and the endosperm and germinated embryo were separated mechanically. Each was submitted separately to extraction, etc.

RESULTS

Diagnostic Characteristics

Although the spectra of pure sterols (Figs. 1-7 and Table I) are different from each other, and constitute a "finger print" which allows unequivocal structural elucidation, we observed some particular differences which are useful for analysis of mixtures. Of special importance is the lack of any peak for sitosterol and clionasterol near 168 Hz, which is just to the left of the singlet for C-18. It is here that campesterol and dihydrobrassicasterol have peaks and also ones which are configurationally diagnostic even in the presence of the 24-ethylcholesterols. Campesterol exhibited a single peak at 167 Hz, while, in dihydrobrassicaterol, two peaks occurred near 168 and 169 Hz. In an authentic mixture (Fig. 8) of sitosterol, campesterol, and dihydrobrassicasterol, slight overlapping of the three theoretical peaks yielded two experimental peaks at ca. 167 and 168 Hz. In the absence of any sitosterol, campesterol could also be distinguished in the presence of dihydrobrassicasterol by a peak at 180 Hz, at which point dihydrobrassicasterol has a minimum. The epimers also exhibit different positions for the C-21 doublet. In the 24-ethyl epimers (sitosterol and clionasterol), a shift of 2 Hz in the left hand portion of the triplet for C-29 is especially diagnostic, along with the apparent disappearance of the right hand portion of the triplet and a small shift in the C-21 doublet. In mixtures, the two left hand portions of the triplets combine to produce a peak with a shoulder. This is seen in the Bergmann sample of "clionasterol" (Fig. 9), which presumably then is a mixture of clionasterol and sitosterol, with the latter the major component. The presence of the Δ^{22}-bond could be ascertained by the large downfield shift of 22 Hz in the doublet for C-21, as well as in a smaller effect (4 Hz) on the singlet for

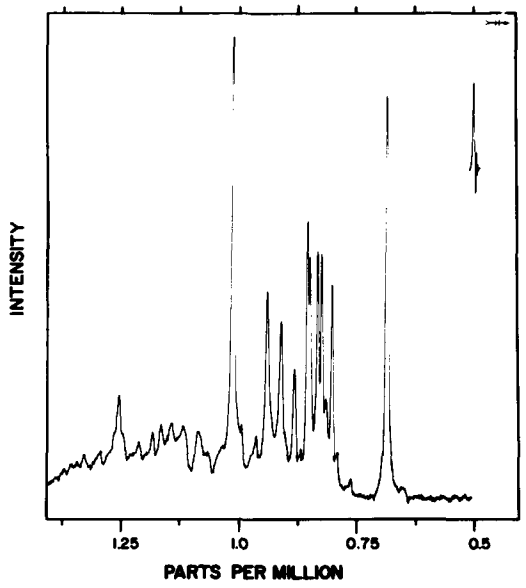


FIG. 10. Nuclear magnetic resonance spectrum of the 4,4-desmethylsterol from fronds of the fern *Dryopteris novaboracensis.*

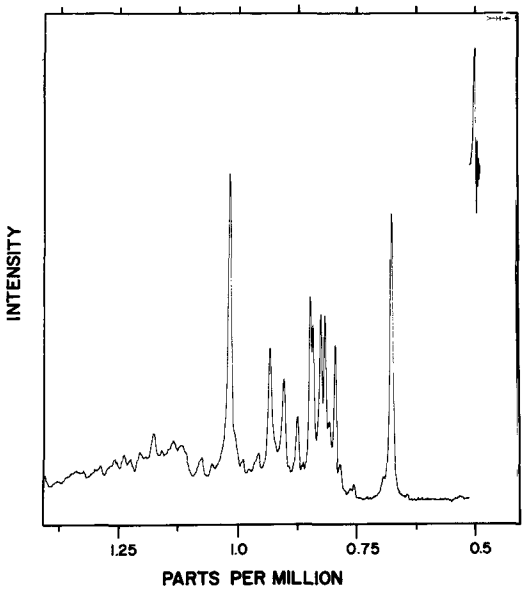


FIG. 11. Nuclear magnetic resonance spectrum of the 4,4-desmethylsterol from the leaves of the tulip tree *Liriodendron tulipifera.*

C-18. The 24β-ethyl configuration in the Δ^{22}-derivatives was readily distinguished by two resolved peaks near 185 Hz and a single strong peak near 161 Hz. The situation was reversed in the 24α-ethyl case. Near 185 Hz, a broad peak with a slight shoulder was found, and near 161 Hz, two peaks occurred.

Nuclear Magnetic Resonance

The spectrum of the fern sterol (Fig. 10) was essentially superimposable on that of authentic sitosterol and was distinctly different from that of clionasterol. Very weak peaks corresponding to 24-methylcholesterol were observable. Near 168 Hz, the two peaks diagnostic for both 24α- and 24β-configurations were present. As the evolutionary hierarchy was climbed, no configurational changes occurred. The sterols from the pine embryo, pine endosperm, tulip tree, cabbage, pea, and soybean all showed spectra essentially identical to that of the fern, except that the amount of 24-methylcholesterol was somewhat greater in some. There was an unmistakable peak close to 167 Hz for campesterol and a weaker peak or shoulder at 168-169 Hz for dihydrobrassicasterol. This is illustrated in Figure 11 for the tulip tree sterol and Figure 12 for the soybean sterol. Numerical data for all cases is given in Table II. No 24-methylcholesterol was observable in the May apple sterols, but a weak peak (ca. 12% of the C-18 peak) occurred at 154 Hz, corresponding to the presence of a Δ^{22}-sterol. In the mass

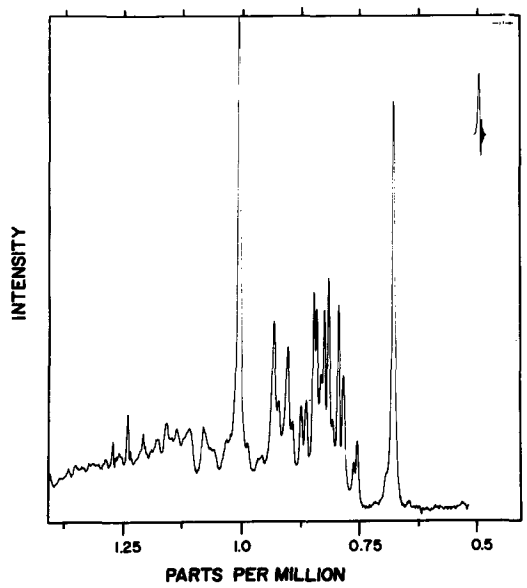


FIG. 12. Nuclear magnetic resonance spectrum of 4,4-desmethylsterol from soybeans, *Glycine max,* after removal of stigmasterol.

spectrum, a minor component with m/e 412 occurred, corresponding to M^+ for stigmasterol. Otherwise the spectrum was identical with that of sitosterol. A similar weak peak was seen in the spectra of the tulip tree and the pea sterol, but none was observable in the sterols from the

TABLE II

Positions of Nuclear Magnetic Resonance Absorption at 220 MHz of Sterols Isolated from Plants[a]

Plant	Part	Sterol	C-18	C-19	C-21	C-28	C-29	C-26,27
Dryopteris novaboracensis	Frond	24-Et-Δ^5(c)	150s	222s	203d	-	186t	184d, 179d
Dryopteris novaboracensis	Frond	24-Me-Δ^5(c)	150s	222s	203d 200d	177d 172d	-	187d, 170d 188d, 171d
Pinus pinea	Endosperm	24-Et-Δ^5(m)	150s	222s	202d	-	186t	184d, 179d 170d, 172d
Pinus pinea	Embryo	24-Et-Δ^5(m)	150s	222s	202d	-	186t	184d, 179d 170d, 172d
Liriodendron tulipifera	Leaf	24-Et-Δ^5(m)	150s	222s	203d	-	186t	183d, 179d 170d, 172d
Podophyllum peltatum	Leaf	24-Et-Δ^5	150s	222s	203d	-	186t	184d, 179d
Pisum sativum	Seed	24-Et-Δ^5(m)	150s	222s	203d	-	186t	184d, 179d 170d, 172d
Brassica oleracea	Leaf	24-Et-Δ^5(c)	150s	222s	203d	-	186t	183d, 179d
Brassica oleracea	Leaf	24-Me-Δ^5(c)	150s	222s	203d, 200d	177d, 172d	-	187d, 170d 188d, 171d
Glycine max	Seed	24-Et-Δ^5(c)	150s	222s	203d	-	186t	184d, 179d
Glycine max	Seed	24-Me-Δ^5(c)	150s	222s	203d, 200d	177d, 172d	-	187d, 170d 188d, 171d

[a]vw = Very weak, m = containing some 24-methyl -Δ^5-sterol, c = chromatographically pure, s = singlet, d = douplet, t = triplet. Only the center is given. For the douplets J=6, and for the triplets J=7. The values can be converted to ppm for comparison with the figures by dividing by 220.

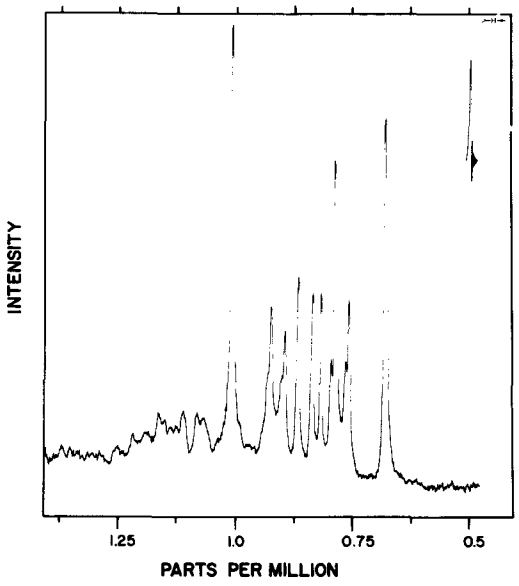


FIG. 13. Nuclear magnetic resonance spectrum of the purified 24-methylcholesterol from soybeans, *Glycine max.*

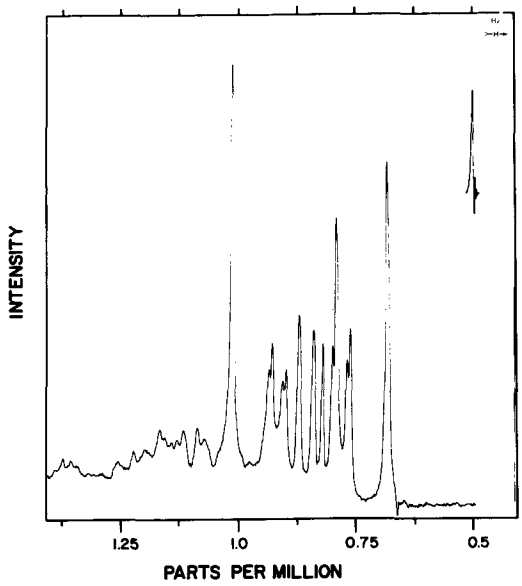


FIG. 14. Nuclear magnetic resonance spectrum of a 1:1 mixture of authentic campesterol and dihydrobrassicasterol.

fern, cabbage, pine embryo, or pine endosperm. The latter two spectra were identical, indicating no generational effect in this organism. In the spectra of the sterols from the cabbage and soybean, the two peaks at 168 and 169 Hz for a mixture of 24α- and 24β-methylcholesterol were especially clear due to higher concentrations of the 24-methyl component.

After separation from sitosterol, the 24-methylcholesterol from the fern, cabbage, and soybean exhibited spectra which, in each case, were an exact composite of those for campesterol and dihydrobrassicasterol, as illustrated in Figure 13 for soybean 24-methylcholesterol and Figure 14 for an authentic mixture. The epimeric character is most readily seen (a) by the presence of two doublets for C-21, with the two peaks for campesterol appearing at 204 and 198 Hz and the two for dihydrobrassicasterol appearing as distinct shoulders 1-2 Hz downfield with about 60% the intensity of the ones for campesterol; and (b) by the presence of the two well resolved peaks at 169 and 168 Hz. Similar clarity in the spectra of the pure sitosterol from the fern, cabbage, and soybean separated from the 24-methyl component also unequivocally showed this compound to have only the 24α-configuration. This is illustrated in Figure 15 for the soybean sample.

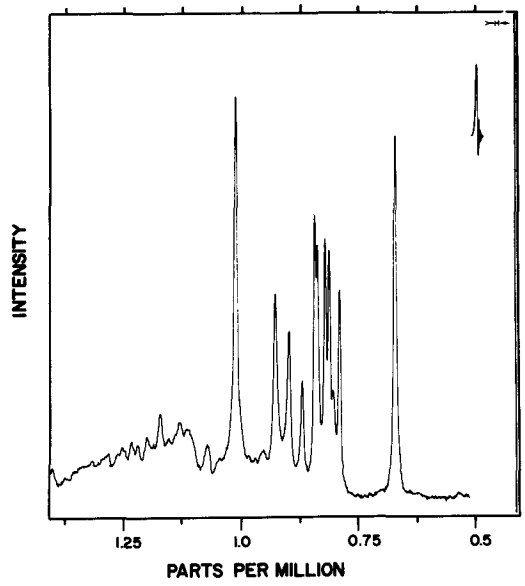


FIG. 15. Nuclear magnetic resonance spectrum of the purified 24-ethylcholesterol from soybeans, *Glycine max.*

DISCUSSION

From the PMR spectra of the steranes derived from the sterols of corn and soybeans, Mulheirn (10,11) has concluded that these plants possess both 24α- and 24β-methylsterols. Our own studies show that the Δ^5-sterols themselves are equally amenable to configurational analysis by PMR spectroscopy, that the epimeric mixture exists naturally as 24-methyl-

cholesterol, that the epimeric mixture occurs widely in tracheophytes, but that the 24-ethylcholesterol, which in all cases was present and dominant, is a single epimer (sitosterol) with the α-configuration. Whereas 25-dehydroporiferasterol is present in a species of *Clerodendrum* (5,6) and its Δ^7-analog in Cucurbitaceae (7,8), 24β-ethylcholesterol itself remains unknown in plants higher than algae. Thus, although vascular plants can biosynthesize sterols with both configurations at C-24, there appears to be a complete selection at the 24-C_2 level for the α-configuration in the dominant compounds of ferns and plants higher in the evolutionary hierarchy when the side chain lacks a Δ^{25}-bond. This is in sharp contrast to nonvascular plants in which 24β-ethylsterols, e.g., clionasterol and poriferasterol, appear to be common.

ACKNOWLEDGMENTS

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Addition of *N*-Acetylcysteine to Linoleic Acid Hydroperoxide[1]

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ABSTRACT

Catalyzed by 10^{-5}M ionic iron in 80% ethanol, *N*-acetylcysteine added to linoleic acid hydroperoxide, forming a thiobond. Reaction of a specific isomer of the hydroperoxide, 13-hydroperoxy-*trans*-11,*cis*-9-octadecadienoic acid, and *N*-acetylcysteine, forms a number of products, of which two were identified as addition compounds. One addition product was 9-*S*-(*N*-acetylcysteine)-13-hydroxy-10-ethoxy-*trans*-11-octadecenoic acid, and the other was 9-*S*-(*N*-acetylcysteine)-10,13-dihydroxy-*trans*-11-octadecenoic acid.

INTRODUCTION

Lipid peroxides degrade proteins by reacting with them to form products by scission (1), protein-protein crosslinking, and covalent bonding with the lipid peroxide (2). Although a secondary product of lipid peroxidation, malonaldehyde, has been implicated as one of the agents responsible for protein crosslinking through Schiff base formation, Roubal (3) showed that radical production is probably the factor most responsible for destruction of amino acid residues. In proteins, among the number of amino acid residues particularly labile are the sulfur amino acids (4,5). Sulfhydryl enzymes are inactivated rapidly by lipid peroxides (6,7) and demonstrate the oxidative sensitivity of the sulfhydryl group. Thiyl radicals are observed in proteins with free sulfhydryl groups after exposure to peroxidized lipid; disulfide bonds do not generate radicals (8). Studies with small thiols, including cysteine, show that thiols are oxidized to disulfide and other products by lipid peroxide (5,9), and the presence of transition metal ions or metalloprotein accelerates oxidation (10,11). Formation of disulfides implies that thiyl radicals are present. Even though thiyl radicals are known to add to olefins (12), no addition products of thiol amino acids and unsaturated lipid peroxide have been characterized.

A number of products result from reaction of linoleic acid hydroperoxide (LOOH) with cysteine as catalyzed by ionic iron (11). These products were characterized as nine oxygenated fatty acids, as well as about an equal number of ninhydrin-reactive lipids (13). To explain the formation of the products, a free radical Fe(II)-Fe(III) redox cycle was proposed by Gardner (14) in which thiyl radicals were generated from cysteine and alkoxy radicals from LOOH. We extended these observations and now report structures of products from reaction of *N*-acetylcysteine and a specific isomer of LOOH, 13-hydroperoxy-*trans*-11,*cis*-9-octadecadienoic acid (13-LOOH).

METHODS

Reaction Conditions

The reaction solution was 3.2 mM 13-LOOH, 13.0 mM *N*-acetylcysteine (Nutritional Biochemicals Corp., Cleveland, OH), and 10^{-5}M $FeCl_3$ in 80% ethanol. The 13-LOOH was prepared 99+% pure by column chromatography (15). The reaction proceeded for 1 hr at room temperature under a nitrogen atmosphere. Products were extracted with $CHCl_3$ as before (13).

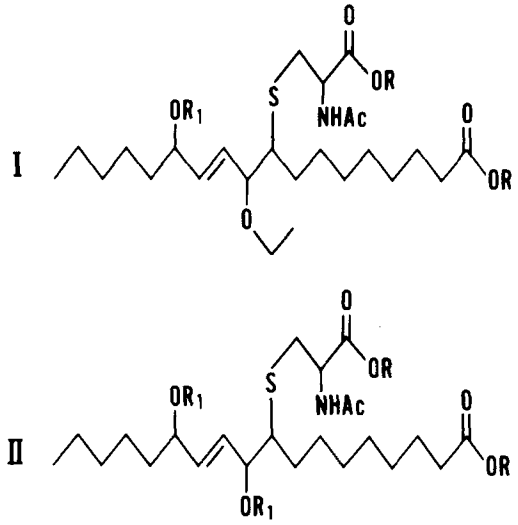
In one experiment a mixture of 79% 13-LOOH and 21% 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid (9-LOOH) was synthesized by the method of Gardner et al. (13), and subsequently used in a reaction with *N*-acetylcysteine.

Chromatography

Column chromatography: The product mixture, containing 9-*S*-(*N*-acetylcysteine)-13-hydroxy-10-ethoxy-*trans*-11-octadecenoic acid (I) and 9-*S*-(*N*-acetylcysteine)-10,13-dihydroxy-*trans*-11-octadecenoic acid (II) (Fig. 1), was methylated with diazomethane, subsequently slurried with 2 g Mallinckrodt SiliCAR CC7 in $CHCl_3$ and applied to a column (inside diameter 2.5 cm) packed with 50 g SiliCAR CC7 in $CHCl_3$. The column was eluted stepwise with 200 ml $CHCl_3$ and 300 ml each of $CHCl_3$ solutions containing 0.5% (v/v), 0.75%, 1.5%, 2%, 3%, and 4% CH_3OH. The 10-ml fractions collected were assayed by thin layer chromatography (TLC). If more separations were required, fractions from the column were purified further by TLC.

TLC: Silica Gel G plates 20 x 20 cm and 250 μ thick were used for analytical and preparative TLC. Quantities in excess of ca. 25 mg

[1]Presented at the AOCS 49th Annual Fall Meeting, Cincinnati, September-October, 1975.



I or II: R and R_1 = H

Ia or IIa: R = CH_3, R_1 = H

Ib or IIb: R = CH_3, R_1 = $Si(CH_3)_3$

FIG. 1. Numerical key and structures of addition products and derivatives from the reaction of *N*-acetylcysteine and 13-hydroperoxy-*trans*-11,*cis*-9-octadecadienoic acid.

were separated on preparative plates 0.5 mm thick. Triple development (air dried between development) with $CHCl_3$:CH_3OH 98:2 in a tank lined with Whatman 3 MM filter paper served for all preparative separations. Products Ia and IIa were located by the presence of a long wave ultraviolet fluorescence which migrated in a band just in front of them.

Fractions from column chromatography were assayed by TLC. From each fraction a 40 μl sample was spotted, and then the plate was developed with $CHCl_3$:CH_3OH 95:5 in a filter paper lined tank. The spots were charred by heat after spraying the plates with 50% H_2SO_4.

Trimethylsilyloxy (TMS) derivatives, Ib and IIb, were separated from the reagents and other impurities by TLC with hexane:anhydrous ether 1:1 solvent (R_f = 0.08 and 0.13 for Ib and IIb, respectively). Long wave fluorescence indicated the front of the compound. TLC scrapings were eluted with anhydrous ether, care being taken to maintain dryness.

Spectroscopy

Mass spectroscopy (MS) was done on a Dupont 21-492-1 mass spectrometer by probe insertion. Ionization was completed with 70 electron volts.

Infrared (IR) spectra were determined as described previously (13). Nuclear magnetic resonance (NMR) spectra were recorded with a Varian Model HA-100 at 100 MHz or with a Bruker WH-90 operating at 90 MHz. The samples were dissolved in $CDCl_3$ with 1% tetramethylsilane as the internal reference. Sample temperature was 29 C, and absorptions were measured by first order analysis. When compounds containing TMS group(s) were analyzed by 100 MHz NMR, the internal lock was provided by 1% $CHCl_3$. A trace of tetramethylsilane served to measure δ 0 accurately.

Derivatives

Diazomethane was used to esterify (16). Hexamethyldisilazane:trimethylchlorosilane:pyridine 2:1:1 (v/v/v) formed TMS ethers from hydroxyl groups. Standing at room temperature was sufficient to form TMS ethers when the compound was ca. a 10% solution in the reagent.

RESULTS

The Reaction

N-Acetylcysteine was allowed to react with 13-LOOH by introducing 10^{-5} M $FeCl_3$ into the reaction mixture. The reaction was essentially complete within 10-20 min. The products were treated with diazomethane and then separated by column chromatography (Table I). Ia and IIa (Fig. 1) were products in which *N*-acetylcysteine had added to the *cis* double bond of 13-LOOH. Besides Ia and IIa, two other compounds, Ia′ and IIa′, were isolated. By all criteria, except minor differences in NMR spectra, Ia′ and IIa′ were indistinguishable from Ia and IIa, respectively. Because two functional groups had added to vicinal carbons, *erythro-threo* isomerism is the most probable reason there are two isomeric forms for each addition product. Other compounds had spectral characteristics typical of both a fatty ester and an *N*-acetylcysteine methyl ester, indicating that more addition products were present; since these components were minor, they were studied no further. As can be seen from Table I, *N,N′*-diacetylcystine dimethyl ester was also observed. On the basis of replicate experiments, product yields vary somewhat, but always I (and I′), II (and II′), and *N,N′*-diacetylcystine are present as major products.

Structure Determination

Products I and I′: Products I and I′ were isolated as their methyl esters, Ia and Ia′. Product Ia was isolated by column chromatography (Table I), but Ia′ had to be purified

TABLE I

Column Chromatography[a] of Linoleic Acid Hydroperoxide:*N*-Acetylcysteine Reaction Products after Methyl Esterification

Eluant volume (ml)	Wt (mg)	Identity of component[b]
0-320	83	Mostly fatty esters
320-430	132	Mostly *S*-methyl-*N*-acetyl-cysteine methyl ester[c]
430-540	20	Unidentified addition products
540-640	32	Ia
640-720	75	Ia, Ia′, and a minor amount of unidentified material
720-900	15	Unidentified addition products
900-1070	33	*N,N′*-Diacetylcystine dimethyl ester and unidentified products
1070-1190	15	IIa
1190-1320	12	IIa′
1320-1790	4	Unidentified

[a]Crude product (550 mg) was applied to a silica column and eluted with $CHCl_3$-CH_3OH.

[b]Ia and IIa are identified in Figure 1. Ia′ and IIa′ were isomers of Ia and IIa that were identical in properties except for minor differences in NMR spectra.

[c]Identified by comparison to a standard synthesized by treatment of *N*-acetylcysteine with diazomethane.

TABLE II

Infrared Spectral Assignments[a] of Addition Products and Their Respective Isomers[b]

Absorption (cm^{-1})		
Ia, Ia′	IIa, IIa′	Assignment
3430(sharp), 1675	3430(sharp), 1665	Amide
3360(broad)	3330(broad)	Amide and hydroxyl
1500	1520	Monosubstituted amide
1085	---	Ether C-O stretch
1020	1020	Secondary alcohol
975	975	Isolated *trans* olefin

[a]Not tabulated are absorption characteristics of fatty esters (e.g., methyl stearate).

[b]Ia′ and IIa′ are isomeric forms of Ia and IIa postulated to be *erythro* and *threo* about carbons 9 and 10 of the fatty ester chain. No difference could be observed in IR spectra between the isomeric forms.

further by TLC after column chromatography.

Spectral data of Ia and Ia′ appear in Table II for IR and in Table III for NMR. The MS of Ib (Fig. 2) is identical to the MS of Ib′ within operating parameters. The MS subtraction ions from Figure 2 are identified in Table IV. Structural assignments are based on these spectral data and on additional experiments outlined below.

The presence of one hydroxyl group was demonstrated by analyzing derivative Ib and Ib′ by NMR. An absorption at δ 0.11 (s, 9 H) indicated the presence of one TMS group. The IR spectra of Ib and Ib′ had decreased absorption at 3360 and 1020 cm^{-1}, compared to the spectra of Ia and Ia′, showing the loss of a hydroxyl group; however, an absorption at 3430 cm^{-1} (sharp) and 3340 cm^{-1} (broad) indicated a free amido group. Absorption at 975 cm^{-1} was evidence of a *trans* double bond (see Table II).

NMR decoupling experiments established relative positions of the functional groups. Decoupling results were identical for both Ia and Ia′. Irradiation of either proton-7 or -5 (Table III) at δ 4.12 or δ 3.74, respectively, decoupled olefinic absorption. Decoupling of proton-4 had no visible effect on spectra; proton-5 could not be observed because it was obscured by protons-13. Irradiation of proton-10 collapsed the doublet of doublets for each of the geminal protons-9 to a doublet and, thus, simplified analysis of the cluster of absorptions at about δ 3.3.

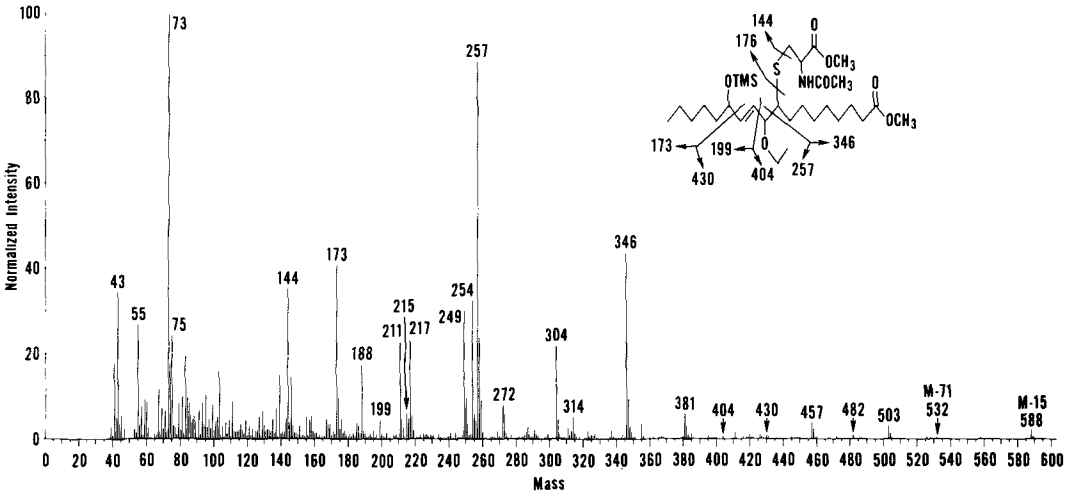
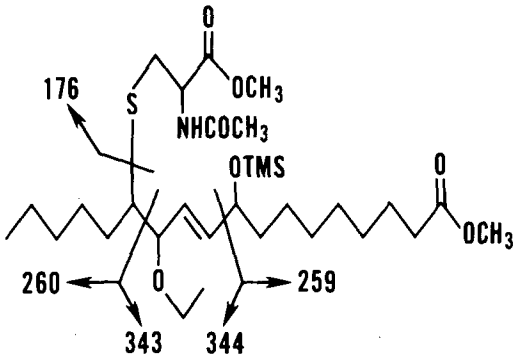


FIG. 2. Mass spectrum of Ib. See Table IV for subtraction ions.

Spectral differences between Ia and Ia′ were insignificant, except for the NMR analyses. The most pronounced differences in Ia and Ia′ NMR absorptions were protons-6, -9, and -10 (Table III). There were other subtle differences inasmuch as NMR analyses of mixtures of Ia and Ia′ revealed that the absorption of protons-15 was a split triplet, absorption of protons-13 was a split singlet, and absorption of protons-14 was more complex than a simple quartet. Possibly the minor differences in chemical shift, reported in Table III, for these protons are not experimental errors. There is no obvious reason for the difference in Ia and Ia′ other than *erythro-threo* isomerism about carbons 9 and 10 of the fatty chain. Conceivably, perturbation of the molecular environment near these groups causes the minor differences in NMR spectra.

MS spectrum of Ib (same as Ib′) in Figure 2 established that the TMS group was at carbon 13 of the fatty ester chain and that the *N*-acetylcysteinethio methyl ester group was at carbon 9. The position of the other groups was confirmed as well.

As additional structural evidence, a mixture of LOOH isomers, 21% 9-LOOH and 79% 13-LOOH, was used as a reactant with *N*-acetylcysteine. As expected, I and I′ from this reaction were mixtures of positional isomers (79% 9-*S*-[*N*-acetylcysteine]-13-hydroxy-10-ethoxy-*trans*-11-octadecenoic acid and 21% 13-*S*-[*N*-acetylcysteine]-9-hydroxy-12-ethoxy-*trans*-10-octadecenoic acid). An MS spectrum of Ib′, which had originated from this reaction, had fragment ions characteristic of both isomeric forms. In addition to the ions shown in Figure 2, the ions *m/e* 259, 260, 343, and 168 (344-176) were observed between 10 and 20% relative intensity, as shown in the following structure:



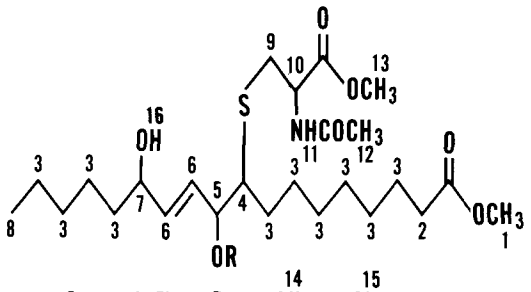
Products II and II′: Products II and II′ were isolated as their methyl esters, IIa and IIa′. Column chromatography separated IIa and IIa′ from the mixture (Table I).

Spectral results for IIa and IIa′ are shown in Tables II and III. The MS spectrum of IIb (Fig. 3) is identical to the MS of IIb′ within experimental error. Table IV explains the subtraction ions from Figure 3. These data and additional experiments outlined below were used to investigate the structure.

Two hydroxyls were indicated by NMR analysis of the derivative, IIb′. Absorptions at δ 0.11 (s, 9 H) and δ 0.13 (s, 9 H) were due to two TMS groups. Compared to the IR of IIa and IIa′, IR spectra of IIb and IIb′ had greatly decreased absorptions at 3350 and 1020 cm^{-1}, indicating the loss of hydroxyl groups; absorp-

TABLE III

Nuclear Magnetic Resonance Spectra of Addition Products and Their Respective Isomers[a]



Proton	δ (Multiplicity, J, Hz)			
	Ia	Ia′	IIa	IIa′
1 (3H)	3.64(s)[b]	3.64(s)	3.66(s)	3.66(s)
2 (2H)	2.29(t)[b]	2.29(t)	2.30(t)	2.30(t)
3 (20H)	1.28(m)[b]	1.28(m)	1.30(m)	1.30(m)
4 (1H)	2.72(m)	2.73(m)	2.74(m)	2.76(m)
5 (1H)	Obscured by 13	Obscured by 13	4.16(m)	4.19(m)
6[c](2H)	5.66(m)	5.69(m)	5.74(m)	5.74(m)
7 (1H)	4.11(m)	4.13(m)	4.16(m)	4.19(m)
8 (3H)	0.87(t)	0.87(t)	0.88(t)	0.88(t)
9 (2H)	2.95(dd,5,14)[b]	2.88(dd,7,13)	2.90(dd,5,14)	2.91(dd,6,14)
	3.18(dd,5,14)	3.24(dd,6,13)	3.11(dd,5,14)	3.20(dd,6,14)
10 (1H)	4.78(m)	4.71(m)	4.88(m)	4.80(m)
11 (1H)	6.81(d)[b]	6.51-6.60(d)	6.82-6.87(d)	6.45-6.85(d)
12 (3H)	2.02(s)	2.02(s)	2.04(s)	2.05(s)
13 (3H)	3.74(s)	3.73(s)	3.76(s)	3.79(s)
14 (2H)	3.42(q)[b]	3.43(q)[d]	---	---
15 (3H)	1.18(t)	1.17(t)	---	---
16[e]				

[a]Ia′ and IIa′ are isomeric forms of Ia and IIa postulated to be *erythro* and *threo* about carbons 9 and 10 of the fatty ester chain.

[b]d = doublet, dd = doublet of doublet, m = multiplet, q = quartet, s = singlet, t = triplet.

[c]The absorption differed between Ia and Ia′ in the spacing of the peaks: Ia δ 5.69, 5.66, and 5.62; Ia′ δ 5.73, 5.68, and 5.66. Because the absorptions of protons-6 were compact, J could not be measured.

[d]Some hyperfine splitting observed.

[e]The chemical shift was variable and broad. Usually, absorption was difficult to locate and was more visible in spectra of IIa and IIa′ than of Ia and Ia′.

tions at 3430 cm^{-1} (sharp) and 3340 cm^{-1} (broad) were due to a free amido group. IR absorption at 975 cm^{-1} indicated the presence of a *trans* olefin (see Table II).

Relative positions of the functional groups were ascertained by NMR decoupling experiments. Results were identical for IIa and IIa′. Irradiation of protons-5 and -7 (Table III) centered at δ 4.16 (4.19) decoupled the olefinic absorption. Irradiation of proton-4 at δ 2.74 (2.76) affected the downfield portion of the absorption at δ 4.16 (4.19), which was undoubtedly due to decoupling of proton-4 from proton-5. Because the upfield portion of the absorption centered at δ 4.16 (4.19) was unaffected by the decoupling experiment, this absorption is probably due to proton-7.

Slight differences in the NMR spectra of IIa and IIa′ were indicative of isomerism as observed for Ia and Ia′ (Table III). The most striking difference was the absorption of the nonequivalent geminal protons-9. Whereas the absorption of these protons was well separated as two doublets of doublets in the spectrum of IIa′, a smaller difference in chemical shift made the two innermost peaks in the IIa spectrum

TABLE IV

Subtraction and Rearrangement Ions Observed in Mass Spectra of Ib and IIb

Observed ion (*m/e*)	Subtracted from parent ion[a]	
	Ib	IIb
211	257-46	301-90
215	404-(176 + 45)	
216	404-(176 + 46)	
217	249-32	249-32
249	176 + 73 R	176 + 73 R
254	430-176	
272	346-(43 + 31)	346-(43 + 31)
298		474-176
304	346-43 + 1	346-43 + 1
314	346-32	346-32
419		346 + 73 R
422		576-(90 + 32 + 32)
482	M - (90 + 31)	
503	M - 173 + 73 R	
526		M - (90 + 31)

[a]Moieties subtracted are 176, 173, and 144, see Figure 3; 43, acetyl; 31 or 32, ester methoxy; 45 or 46 ether ethoxy; 90, trimethylsilyloxy + 1; and 73, trimethylsilyl. R represents a rearrangement ion according to Kleiman and Spencer (17).

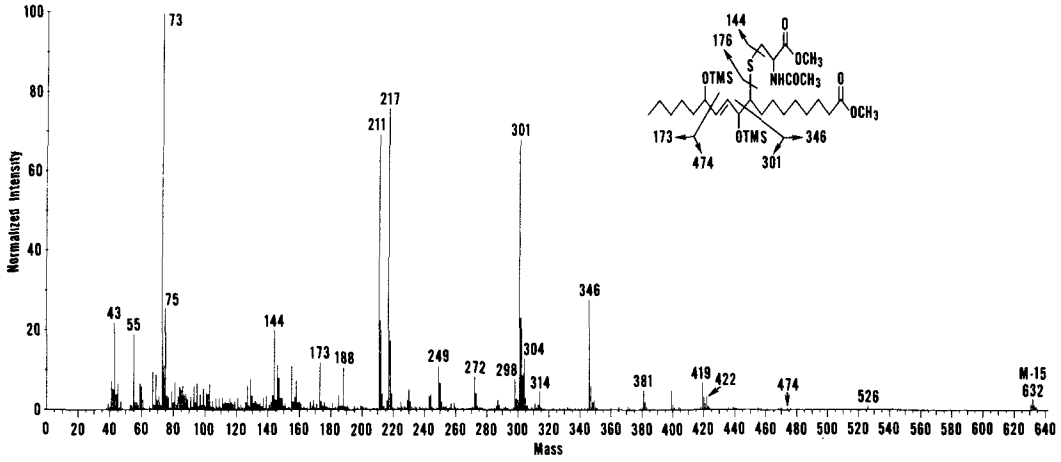
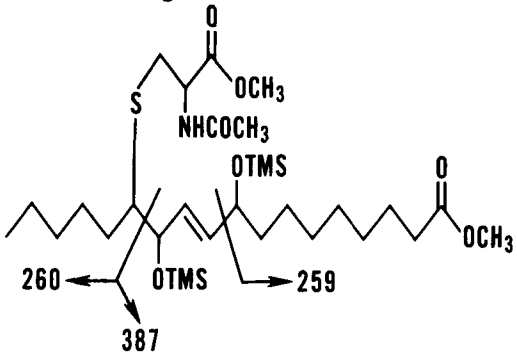


FIG. 3. Mass spectrum of IIb. See Table IV for subtraction ions.

overlap. Decoupling proton-10 resolved the overlap. Because most of the observed differences in IIa and IIa′ are centered about the assymetric center of carbons 9 and 10 of the fatty ester chain, *erythro-threo* isomerism is indicated but not proved.

The MS spectrum of IIb (same as IIb′) appears in Figure 3. TMS groups are indicated for carbons 13 and 10 on the fatty ester chain, and an *N*-acetylcysteinethio methyl ester group at carbon 9.

As was shown for Ia and Ia′, additional evidence was obtained by using mixed hydroperoxides, 21% 9-LOOH and 79% 13-LOOH, as reactants with *N*-acetylcysteine. IIb′ derived from this reaction was a mixture of positional isomers. Besides obtaining the fragment ions in Figure 3, ions at 259, 260, 387, 333 (260 + 73R), and 297 (387-90) *m/e* with relative intensity between 5-10% indicated the presence of the following structure:



N,N'-Diacetylcystine: *N,N'*-Diacetylcystine dimethyl ester was isolated from the product mixture by column chromatography followed by TLC. Final purification was by crystallization from ether-methanol. The isolated material yielded an IR spectrum ($CHCl_3$) with characteristic absorptions due to a monosubstituted amide at 3430, 3320, 1665, and 1500 cm^{-1}, and those due to an ester carbonyl at 1740 cm^{-1}. NMR gave the following absorptions: acetyl, δ 2.04 (s, 6 H); methylene, δ 3.16 (d, 4 H); ester methoxy, δ 3.73 (s, 6 H); amide methine, δ 4.81 (m, 2 H); and amide, δ 7.09 (d, 2 H). An MS spectrum had the following intense ions: M(*m/e* 352), M - 59, 176 (disulfide cleavage), 144 (176-32), 134, 118, 102, 88, 84, 60, and 43.

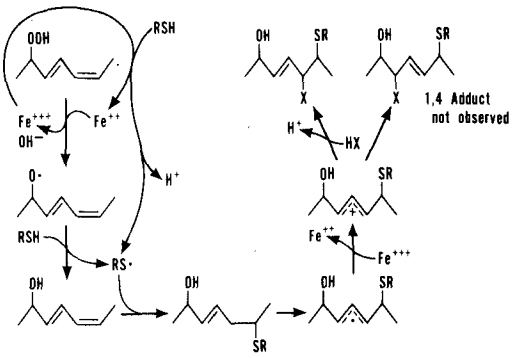


FIG. 4. Pathway proposed for formation of addition products. HX, solvent; RSH, *N*-acetylcysteine; fatty acid structures are abbreviated.

DISCUSSION

As a reactant, *N*-acetylcysteine was selected as a derivative of cysteine that would more closely resemble a model of this amino acid in a protein. Some of our unpublished results (18) indicate that *N*-acetylcysteine and cysteine behave similarly regarding the addition reaction described here, but that cysteine also yields products from a browning reaction which is probably due to Schiff base formation between the amino group and the aldehydes produced as secondary products of LOOH decomposition (19). Additionally, since metal ions form bidentate complexes with the thiol and amino groups of cysteine (20), using *N*-acetylcysteine demonstrated that the presence of an amino group is not necessary for the reaction to take place. The presence of 10^{-5}M ionic iron is necessary to complete the reaction within the time allotted. Without catalyst, no significant reaction was observed. Generally, complexes of cysteine and Fe(III) undergo an intramolecular electron shift from sulfur to a vacant *d*-orbital of iron, giving an Fe(II) species complexed with a thiyl radical (20). Taylor et al. (21) indicated there was also a possibility for an ionic mechanism.

Thiyl radicals are indicated because *N*-acetylcysteine added to the conjugated diene by anti-Markownikoff rule (12). Thiyl radicals additionally were indicated by production of the disulfide, *N,N'*-diacetylcystine. After thiyl addition to the conjugated diene, an available hydrogen radical usually adds at either end of the resultant allyl radical. Instead, an oxygenated function added; i.e., hydroxy or ethoxy. In a previous study (13) of this reaction, it appeared that hydrogen radicals were unimportant in addition reactions, but rather doubly and triply oxygenated fatty acids resulted. In formation of products II and II', hydroxyl could add as a result of the expected presence of hydroxy radicals with Fenton reagents (22). Conditions for Fenton reactions presumably are possible through interaction of Fe(II) and hydroperoxide. However, addition of an ethoxy group (products I and I') presents a mechanistic problem. The energetics of ethoxy radical formation are usually unfavorable. Although transition metal ions often catalyze alkoxy radical formation from highly substituted alcohols, primary alkanols are usually converted into carbon-centered radicals (23). Instead, solvolysis is indicated in which water (II and II') and ethanol (I and I') have participated in a substitution reaction. Substitution can occur by metal ion oxidation of alkyl radicals to carbonium ions (24), and this appears to be responsible for the origin of the hydroxyl or ethoxy group α to the *N*-acetylcysteinethio group. A possible pathway is shown in Figure 4. In metal-catalyzed oxidation of allylic radicals, 1,2 adducts always predominate over 1,4-adducts because the metal ion coordinates with the Π-bond of the allylic radical (25); consequently, the absence of the 1,4 addition products in this study is explained.

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Dual-Labeled Technique for Human Lipid Metabolism Studies Using Deuterated Fatty Acid Isomers[1]

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ABSTRACT

Two deuterated fatty acids, elaidate-d_2 and oleate-d_4, were fed simultaneously to a human subject as a mixture of trielaidin-d_6 and triolein-d_{12}. Periodically, blood samples were drawn, and red blood cells were separated from the plasma. Red blood cells and plasma lipids were fractionated and analyzed by combined gas chromatography–multiple ion mass spectroscopy. Dual deuterium-labeling allows rate and extent of fatty acid incorporation to be followed in various plasma and red cell neutral and phospholipid fractions. Maximum amount of deuterated fat varied from 4% in cholesterol ester to 64% in phosphatidyl ethanolamine. The highest levels of deuterated fat occurred in either 6-, 8-, or 12-hr samples; generally, <1% labeled fatty acids could be detected in 72-hr samples. Because the method is based on dual-labeling, differences in the relative incorporation of both fatty acid isomers can be compared directly. Differences in rates of incorporation, rates of removal, and extent of incorporation of labeled fatty acids into blood plasma can also be determined reliably. Our experimental labeling of fats with deuterium permits for the first time the metabolism of two fatty acid isomers to be compared simultaneously in human subjects. This new method should be applicable to a variety of other lipid metabolic studies.

INTRODUCTION

An effective method for studying the metabolism of individual fats and fatty acid isomers uses a dual-labeled approach that involves simultaneously feeding carbon-14 and tritium-labeled fats (1-4). Dual labeling produces more accurate results than single labeling. Basically, each experiment contains an internal standard that is subjected to exactly the same biological variations as the sample being studied. In this way, a series of dual-labeled experiments will contain a built-in common denominator provided the same internal standard is used in all experiments. In a series with several different fatty acid isomers, biological variation can be statistically eliminated without repeating any specific experiment more than two or three times once nine or more subjects have been studied (1,5).

The radioactive hazard of carbon-14 and tritium obviously restricts their use to experiments with animals and terminal patients. Research with terminal human subjects or laboratory animals is often undesirable because their lipid metabolism is likely to differ from healthy normal humans.

The metabolism of many single-labeled stable isotope (^2H, ^{13}C, ^{15}N, ^{18}O) compounds has been studied in human subjects, but no investigation has been reported based on a dual-labeled isotope stable approach.

Because deuterium is economical and readily available, the problem was how to use this stable isotope in a dual-labeled approach as the only tracer. Obviously, for experiments that compare the metabolism of two fatty acid isomers, each fatty acid isomer must be labeled with a specific but different number of deuterium atoms per molecule. On the basis of different mol wts, mass spectroscopy (MS) can quantitatively measure the amounts of each deuterated fatty acid and distinguish labeled from nonlabeled fatty acids.

Initially, two deuterated fatty acids (dideuteroelaidate and tetradeuterooleate) were simultaneously fed to test the feasibility of the dual-labeled concept for studying lipid metabolism in a single human subject.

Although it is possible to compare the metabolism of these deuterated fatty acids, this study illustrates the type of results obtainable by the dual-labeled stable isotope approach. We will confirm these results by additional experiments in order to reduce the possibility of biological variation.

EXPERIMENTAL PROCEDURES

Synthesis of Deuterated Fats

All synthetic work involving preparation of

[1]Presented at the AOCS meeting, Dallas, Texas, April 27-30, 1975.

TABLE I

GC-MIMS[a] Analysis of Synthetic Deuterated Fat Mixtures

Item	Synthetic mixture			
	A	B	C	D
% Total label in mixture	100	16.6	10.7	5.8
% El-d_2[b] in label	48.7	48.7	49.6	46.4
Standard deviation	0.35	0.41	0.85	0.85
95% Confidence limits for % El-d_2	48.0-49.4	-----	46.4-50.0[c]	-----
Number of replicate analyses	16	3	3	3

[a]Gas chromatography combined with multiple ion mass spectrometry.

[b]Elaidate-d_2.

[c]Confidence limits for B, C, and D pooled with 6 degrees of freedom.

methyl *trans*-9-octadecenoate-9,10-d_2 (El-d_2) and *cis*-9-octadecenoate-12,12,13,13,-d_4(Ol-d_4) was done at the Northern Regional Research Laboratory, Peoria, IL. Oleate-d_2 (Ol-d_2) was prepared by deuteriation of methyl stearolate (6) with Lindlar catalyst (1,7,8) and deuterium gas. The Ol-d_2 was isomerized to El-d_2 with nitrous oxide (9), and the El-d_2 was separated from unisomerized Ol-d_2 by silver resin chromatography (10). The purity of El-d_2 was determined by epoxy gas chromatography (GC) (11), as well as silver nitrate thin layer chromatography (TLC), and double-bond position was examined by reductive ozonolysis (12). Deuterium distribution, as measured by MS, for El-d_2 showed 87.1% of the sample contained two deuterium atoms per molecule, 12.9% contained one deuterium atom per molecule, and 0.1% contained three deuterium atoms.

Trielaidin-d_6 was prepared from glycerol, El-d_2 acid, and catalytic amounts of *p*-toluenesulfonic acid (13). Rapid bubbling of nitrogen through the mixture removed water formed during the reaction.

Methyl *cis*-9-octadecen-12-ynoate (14) was extracted from *Crepis alpina* seed with hexane, the triglycerides were transesterified with methanol sodium methoxide, and then the methyl crepenynate was separated by countercurrent distribution.

The methyl crepenynate was selectively brominated (15) to protect the olefinic bond at the 9 position. The remaining acetylenic bond in the 12 position was fully deuterated at 30 C with 5% platinum-on-carbon and deuterium gas at 500 psi. The resulting methyl tetradeuterodibromooctadecenoate was refluxed for 45 min with zinc powder in absolute ethanol (16). Debromination gave a mixture of 17% stearate-d_6, 67% Ol-d_4, and 16% El-d_4. The mixture was separated by silver resin chromatography (10), and the El-d_4 and Ol-d_4 fractions were analyzed by IR, TLC, and GC. The double bond position was determined by reductive ozonolysis (12) and found to remain in the 9 position. Analysis by MS indicated that platinum-catalyzed deuteriation produced a wide deuterium distribution in the Ol-d_4 and confirmed previous reports (17,18). The Ol-d_4 actually contained 2% Ol-d_1, 9% Ol-d_2, 22% Ol-d_3, 38% Ol-d_4, 13% Ol-d_5, 6% Ol-d_6, 4% Ol-d_7, 4% Ol-d_8, and 2% Ol-d_9. Fortunately, analysis of standard mixtures containing different amounts of Ol-d_0, El-d_2, and Ol-d_4 (Table I) showed that multiple ion mass spectroscopy (MIMS) could analyze these mixtures quantitatively (19).

Triolein-d_{12} was prepared from glycerol and Ol-d_4 acid with *p*-toluenesulfonic acid catalyst (13). Bubbling nitrogen rapidly through the reaction mixture removed any water formed during the reaction.

Feeding El-d_2:Ol-d_4 Mixture

Feeding the deuterated fats and drawing blood samples were conducted at Georgetown University Medical School, Washington, DC. The El-d_2 and Ol-d_4 were fed as trielaidin-d_6 and triolein-d_{12}. A mixture (36.5 g) containing 17.8 g of trielaidin-d_6 and 18.7 g of triolein-d_{12} was blended with 50 g of calcium casein, 50 g of maltose-dextrose, 25 g of sucrose, and enough water to reproduce the consistency of a milk shake. This deuterated milk shake was fed to a 23-year old male in place of breakfast after an overnight fast. This amount of deuterated fat (36.5 g) represents about one-third of the daily fat consumed by the average American (20). The subject was a 5 ft 10 in., 160 lb Caucasian in good health. His diet was not controlled before or after feeding. Blood samples (30 ml) were drawn at 0-, 1-, 2-, 4-, 8-, 12-, 24-, 48-, and 72-hr intervals after feeding. All samples were immediately centrifuged at 1500 *g* for 30 min. After the plasma layer was removed, red blood cells (RBC) were washed three times with isotonic saline and then lysed with distilled water (21).

Extraction and Separation of Blood Lipids

At the USDA Lipid Nutrition Laboratory, Beltsville, MD, blood lipids were separated and prepared for analysis. Plasma and RBC lipids were extracted by Folch's procedure (22). The plasma lipids were separated by unidimensional preparative TLC methods (23,24) into triglycerides (TG), free fatty acids (FFA), cholesterol esters (CE), phosphatidyl choline (PC), lysophosphatidyl choline (lyso PC), phosphatidyl ethanolamine (PE), phosphatidyl serine-phosphatidyl inositol (PS + PI), and sphingomyelin (SM) fractions. Similarly, RBC lipids were separated by TLC into TG, PC, SM, PE, and PS + PI fractions. A portion of the PC fraction was hydrolyzed with phospholipase-A (phosphatidate 1-acylhydrolase, EC 3.1.1.32) (25), and products were separated by TLC, Individual plasma and RBC fractions were then converted to their methyl esters for combined GC-MIMS analyses.

Analysis of Blood Lipid Fractions

Mass spectral analysis of the blood lipids was performed at the Northern Regional Research Laboratory. A Packard Model 7400 GC, equipped with a 6-ft 10% Apiezon-L on 100/120 mesh Chromasorb-W column, and a Nuclide Model 12 90 DF mass spectrometer, equipped with a silicon rubber membrane separator (19), were used to analyze all samples except the TG fractions. The TG fractions contained enough sample to permit analysis without use of the membrane separator. The MIMS technique allowed the amounts of Ol-d_0 (m/e = 296), El-d_2 (m/e = 298), and Ol-d_4 (m/e = 300) in the blood lipid fractions to be determined quantitatively. Experimental details of the MIMS technique have been described (19).

RESULTS

Plasma Neutral Lipids

GC-MIMS analysis of plasma neutral lipids showed extensive incorporation of deuterated fat into TG (Fig. 1) and FFA but low incorporation into CE. The 6-hr sample contained the largest amount of labeled fat (54.9%) in the plasma TG fractions. The curves in Figure 1 representing the total labeled fat and total monoene content have distinct inflection points at 6 hr and 12 hr. These inflections are also observed in the Ol-d_4 and El-d_2 curves. The percentages of Ol-d_4 and El-d_2 in plasma TG samples are essentially the same as in the mixture fed. Samples analyzed at 48 hr and 72 hr contained < 1% deuterated fat. The total octadecenoate content of plasma TG increased from 44% to a maximum of 63% in the 6-hr sample and then returned to 44% in the 24-hr sample.

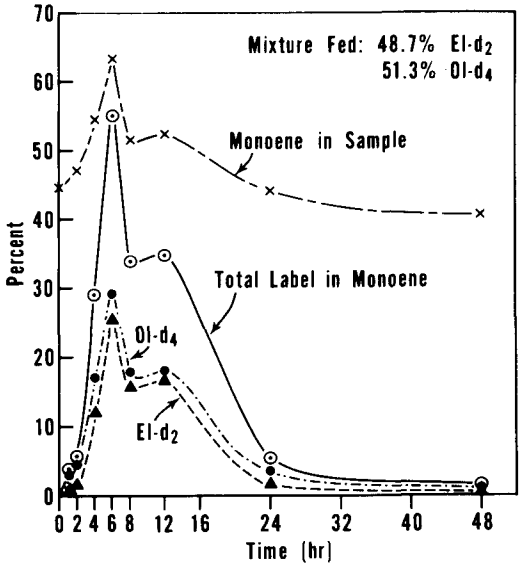

FIG. 1. Incorporation of El-d_2 (elaidate-d_2) and Ol-d_4 (oleate-d_4) into plasma triglycerides.

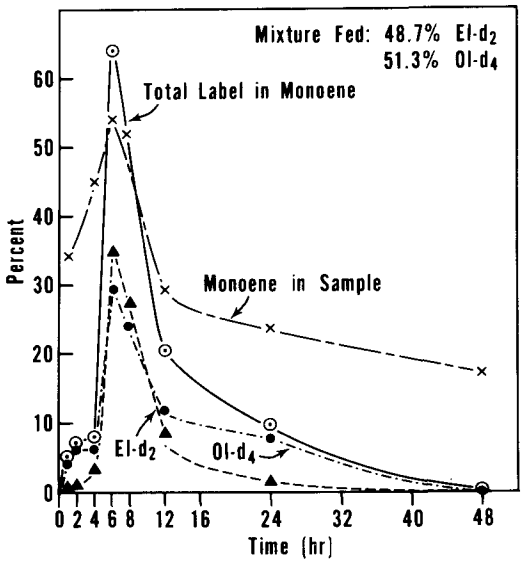

FIG. 2. Incorporation of El-d_2 (elaidate-d_2) and Ol-d_4 (oleate-d_4) into plasma free fatty acids.

The 6-hr plasma FFA fraction contained the maximum amount (64.2%) of deuterated fat found in any neutral lipid or phospholipid fraction. Total deuterated fat increased rapidly but cleared just as rapidly from the plasma FFA (Fig. 2). Initially, Ol-d_4 and El-d_2 are incorporated into plasma FFA at similar rates, but El-d_2 is incorporated considerably more than

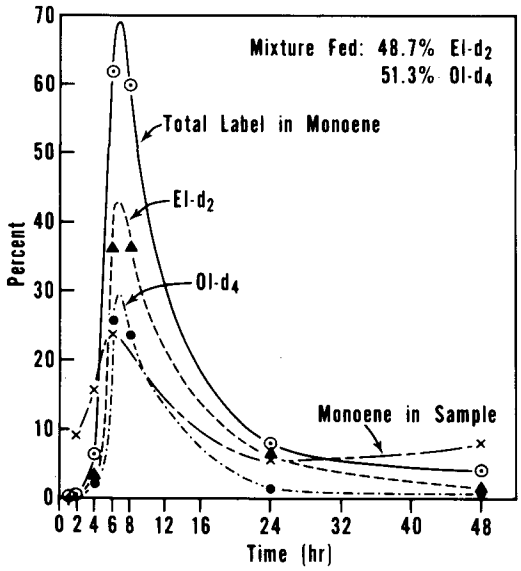

FIG. 3. Incorporation of El-d_2 (elaidate-d_2) and Ol-d_4 (oleate-d_4) into plasma phosphatidyl ethanolamine.

Ol-d_4 in the 6-hr sample. Data on the 12- and 24-hr samples indicate that El-d_2 is cleared from FFA faster than Ol-d_4. Octadecenoate also increased from an initial 34% to 54% in the 6-hr sample and then decreased to 17% in the 48-hr sample.

Relatively low levels of deuterated fat were found in all plasma. CE samples compared to plasma phospholipids. The 24-hr plasma CE fraction contained the most deuterated fat (4.1%), of which 85.4% was Ol-d_4. Thus the enzymes, such as phosphatidyl choline:cholesterol acyltransferase (EC 2.3.1.43) and acyl-CoA:cholesterol *O*-acyltransferase (EC 2.3.1.26), which are responsible for cholesterol esterification appear to be less active but much more selective than those enzymes responsible for phospholipid formation.

Plasma Phospholipids

Deuterated oleate and elaidate were incorporated into all plasma phospholipids Extent of incorporation varied from a maximum of 61.8% in PE down to 11% in SM.

The GS-MIMS analyses of plasma PE fractions are plotted in Figure 3. The 6-hr plasma PE fraction contained the largest percentage of deuterated fat (61.8%) of any phospholipid fraction. Because deuterated monoene in 6- and 8-hr PE fractions contained about 60% El-d_2 compared to 48.7% in the mixture fed, preferential incorporation of El-d_2 over Ol-d_4 is indicated. El-d_2 increased to 85% of the deuterated monoene in the 24-hr PE sample due to a more rapid removal of Ol-d_4 from plasma PE. Total monoene in plasma PE increased from 9.1% (0 hr) to 23.8% (6 hr) and then decreased to 5.6% in the 24-hr sample. Total monoene curve in Figure 3 resembles the curve showing the total deuterated fat content of the samples. Obviously, this increase in total monoene occurs because deuterated monoene has been incorporated into plasma PE.

In Figure 3, the peaks of the curves representing labeled fatty acids have been drawn on the basis that these curves must fit a continuous mathematical function.

Analysis of the plasma PS + PI samples is given in Table II. The PS + PI 12-hr sample may have contained a higher percentage of deuterated fat than the 8-hr fraction (39.2%), but the PS + PI 12-hr sample was lost during processing. A selective incorporation of Ol-d_4 was noted in plasma PS + PI. The 6- and 8-hr samples contained 60.7% and 57.0% Ol-d_4 compared to 51.3% Ol-d_4 in the mixture fed. The percentages for Ol-d_4 and El-d_2 (Table II) indicate both fatty acids were removed at similar rates from the PS + PI fraction.

Human blood plasma phospholipids contain ca. 70% PC (26). Selection of El-d_2 over Ol-d_4 in plasma PC is apparent from the curves in Figure 4. The percentage of El-d_2 in PC is 9-11% higher than the 48.7% El-d_2 in the mixture fed. The rate and extent of incorporation of deuterated oleate and elaidate into

TABLE II

Analysis of Phosphatidyl Serine and Phosphatidyl Inositol Samples[a] from Human Blood Plasma Phospholipids

Sample time (hr)	Total label in monoene (%)	El-d_2 in label (%)	Ol-d_4 in label (%)	Total monoene in sample (%)
1	0.0	---	---	11.3
4	0.0	---	---	13.1
6	26.5	39.2	60.8	14.9
8	39.2	43.0	57.0	15.0
24	24.5	46.4	53.6	12.4
48	3.6	43.1	56.9	10.4

[a]Mixture fed: 48.7% El-d_2 (elaidate-d_2) and 51.3% Ol-d_4 (oleate-d_4).

plasma PC were slower than into plasma PE. Maximum deuterated monoene content in PC was 24.8% for the 12-hr sample compared to 61.8% for the 6-hr PE sample. Clearing of deuterated fat from plasma PC was also slower than from plasma PE. The 24-hr PC sample still contained 15.5% labeled monoene, whereas the 24-hr PE sample contained 7.8% labeled monoene. This difference indicates that the 8-hr PE fraction, which had more than three times as much deuterated fat as the 8-hr PC fraction, clears deuterated fat much more rapidly than the PC fraction.

GC analysis of plasma TG, FFA, and PE showed that the percentage total monoene increased as the percentage deuterated fat increased. This correlation was not so dramatic in PC samples. The percentage total monoene in PC remained constant between 18.5-21.5%, whereas the percentage deuterated fat increased from 0.0 to 24.8% in the 12-hr sample. These results demonstrate that deuterated monoenes are directly replacing nondeuterated monoenes.

Phospholipase-A was used to hydrolyze the fatty acid in the 2 position of plasma PC. Analysis of the remaining fatty acid in the 1 position of plasma PC is given in Figure 5. Preferential incorporation of El-d_2 into the 1 position of PC, as seen in Figure 5, has been observed in other studies (27,28). A maximum of 35% deuterated fat was found in the 1 position of the 12-hr PC fraction (PC-1), which is 11% more label than was in the total PC sample. Because El-d_2 in the 8-, 12-, and 24-hr PC-1 samples averaged ca. 75%, elaidate incorporation is mainly in the 1-position. GC analysis of PC-1 did not indicate a major replacement of saturates by El-d_2 as others (29) have reported; instead, unlabeled monoene was apparently replaced by El-d_2.

GC-MIMS data were not consistently satisfactory for the fatty acids obtained by phospholipase-A hydrolysis of PC samples. The fatty acids in the 2 position of the 12-hr PC sample (PC-2) contained 15.9% total deuterated fat, of which 56% was Ol-d_4. This 56% Ol-d_4 is 4.7% higher than that fed and demonstrates that

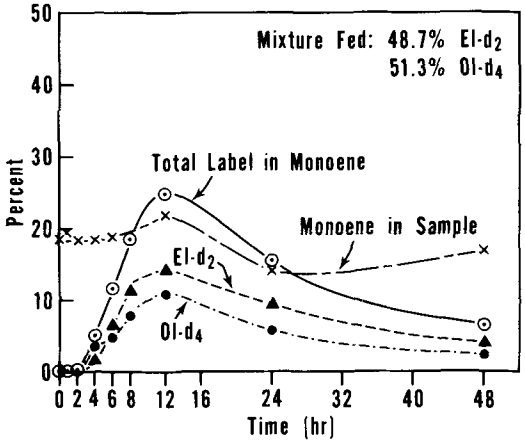

FIG. 4. Incorporation of El-d_2 (elaidate-d_2) and Ol-d_4 (oleate-d_4) into plasma phosphatidyl choline.

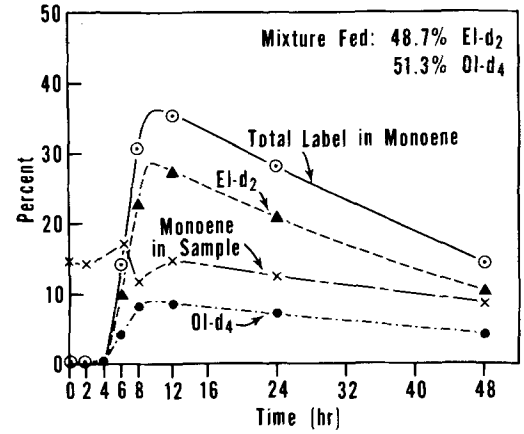

FIG. 5. Incorporation of El-d_2 (elaidate-d_2) and Ol-d_4 (oleate-d_4) into the one position of plasma phosphatidyl choline.

TABLE III

Analysis of Lysophosphatidyl Choline Samples[a] from Plasma Phospholipids

Sample time (hr)	Total label in monoene (%)	El-d_2 in label (%)	Ol-d_4 in label (%)	Total monoene in sample (%)
0	---	---	---	15.9
2	0.5	---	---	14.5
4	5.1	29.1	70.9	13.9
6	13.0	35.2	64.8	15.3
8	21.0	41.0	59.0	17.6
12	19.5	50.8	49.2	17.7
24	9.6	56.2	43.8	14.0
48	6.0	35.7	64.3	15.0

[a]Mixture fed: 48.7% El-d_2 (elaidate-d_2) and 51.3% Ol-d_4 (oleate-d_4).

TABLE IV

Maximum Level of Deuterated El-d_2 and Ol-d_4 in Plasma Fractions[a]

Plasma fraction[b]	Time (hr)	Total El-d_2 and Ol-d_4 (%)	El-d_2 in monoene (%)	Ol-d_4 in monoene (%)	El-d_2 in label (%)
FFA	6	64.2	34.7	29.5	54.0
PE	6	61.8	36.3	25.5	58.7
TG	6	54.9	25.8	29.1	46.9
PS + PI	8	39.2	16.9	22.3	43.1
PC-1	12	35.2	27.9	8.3	78.6
PC	12	24.8	14.1	10.7	56.8
Lyso-PC	8	21.0	8.5	12.5	40.3
PC-2	12	15.9	7.0	8.9	44.0
SM	12	11.0	5.9	6.1	53.6
CE	12	4.1	0.6	3.5	14.6

[a]Composition of mixture fed: 48.7% El-d_2 (elaidate-d_2) and 51.3% Ol-d_4 (oleate-d_4).

[b]FFA = free fatty acid, PE = phosphatidyl ethanolamine, TG = triglycerides, PS + PI = phosphatidyl serine and phosphatidyl inositol, PC = phosphatidyl choline, PC-1 and PC-2 = fatty acid from phosphatidyl choline positions 1 and 2, lyso-PC = lysophosphatidyl choline, SM = sphingomyelin, CE = cholesterol esters.

selective fatty acid incorporation into the PC-2 position is much less than was observed for the PC-1 position.

Plasma phospholipids contain only 6% lyso-PC (26), but all lyso-PC fractions contained sufficient sample for GC-MIMS analysis (Table III). In general, lyso-PC results do not resemble PC or PC-1 data except that monoene increased little as percentage of deuterated fat increased. A maximum of 21.1% deuterated fat was found in the 8-hr sample, and 41% of it was El-d_2. This apparent selectivity for Ol-d_2 in 6- and 8-hr samples was not observed in 12- and 24-hr samples.

In general, plasma SM fractions could not be accurately analyzed due to extraneous impurities and small sample size, but accurate GC-MIMS analyses were obtained for the 8- and 12-hr SM samples. The 12-hr sample contained 10.9% total deuterated fat, of which 54.1% was El-d_2. Amounts of deuterated fat were smaller in the 8- and 24-hr samples, and no label was detected in the 4- and 24-hr samples.

Deuterated fat was found in all plasma lipid fractions; these data are summarized in Table IV, which lists those plasma fractions that contained the maximum levels of deuterated fat. Distribution of El-d_2 and Ol-d_4 in these fractions is also summarized in Table IV. All plasma fractions contained enough labeled fat for reliable detection.

RBC Samples

Methyl esters of TG, CE, and FFA from RBC neutral lipids were analyzed. RBC-CE and RBC-FFA samples incorporated <5% and <2% total deuterated fat, respectively. The small deuterated fat level plus insufficient sample prevented an accurate analysis.

The RBC-TG fractions had a maximum of 23% deuterated fat in the 8-hr sample. No preferential selection of El-d_2 or Ol-d_4 occurred during incorporation and removal of the labeled fats from RBC.

The RBC-phospholipid methyl esters incorporated <5% deuterated fat into PC-1 and SM fraction and <2% into the various other phospholipid fractions. This level of deuterated fat is too low for accurate measurements by our present analytical method.

DISCUSSION

Our major purpose was to establish the limitations of the dual deuterium-labeled method by identifying which blood lipid fractions can be studied. We assumed that deuterium in El-d_2 and Ol-d_4 was not susceptible to H-D exchange in the biological system, because in vivo experiments with similarly labeled tritiated fatty acids showed no loss of label (2). Essentially all plasma lipid fractions incorporated enough deuterated fat for quantitative detection (Table IV). The CE fraction was the only one that contained a marginal level of labeled fat. The maximum level of labeled fat in the various plasma fractions occurred at different sampling times. Possibly interconversion kinetics and half-life of the plasma lipid fractions could be calculated from these differences.

Accuracy of the data in Table IV decreases as the percentage label in each fraction decreases, as illustrated in Table I, which shows the standard deviation and 95% confidence limits for the percentage El-d_2 in synthetic mixtures. Accuracy of the data deteriorates below 15% labeled fat.

The original mixture fed (Table I, mixture

A) was analyzed by GC-MIMS 16 times and gave a mean of 48.7% El-d_2 in the mixture with a standard deviation of 0.35. When mixture A was diluted with pure methyl oleate to a concentration of 16.6% label in monoene, the mean value El-d_2 was 48.7% with a standard deviation of 0.41. Further dilution to 5.8% label in monoene gave a mean of 46.4% El-d_2 in label with a standard deviation of 0.85.

Small sample size and the many blood fractions prevented extensive analytical replication. Projecting errors in measurement of known mixtures onto blood lipid data suggests that for samples containing $>16.6\%$ label, measured values are probably more accurate than those having 5.8-16.6% label. The value of 46.4% El-d_2 in label at the 5.8% level, plus some other measurements, indicates that, as the percentage label goes below 5.8% El-d_2 values tend to be slightly lower than they should be owing to a small systematic error. The statistical analyses will be included in a separate publication along with experimental details.

In Figures 1-3, the "total label in monoene" curves from GC-MIMS data coincide with the "monoene in sample" curves from GC analysis. The similarity in these curves from two different methods of analysis supports data obtained by the dual deuterium-labeled method.

Curves in Figures 1-5 and data in Tables II and III demonstrate applicability of the dual deuterium-labeled method for following incorporation of ingested fat into various blood lipids and for measuring the clearing times of individual plasma lipid fractions. These tables and figures also show that dual deuterium labeling can be used for measuring the relative dynamic metabolic activity of the various lipid fractions in blood plasma. Although plasma-FFA and PE fractions constitute only a small percentage of total plasma lipid, they are apparently active metabolic intermediates inasmuch as $>60\%$ deuterated monoene was incorporated into these fractions 6 hr after ingestion of the deuterated fat. In contrast, the PC fraction, which constitutes ca. 70% of plasma phospholipids, contained a maximum of only 25% deuterated monoene 12 hr after ingestion of labeled fat. Such slow incorporation and slow clearing of the labeled monoene from PC reflect either its less active role in metabolic processes or a large PC pool in body tissue. Analysis of phospholipase-A treated PC showed that plasma-PC-1 fractions were unique. These fractions were the only set of samples that showed a strong preference for incorporation of El-d_2 but did not show a large increase in total monoene content. The change in El-d_2:Ol-d_4 ratio in the lyso-PC fractions shown in Table III may be caused by an experimental artifact or may reflect an actual selective incorporation of Ol-d_4 at low monoene concentrations. Lyso-PC data illustrate that small differences in metabolism of similar compounds can be compared by dual deuterium labeling. Normally these extremely small differences at low levels of isotope incorporation would be lost because of experimental variation if the labeled fats were fed separately.

Good GC-MIMS data were not obtained for all PC-2 and SM fractions because of insufficient sample size or extraneous and interfering impurities. Such problems are not expected to recur and should not be a factor in future experiments.

RBC lipids incorporated little labeled fat into RBC membrane, although esterification or exchange of human RBC lipids with plasma lipids has been reported (30) and mature erythrocytes from rats are known to incorporate labeled fat into their phospholipids (31). The RBC-TG fraction was the only RBC lipid that contained appreciable amounts (23% maximum) of deuterated fat. Other RBC lipid fractions had $<5\%$ deuterated fat, an amount which limits the effectiveness of dual deuterium labeling for following lipid incorporation into RBC.

In summary, we have demonstrated successfully that human metabolism of two fatty acid isomers can be studied directly under identical biological and experimental conditions. The natural fatty acid isomer, oleic acid, was fed as triolein and compared with the unnatural fatty acid isomer, elaidic acid, fed as trielaidin. Total monoene content of plasma lipids paralleled the total deuterated fat incorporation. FFA, TG, and PE fractions contained the largest amounts of deuterated fat. PE and the one acyl position of PC were the most selective fractions for preferential accumultion of El-d_2. The PS + PI fraction was the most selective for Ol-d_4 incorporation.

Comparisons made between the metabolism of elaidate and oleate in human plasma and RBC are intended only to demonstrate how dual deuterium labeling can be applied to solve the problem of human lipid metabolism. Data from additional experiments will be presented in a subsequent paper and oleate-elaidate metabolism in human blood lipids will be compared in detail.

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Effect of Eicosa-5,8,11,14-tetraynoic Acid on Fatty Acid Composition of Selected Organs in the Rat

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ABSTRACT

Eicosa-5,8,11,14-tetraynoic acid or arachidonic acid or no supplement (controls) was given orally to rats maintained on a fat free diet and the fatty acid composition of total lipids of several organs determined. No changes were noted in the total fatty acid concentrations of the organs examined in the various groups. A decrease in the amount of arachidonic acid, 22:4ω6 and 22:5ω6 (as percent of total fatty acids), and an increase in the amount of 20:3ω6 and linoleate were observed in total lipids of several organs. In the group receiving the arachidonate supplement, there was less linoleate and 20:3ω6 and more arachidonate than in the controls. Both eicosa-5,8,11,14-tetraynoic acid and arachidonate supplements resulted in a decrease in 20:3ω9 in most organs studied. Generally, the most marked changes were seen in liver but, of the other organs examined (heart, kidney, testis, brain, and adrenals), only the adrenals failed to show any significant differences between the controls and each of the two supplemented groups. Although the experimental conditions preclude conclusive interpretation of the changes observed, it is suggested that eicosa-5,8,11,14-tetraynoic acid was effective in inhibiting the conversion of linoleate to arachidonate and the conversion of arachidonate to 22:4 and 22:5.

INTRODUCTION

The compound eicosa-5,8,11,14-tetraynoic acid (TYA) is the acetylenic analog of the important polyene, arachidonic acid. TYA has been reported to block cholesterol synthesis between acetate and mevalonic acid and to exert a suppressive effect on sebaceous gland secretion (1). It has also been shown to inhibit prostaglandin biosynthesis by its direct effect on prostaglandin synthetase and by prevention of the inactivation of endogenous inhibitors (2).

The experimental usefulness of such a compound is obvious and, therefore, it becomes important to know what other metabolic reactions may be inhibited, or potentiated, by its presence. It is known, for instance, that polyenoic acids interfere with each other in desaturation reactions (3). We were interested, therefore, in exploring the possibility that TYA might have an effect on conversion of linoleate to arachidonate and on the conversion of arachidonate to 22-carbon polyenes in the intact animal. In this study, the fatty acid composition of total lipids of several organs was determined in rats maintained on a fat free diet and given several doses of this compound for comparison with rats maintained on the same diet but not receiving TYA. In addition, we have also included a group of animals given an amount of arachidonic acid similar to that amount of TYA.

MATERIALS AND METHODS

Weanling male Sprague-Dawley rats were randomly divided into three groups of six each and maintained on a purified diet containing all essential nutrients but no fat. Rats of Group 1 were given by stomach tube 150 mg TYA suspended in 0.5 ml coconut oil twice a week for a total of five doses (750 mg in 17 days). Group 2 was given by stomach tube 150 mg arachidonic acid in 0.5 ml coconut oil twice a week for a total of five doses (750 mg). Group 3 was given by stomach tube 0.5 ml coconut oil twice a week for a total of five doses. All animals were killed 4 days after the last dose, selected organs removed quickly, frozen in liquid nitrogen, and maintained at -65 C until hydrolyzed and extracted. Methods for hydrolysis of tissues, extraction, methylation, and gas liquid chromatographic analysis have been published (4) with the exception that the gas chromatographic column was packed with 10% SP 2340 on 100/120 mesh Supelcoport (Supelco, Inc., Bellefonte, PA) and the analysis done by temperature programming at 4/min from 160 to 225. Identification of compounds was done usually by retention time and reference to compounds identified previously by chemical means (4). When necessary, fractions isolated by gas chromatography were identified after hydrogenation and rechromatography of the saturated derivatives.

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TABLE I

Major Fatty Acid Composition of Liver Total Lipids[a]

Fatty acid[c]	Percent of total fatty acid[b] Group 1 (TYA[d] supplemented)	Group 2 (20:4 supplemented)	Group 3 (Coconut oil only)
16:0	24.0 ± 0.75	20.9 ± 0.48	21.7 ± 1.3
16:1	3.3 ± 0.29	4.3 ± 0.12	5.8 ± 1.3
18:0	14.1 ± 0.52	16.2 ± 0.57	14.9 ± 1.1
18:1	26.5 ± 1.7	20.7 ± 0.72[f]	24.6 ± 0.97
18:2	11.2 ± 1.3[f]	3.5 ± 0.40[g]	6.6 ± 0.50
20:3ω9	4.6 ± 1.1	1.4 ± 0.38[e]	4.7 ± 0.73
20:3ω6	1.8 ± 0.19[f]	0.4 ± 0.13[f]	1.0 ± 0.10
20:4ω6	8.3 ± 0.25[f]	22.5 ± 0.91[g]	13.1 ± 1.3
22:4ω6	0.05 ± 0.05[f]	0.5 ± 0.08	0.3 ± 0.07
22:5ω6	0.3 ± 0.09[f]	2.8 ± 0.26[f]	1.0 ± 0.19
22:5ω3	0.1 ± 0.03[e]	0.3 ± 0.04	0.4 ± 0.09
22:6ω3	6.3 ± 0.27	5.0 ± 0.42	5.4 ± 0.43

[a] Some minor components not given.

[b] Mean ± SE of six rats in each group.

[c] Number of carbons in chain: number of double bonds; omega number denotes the position of the first double bond from the methyl end.

[d] TYA = eicosa-5,8,11,14-tetraynoic acid.

[e] P value vs. coconut oil group <0.05.

[f] P value vs. coconut oil group <0.01.

[g] P value vs. coconut oil group <0.001.

TABLE II

Major Fatty Acid Composition of Heart Total Lipids[a]

Fatty acid[c]	Percent of total fatty acids[b] Group 1 (TYA[d] supplemented)	Group 2 (20:4 supplemented)	Group 3 (Coconut oil only)
16:0	16.0 ± 1.67	17.2 ± 0.81	15.9 ± 0.69
16:1	4.8 ± 0.36[e]	5.4 ± 0.49[f]	3.7 ± 0.25
18:0	14.7 ± 0.68[e]	16.0 ± 0.95	17.2 ± 0.76
18:1	36.8 ± 1.47[g]	33.2 ± 3.7[e]	22.3 ± 0.35
18:2	13.2 ± 0.79	6.2 ± 0.45[g]	12.2 ± 0.99
18:3	0.9 ± 0.07	0.7 ± 0.10	0.6 ± 0.15
20:3ω9	2.0 ± 0.06[f]	1.5 ± 0.16[g]	3.9 ± 0.57
20:3ω6	1.1 ± 0.11	0.2 ± 0.06[f]	1.0 ± 0.12
20:4	5.4 ± 1.3[g]	14.0 ± 3.12	14.6 ± 0.23
22:4	0.2 ± 0.08[e]	0.8 ± 0.18	0.5 ± 0.07
22:5ω6	0.1 ± 0.07[f]	1.4 ± 0.35	1.3 ± 0.14
22:5ω3	0.5 ± 0.15	0.4 ± 0.09[f]	0.8 ± 0.07
22:6	2.4 ± 0.70[e]	2.2 ± 0.66[f]	5.0 ± 0.41

[a] Some minor components not given.

[b] Mean ± SE of six rats in each group.

[c] Number of carbons in chain : number of double bonds; omega number denotes the position of the first double bond from the methyl end.

[d] TYA = eicosa-5,8,11,14-tetraynoic acid.

[e] P value vs. coconut oil group <0.05.

[f] P value vs. coconut oil group <0.01.

[g] P value vs. coconut oil group <0.001.

RESULTS

No significant differences in the total fatty acid concentration of the organs examined were noted among the various groups. Neither the body wt nor the liver wt of either supplemented group was significantly different than that of the nonsupplemented controls (Group 3).

An effect of TYA on fatty acid composition of total lipids was observed in five of the six organs studied. The major fatty acid composi-

TABLE III

Major Fatty Acid Composition of Kidney Total Lipids[a]

Fatty acid[c]	Percent of total fatty acids[b] Group 1 (TYA[d] supplemented)	Group 2 (20:4 supplemented)	Group 3 (Coconut oil only)
16:0	2.0 ± 0.58	22.8 ± 0.90[f]	19.0 ± 0.66
16:1	4.7 ± 0.23	4.3 ± 0.38	4.1 ± 0.17
18:0	12.9 ± 0.34[e]	15.0 ± 0.41	14.4 ± 0.57
18:1	29.4 ± 1.1[e]	22.8 ± 1.2	25.8 ± 1.01
18:2	11.8 ± 0.75[g]	2.3 ± 0.25[g]	6.6 ± 0.24
18:3	0.5 ± 0.05	0.3 ± 0.04[f]	0.5 ± 0.05
20:3ω9	1.9 ± 0.39	0.5 ± 0.12[g]	2.4 ± 0.38
20:3ω6	1.1 ± 0.05	0.5 ± 0.05[g]	1.3 ± 0.10
20:4	14.7 ± 0.45[g]	26.9 ± 1.47[f]	21.4 ± 1.00
22:4	0.1 ± 0.03[g]	0.7 ± 0.05[e]	0.5 ± 0.06
22:5ω6	trace[h]	0.8 ± 0.12[f]	0.4 ± 0.02
22:5ω3	0.30 ± 0.14	0.1 ± 0.03	0.2 ± 0.02
22:6	1.3 ± 0.11[f]	1.7 ± 0.17	1.9 ± 0.14

[a]Some minor components not given.

[b]Mean ± SE of six rats in each group.

[c]Number of carbons in chain : number of double bonds; omega number denotes the position of the first double bond from the methyl end.

[d]TYA = eicosa-5,8,11,14-tetraynoic acid.

[e]P value vs. coconut oil group <0.05.

[f]P value vs. coconut oil group <0.02.

[g]P value vs. coconut oil group <0.001.

[h]Trace, <0.10.

TABLE IV

Major Fatty Acid Composition of Testicular Total Lipids[a]

Fatty acid[c]	Percent of total fatty acids[b] Group 1 (TYA[d] supplemented)	Group 2 (20:4 supplemented)	Group 3 (Coconut oil only)
16:0	42.5 ± 0.82	42.8 ± 1.8	43.4 ± 1.4
16:1	3.7 ± 0.37	3.7 ± 0.56	3.1 ± 0.36
18:0	6.7 ± 0.47	7.3 ± 0.59	7.8 ± 0.52
18:1	22.7 ± 0.72[f]	17.8 ± 1.1	17.7 ± 1.0
18:2	7.0 ± 0.38[g]	1.7 ± 0.11[e]	2.3 ± 0.22
20:3ω9	0.4 ± 0.18[f]	0.3 ± 0.10[f]	1.3 ± 0.32
20:3ω6	2.7 ± 0.45[g]	0.2 ± 0.06[e]	0.5 ± 0.12
20:4	8.2 ± 0.69[f]	11.3 ± 0.66	10.4 ± 0.56
22:4	0.2 ± 0.04	0.7 ± 0.20	0.4 ± 0.11
22:5ω6	3.1 ± 0.32[g]	11.4 ± 0.89	10.4 ± 0.92
22:6	1.4 ± 0.25	0.9 ± 0.09	1.1 ± 0.20
24:4	0.9 ± 0.09	0.7 ± 0.14	0.7 ± 0.10
24:5	0.2 ± 0.05[f]	0.7 ± 0.25	0.7 ± 0.13

[a]Some minor components not given.

[b]Mean ± SE of six rats in each group.

[c]Number of carbons in chain : number of double bonds; omega number denotes the position of the first double bond from the methyl end.

[d]TYA = eicosa-5,8,11,14-tetraynoic acid.

[e]P value vs. coconut oil group <0.05.

[f]P value vs. coconut oil group <0.01.

[g]P value vs. coconut oil group <0.001.

tion of these five organs (liver, heart, kidney, testis, and brain) is shown in Tables I-V, respectively. In each case, comparison of results on each of the two supplemented groups (Group 1, TYA; Group 2, arachidonic acid) is made with those obtained for control rats given only the coconut oil carrier by stomach tube (Group 3). The data are reported as percent of total fatty

TABLE V

Major Fatty Acid Composition of Brain Total Lipids[a]

Fatty acid[c]	Percent of total fatty acids[b] Group 1 (TYA[d] supplemented)	Group 2 (20:4 supplemented)	Group 3 (Coconut oil only)
16:0	19.4 ± 0.35	19.1 ± 0.55	18.3 ± 0.28
16:1	1.0 ± 0.12	1.1 ± 0.17	1.4 ± 0.05
18:0	15.4 ± 0.47[f]	17.4 ± 0.41	17.5 ± 0.36
18:1	22.3 ± 0.33	20.8 ± 0.42	21.6 ± 0.37
18:2	1.0 ± 0.05[g]	0.2 ± 0.03[g]	0.4 ± 0.03
20:1	1.8 ± 0.19	1.4 ± 0.03[e]	1.8 ± 0.09
20:3ω9	0.4 ± 0.09[e]	0.3 ± 0.04	0.7 ± 0.10
20:3ω6	1.1 ± 0.07[g]	0.3 ± 0.04[f]	0.5 ± 0.04
20:4	10.0 ± 0.24[e]	11.2 ± 0.22	10.8 ± 0.16
22:4	3.1 ± 0.10	3.5 ± 0.16[e]	3.1 ± 0.10
22:5ω6	0.8 ± 0.04	1.2 ± 0.09[e]	0.9 ± 1.2
22:5ω3	0.4 ± 0.07	trace[h]	0.2 ± 0.04
22:6	15.8 ± 0.26	15.5 ± 0.24	15.3 ± 0.25

[a]Some minor components not given.

[b]Mean ±SE of six rats in each group.

[c]Number of carbons in chain : number of double bonds; omega number denotes the position of the first double bond from the methyl end.

[d]TYA = eicosa-5,8,11,14-tetraynoic acid.

[e]P value vs. coconut oil group <0.05.

[f]P value vs. coconut oil group <0.01.

[g]P value vs. coconut oil group <0.001.

[h]Trace, <0.10.

acids. There was a decrease in Group 1 compared to Group 3 in the following fatty acids: stearic acid in heart, kidney, and brain; 20:3ω9 in heart, testis, and brain; arachidonate in all five organs; 22:4ω6 in liver, heart, and kidney; 22:5ω6 in all but the brain; and 22:6 in heart and kidney. There was an increase in Group 1 compared to Group 3 in the following: oleate in heart, kidney, and testis; linoleate in liver, kidney, testis, and brain (also in heart, but this was not statistically significant [Table II]); and 20:3ω6 in liver, testis, and brain. Changes in fatty acid composition also occurred with arachidonate supplementation (Group 2) compared to the nonsupplemented rats (Group 3). A decrease in Group 2 compared to Group 3 was noted in the following: oleate in liver; linoleate in all five organs; 20:3ω9 in all five organs; 20:3ω6 in all five organs; and 22:6 in heart. An increase in Group 2 compared to Group 3 occurred in the following: oleate in heart; arachidonate in liver and kidney; 22:4ω6 in kidney and brain; and 22:5ω6 in liver, kidney, and brain.

No significant changes were observed in any fatty acids of total lipids of adrenals in either Group 1 or 2 compared to Group 3.

DISCUSSION

The most significant finding in these studies was the change in relative concentrations of linoleate and arachidonate levels (and docosapentaenoic acid in testis) in the different groups. Arachidonate levels decreased in all organs examined except adrenals, and there was a corresponding increase in linoleate levels in most of the organs and an increase in the amount of the intermediate 20:3ω6 in some of the organs examined. Interpretation of these results has to be guarded because of the complexities of the dietary conditions involved (fat free diet in all animals and arachidonate supplementation in one group) and because of changes which occur during rapid growth of the animals. However, the results suggest that TYA was inhibiting the conversion of linoleate to arachidonate and the conversion of arachidonate to 22:4 and 22:5. The latter was particularly noticeable in the testis (22:5ω6 level in Group 1 of 3.1 compared to 11.4 in Group 2 and 10.4 in Group 3). This fatty acid is known to accumulate in testicular lipids during sexual maturation of the rat (5). TYA was administered to these rats at 4 and 5 weeks of age when accumulation of this polyene was proceeding at a rapid rate as shown by the levels of 22:5ω6 in Groups 2 and 3).

TYA apparently also interfered with conversion of oleate to 20:3ω9, for there was a decrease of this trienoic acid in some organs and an increase in the concentration of oleate

in Group 1 compared to Group 3. A decrease in concentration of 20:3ω9 also occurred with arachidonate supplementation (Group 2) but, in this case, oleate concentration increased only in the heart. It was expected that arachidonate supplementation would influence the disappearance in liver of the triene characteristic of essential fatty acid deficiency, but it was effective also in other organs, including brain.

Changes in the fatty acid composition of adrenal lipids did occur in Groups 1 and 2 compared to 3, but they were not statistically significant because of the large variation between different samples of the same group. The variation may be due, at least in part, to contamination of adrenal samples with surrounding tissue. The changes were similar to those seen in other organs. Thus, the amount of arachidonate decreased and that of linoleate increased in the group receiving TYA.

The inhibition of prostaglandin synthesis by TYA has been shown (2,6,7). Results reported in this paper indicate that other metabolic reactions involving arachidonic acid, its precursors, and its metabolic derivatives are also affected. Elucidation of the site and mode of action of TYA in these reactions awaits additional investigations.

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Studies on the 12α and 26-Hydroxylation of Bile Alcohols by Rabbit Liver Microsomes

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ABSTRACT

12α-Hydroxylation of two C_{27}-steroids by rabbit liver microsomes was studied. Optimal assay conditions were determined with 7α-hydroxy-4-cholesten-3-one and 5β-cholestane-3α,7α-diol as substrates. The rate of 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one was found to be greater than that of 5β-cholestane-3α,7α-diol by ca. 60%. Microsomal 26-hydroxylation of 5β-cholestane-3α7α-diol was also measured, and the ratio of 26-hydroxylation to 12α-hydroxylation of 5β-cholestane-3α,7α-diol was found to be ca. 0.4. Rabbit liver 12-αhydroxylase was more active than that of three other species (man, rat, monkey), explaining in part the predominance of cholic acid in rabbit bile.

INTRODUCTION

The mechanism whereby cholesterol is converted into bile acids in vertebrates has been studied extensively in recent years (1). The initial steps in the formation of the main primary bile acids, cholic acid and chenodeoxycholic acid, are probably identical: either 5β-cholestane-3α,7α-diol (I) or 7α-hydroxy-4-cholesten-3-one (II) is the last precursor common to both of the primary bile acids. At present it is not known with certainty whether the diol (I) or the unsaturated ketone (II) is the preferred substrate in the formation of cholic acid (2,3). Presumably 26–hydroxylation of I and II would yield predominantly chenodeoxycholic acid, whereas 12α-hydroxylation, followed by 26-hydroxylation, would result in the formation of cholic acid. It is currently assumed that the relative activities of the 12α- and 26-hydroxylases determine the cholic acid to chenodeoxycholic acid ratio in biliary bile acids (3,4).

Rabbit bile has an unusually high ratio of cholic acid (plus its bacterial metabolite, deoxycholic acid) to chenodeoxycholic acid, compared to that of most other mammalian species (5). It seemed of interest therefore to determine relative rates of 12α- and 26-hydroxylations of 7α-hydroxy-4-cholesten-3-one and 5β-cholestane-3α,7α-diol by rabbit liver microsomes to find out whether biliary bile acid composition reflects the enzyme activities.

MATERIALS AND METHODS

Labeled Substrates

[^{3}H]-5β-Cholestane-3α,7α-diol was synthesized according to Björkhem et al. (6) from [^{3}H]-chenodeoxycholic acid by electrolytic coupling with isovaleric acid, and purified by column chromatography on aluminum oxide grade III (Bio-Rad Labs, Richmond, CA). Elution with increasing concentrations of ethyl acetate-benzene yielded white crystals, mp 80 C, sp act 0.8 μCi/mg, radioactive purity 97% (Checked by thin layer chromatography [TLC] on 0.25 mm alumina G plates [Analtech Wilmington, DE], solvent system: chloroform: acetone:methanol 7:5:0.3 v/v/v).

[^{3}H]-7α-Hydroxy-4-cholesten-3-one was prepared as described by Björkhem et al. (6): [^{3}H]-5β-Cholestane-3α,7α-diol obtained as above was oxidized with aluminum-tertbutoxide; the resulting [^{3}H]-7α-hydroxy-5β-cholestane-3-one was purified by column chromatography on aluminum oxide grade III and dehydrogenated with SeO_2 in ethanol. The crude reaction product was purified by column chromatography on aluminum oxide grade IV and eluted with increasing concentrations of ethyl acetate in benzene; crystals, mp 181 C, sp act 0.75 μCi/mg, radioactive purity 98% (TLC on 0.25 mm plates of Silica Gel G [Analtech, Wilmington, DE], solvent system: benzene:ethyl acetate 6:4 v/v).

Unlabeled Reference Compounds

5β-Cholestane-3α,7α-diol and 7α-hydroxy-4-cholesten-3-one were synthesized in the same manner as the labeled substrates.

5β-Cholestane-3α,7α,12α-triol was prepared by electrolytic coupling of cholic acid and isovaleric acid, as described by Bergström and Krabisch (7), mp 184-185 C.

7α,12α-Dihydroxy-4-cholesten-3-one was

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synthesized as described by Berséus et al. (8), mp 228-229 C.

5β-Cholestane-3α,7α,26-triol was synthesized from chenodeoxycholic acid by electrolytic coupling with methyl-2-methyl-3-carboxypropionate, by the method described by Berséus and Danielsson (9,10), with modifications as follows: Methyl-2-methyl-3-carboxypropionate (3 g) was prepared by refluxing 5 g methyl succinic anhydride (K & K Labs, Cleveland, OH) with 3 ml anhydrous methanol for 1.5 hr; the product was reacted with 500 mg of chenodeoxycholic acid in an electrolytic bath surrounded with ice, containing 200 ml anhydrous methanol, and 25 ml of 0.5 N sodium methoxide: methanol (Applied Science Labs, State College, PA). Platinum electrodes (2 cm diameter) were placed at 0.3 cm distance, and the electrolsis was run 2 hr at ca. 1-1.5 Amp. The reaction mixture was diluted with an equal amount of water and extracted twice with 100 ml ether. The ether extracts were washed twice with 50 ml 5% Na_2CO_3 to dissolve the unreacted free acids, washed with water, dried over anhydrous Na_2SO_4, and evaporated. The crude methyl 3α,7α-dihydroxy-5β-cholestan-26-oate was purified by preparative TLC on Silica Gel G plates, 500 μ thick (Analtech), using known reference compounds as standards. The plates were developed in a solvent system containing chloroform:acetone:methanol (7:5:0.3 v/v/v), and narrow bands along the two edges were sprayed with 3.5% phosphomolybdic acid in isopropanol. The band with the same R_f as the reference compound was scraped off the plates and extracted by stirring 15 min with 100 ml acetone. Evaporation of the solvent and crystallization from acetone: water yielded 60 mg methyl 3α,7α-dihydroxy-5β-cholestan-26-oate (purity by TLC > 99% [mp 50-55 C]). Purified ester (50 mg) was reduced with 50 mg $LiAlH_4$ (Alpha Inorganics, Beverly, MA) in 20 ml anhydrous ether by refluxing the reaction mixture for 2 hr. After decomposition of the unreacted $LiAlH_4$ with ethyl acetate, the reaction mixture was acidified with 2N HCl, the organic layer was separated, washed with water, dried over anhydrous Na_2SO_4 and evaporated. The crude reaction product was purified by preparative TLC, using the same type of plates and solvent system as for the purification of the ester, with pure 5β-cholestane-3α,7α,26-triol and methyl 3α,7α-dihydroxy-5β-cholestan-26-oate applied as external standards on both sides of the plates. The area corresponding to the R_f values of the reference compounds was scraped from the plates and extracted with acetone. Evaporation of the solvent and crystallization of the compounds from acetone: water (1:1 v/v) yielded 12 mg unreacted ester and 18 mg pure 5β-cholestane-3α,7α,26-triol, mp 149-151 C, mass spectrum identical with that published by Björkhem and Gustafsson (11) with main peaks at m/e 546 (M-90), 531 [M-(90+15)], 456 [M-(2x90)], 441 [M-(2x90+15)], 366 [M-3x90)], and 351 [M-(3x90+15)].

It is of practical interest that the purification of most of the intermediate reaction products, as well as that of the final bile alcohols, could be performed by preparative TLC on activated alumina plates, rather than silica gel, as described in the experimental section for the synthesis of 5β-cholestane-3α,7α,26-triol. This procedure avoids time-consuming purification procedures by column chromatography.

Animals and Human Subjects

The rabbits used were male New Zealand whites weighing ca. 1 kg. Rats were males of the Sprague-Dawley strain (Charles River Breeding Laboratories, Wilmington, MA) weighing between 200-250 g. The Rhesus monkeys used were 2 males and 2 females which weighed between 2.7-3.3 kg. All animals were given food and water ad libitum prior to sacrifice. The livers of the animals were always removed between 10 and 11 a.m. to minimize possible effects of diurnal variations. Liver biopsies from the human subjects were kindly provided by G. Salen (12).

Preparation of Microsomes and Assay Conditions

Male rabbits weighing ca. 1 kg were used. Liver homogenates (30% w/v) were prepared and used for preparation of microsomal suspensions of ca. 30% concentration, as described for the preparation of rat liver microsomes (13). Protein concentration was determined by the method of Lowry et al. (14). Standard incubations were performed at 37 C for 20 min; 100 nmol of the substrate dissolved in 50 μl acetone were preincubated for solubilization for 10 min at room temperature with 0.3 ml of a solution containing 0.6 mg bovine serum albumin (Calbiochem, Monsey, NY). The standard assay system for all three microsomal enzymes contained, in a vol of 2.2 ml: 0.3ml of solubilized substrate (100 nmol substrate), 0.5 ml of the 30% microsomal suspension, 1.0 ml of a reduced nicotinamide adenine dinucleotide phosphate generating system (70 mM potassium phosphate buffer, pH 7.4; 2.25 mM $MgCl_2$; 1.25 mM nicotinamide adenine dinucleotide phosphate; 2.5 mM glucose-6-phosphate and 5 units glucose-6-phosphate dehydrogenase), and 0.4 ml of homogenizing medium (0.3 M sucrose, 75 mM nicotinamide, 2 mM ethylenedia

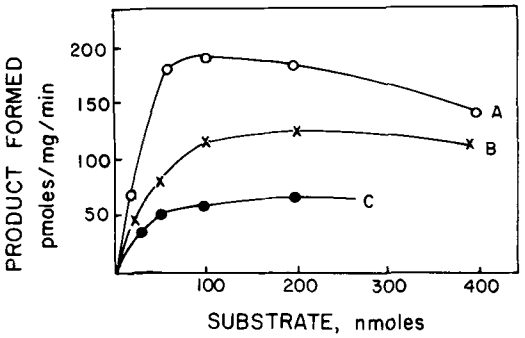

FIG. 1. Effect of substrate concentration 12α- and 26-hydroxylation rates of 5β-cholestane-3α, 7α-diol and 7α-hydroxy-4-cholesten-3-one. Standard assay conditions were used except for substrate concentrations. A = 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one; B = 12α-hydroxylation of 5β-cholestane-3α, 7α-diol; C = 26-hydroxylation of 5β-cholestane-3α,7α-diol. All points represent the average of duplicate determinations.

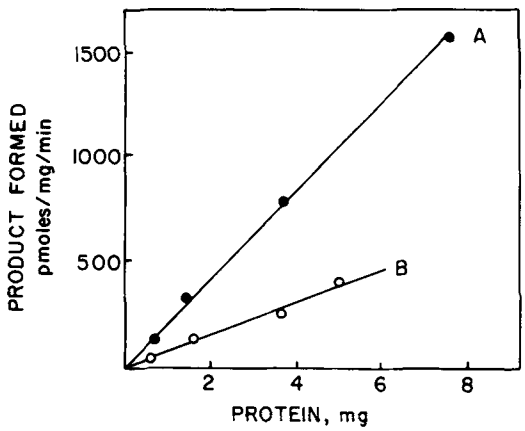

FIG. 2. Effect of microsomal protein concentration on the 12α-hydroxylation of 5β-cholestane-3α,7α-diol and 7α-hydroxy-4-cholesten-3-one. Standard assay conditions (0.6 mg albumin and 100 nmol substrate per tube), except for microsomal protein concentrations. A = 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one; B = 12α-hydroxylation of 5β-cholestane-3α,7α-diol. All points represent the average of duplicate determinations.

minetetraacetic acid, and 20 mM mercaptoethanol).

Incubations were terminated by the addition of 2 ml methanol, and the reaction products were extracted with 20 vols of chloroform: methanol (3:1 v/v). The chloroform extracts were evaporated under nitrogen and the incubation products were separated by TLC on Silica Gel G (Analtech) plates. When [^{3}H]-7α-hydroxy-4-cholesten-3-one was used as substrate, unlabeled 7α-hydroxy-4-cholesten-3-one and 7α,12α-dihydroxy-4-cholesten-3-one were added as carriers and the plates were developed with ethyl acetate:benzene (6:4 v/v). The compounds were made visible by exposing the plates to iodine vapor. Unlabeled carriers for the extracts from the incubation of [^{3}H]-3α,7α-dihydroxy-5β-cholestane used as substrate were 5β-cholestane-3α,7α-diol (R_f 0.90), 5β-cholestane-3α,7α,26-triol (R_f 0.59), and 5β-cholestane-3α,7α,25-triol (R_f 0.64). The solvent system used was chloroform:acetone: methanol (7:5:0.3 v/v/v) on alumina plates, 0.25 mm thick, which were activated by heating 30 min at 100 C. The compounds were made visible by spraying the plates with 3.5% phosphomolybdic acid in isopropanol.

The pertinent spots were scraped from the plates and counted for radioactivity in vials containing 0.3 ml water and 14 ml of scintillation solution (5 g of 2,5-diphenyloxazole and 100 g naphthalene per liter of dioxane). Enzyme activities were expressed in picomoles of product formed per mg protein per min.

RESULTS

Identification of Incubation Products

Incubation products were identified by gas chromatography-mass spectrometry. Retention times and mass spectra of the incubation products 7α-hydroxy-4-cholesten-3-one, 5β-cholestane-3α,7α,12α-triol, and 5β-cholestane-3α,7α-26-triol were identical with those of the authentic reference compounds synthesized as described in the experimental section.

Properties of the Microsomal Assay System

The effects of substrate concentrations on hydroxylation rates are presented in Figure 1 for the following substrates: A = 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one; B = 12α-hydroxylation of 5β-cholestane-3α,7α-diol; C = 26-hydroxylation of 5β-cholestane-3α,7α-diol. In the standard assay systems containing ca. 1-2 mg protein per tube, 100 nmol substrate "solubilized" with 0.6 mg albumin was used in all three hydroxylation reactions studied to ensure that the enzymes were saturated. The relationship between reaction rate and enzyme concentration is illustrated in Figure 2 for the 12α-hydroxylations of the two substrates studied (A = 7α-hydroxy-4-cholesten-3-one; B = 5β-cholestane-3α,7α-diol). Proportionality was observed when the protein concentration ranged up to ca. 8 mg per tube in the standard assay system. The rate of formation of the reaction products was found to be linear with time during a 40 min period, and an incubation time of 20 min was chosen for the standard assay.

The optimum pH for the enzyme activities studied was ca. 7.4. Figure 3 illustrates the relative reaction rates of the hydroxylations studied (A = 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one; B = 12α-hydroxylation of 5β-cholestane-3α,7α-diol; C = 26-hydroxylation of 5β-cholestane-3α,7α-diol), expressed in picomoles reaction products formed per mg microsomal protein per min.

A comparison between 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one catalyzed by rabbit liver microsomes and liver microsomes obtained from other species is illustrated in Figure 4. Liver microsomes of rat, man, and monkey were used for enzyme measurements, and the results indicate that the most active 12α-hydroxylase was that of the rabbit.

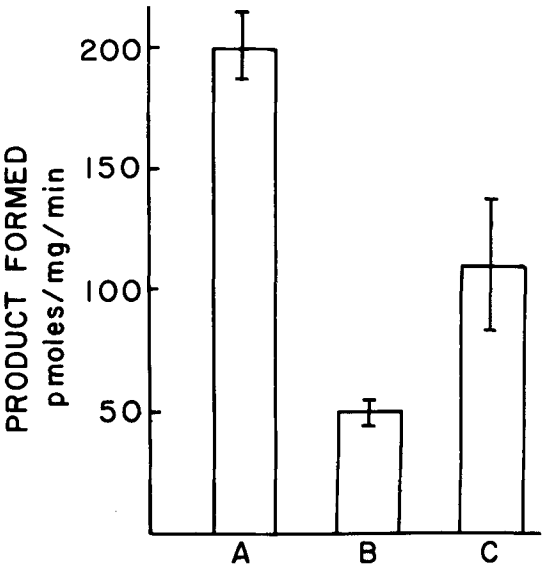

FIG. 3. Relative rates of 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one (A), 26-hydroxylation of 5β-cholestane-3α,7α-diol (B), and 12α-hydroxylation of 5β-cholestane-3α,7α-diol (C), by rabbit liver microsomes. Four determinations were made for each substrate. The vertical lines represent the (±) SEM.

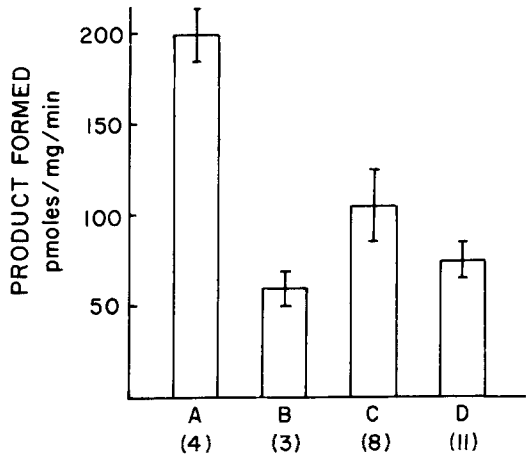

FIG. 4. Species difference in 12α-hydroxylase activity in livers of rabbit (A), man (B), monkey (C), and rat (Sprague-Dawley) (D), determined by using [^{3}H]-7α-hydroxy-4-cholesten-3-one as substrate and the standard assay conditions as described in the experimental section. Enzyme activity is expressed in picomoles 7α,12α-dihydroxy-4-cholesten-3-one formed. The figures in parentheses indicate the number of experiments for each assay. The vertical lines represent the (±) SEM.

DISCUSSION

The rate of 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one by rabbit liver microsomes was found to be ca. 0.2 nmol 12α-hydroxylated product formed per mg protein per min, which is ca. twice as high as the reaction catalyzed by monkey liver and three times higher than that catalyzed by human liver microsomes (Fig. 4). The high 12α-hydroxylase activity is in good agreement with the bile acid composition of rabbit bile, consisting mostly of cholic acid and its bacterial metabolite, deoxycholic acid. A similar correspondence between a high 12α-hydroxylase activity and the predominance of cholic acid in the bile was found in a patient with cerebrotendinous xanthomatosis (15). The 26-hydroxylation of the same substrate by the microsomal fraction of rabbit liver did not occur to a considerable extent under the assay conditions employed.

When one compares the 12α-hydroxylation of the two substrates studied, 7α-hydroxy-4-cholesten-3-one seems to be a more suitable substrate for the microsomal 12α-hydroxylase than 5β-cholestane-3α,7α-diol (Fig. 1). The same figure indicates that 12α-hydroxylation of the diol is much faster than the 26-hydroxylation of the same substrate, but that 26-hydroxylation is nevertheless a noteworthy reaction in the microsomes. This finding is somewhat unexpected inasmuch as according to Björkhem and Gustafsson (11), the ratio of 12α:26-hydroxylation is important in determining the cholic acid:chenodeoxycholic acid ratio in the bile, yet rabbit bile contains practically no chenodeoxycholic acid or its bacterial metabolites. Perhaps in the rabbit, in vivo, the pathway of bile acid synthesis involves 7α-hydroxy-4-cholesten-3-one rather than 5β-cholestane-3α,7α-diol as substrate for the 12α-hydroxylase. This assumption is based on the fact that we found a better correlation between the 12α:26-hydroxylation determined in vitro and

the cholic acid:chenodeoxycholic acid ratio in the bile when 7α-hydroxy-4-cholesten-3-one rather than the diol was used as substrate for the same hydroxylations. These considerations are of course valid only if it is assumed that in vitro measurements of enzyme activity reflect in vivo reaction rates.

ACKNOWLEDGMENTS

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Variations in Lipid Composition and Sound Velocity in Melon from the North Atlantic Pilot Whale, *Globicephala melaena melaena*

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ABSTRACT

Nonpolar lipids and sound velocity at different distances from the skin surface within a sample of pilot whale melon were studied. Thin layer chromatography, sensitive radioisotopic methods, and an ultrasonic echo scanner were used. Wax esters had a maximum concentration at 11-12 cm from the skin surface. Non- and monoisovalero triglycerides both exhibited a minimum at 8-10 cm, whereas the diisovalero species increased steadily from the skin surface towards the center of the pilot whale head. A minimum of sound velocity, ca. 1340 m/s, was found at 9-11 cm, thus coinciding with a region rich in isovaleric acid esterified in wax and diisovalero triglycerides. These findings are compatible with predictions based on the known correlations between lipid structure and sound velocity. Consequently, additional evidence for refraction of sound in pilot whale melon is presented. This is important in understanding the system of echolocation of toothed whales.

INTRODUCTION

Evidence is accumulating that the fat-filled bulge or melon, on the head of dolphins is a collimator of ultrasound emitted by the animal for the purpose of echolocation (1). The chemical composition of the oil constituting 80% of the melon's wt has been studied by several authors (2-4). The lipid composition of this fat pad differs from that of blubber, the fat surrounding the rest of the animal (5-7). A great part of the oil from the melon, or head oil, consists of triglycerides (TGs) with one or two isovaleric acid residues, and isobranched longer fatty acids (2,3). The acoustic properties of the head oil from pilot whales have also been studied (8). The oil had a low sound velocity compared to some other oils, and the sound velocity was lower than that of the surrounding medium, i.e., water. This was considered another indication that the *Globicephala* melon functions as an acoustic lens. This work presumed that conclusions about the acoustic properties of the melon tissue could be drawn from measurements on the head oil. This supposition should be tested because the reliability of the model of the collimating function of the melon depends on the reliability of this presumption.

Recently, papers describing the existence of variations of lipid composition within the *Globicephala* melon have occurred (9). Much of the chemical part of this work was done before these results became known to the present authors.

The present study shows the existence of variations in sound velocity within the melon concomitant with variations of lipid composition. The earlier presumptions regarding the sound velocity of the melon extrapolated from measurements on the head oil (8) seem to be correct.

MATERIALS AND METHODS

The Melon Sample

A piece of tissue from the apex of the melon of a pilot whale (Fig. 1), was cut rectangularly, 13 x 10 cm of skin surface x 18 cm perpendicularly down to the jawbone. The whale was caught off the Faroe Islands in October, 1970. Its sex, wt, and length are unknown. The piece (2090 g) was cut out immediately after capture and stored frozen during transport and until analysis. Oil from points 1 cm apart on a line perpendicular to the skin surface was aspirated

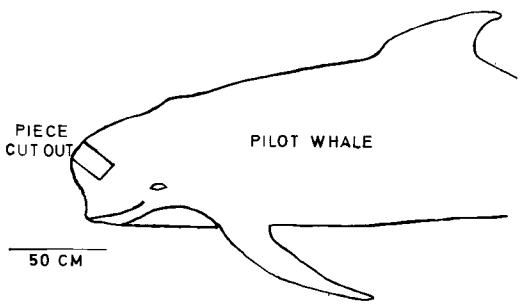

FIG. 1. Schematic diagram showing the position of the sample analyzed in this paper.

with a microliter syringe and spotted directly (0.2 μl) on the thin layer chromatographic plate.

Subsequently, a slice weighing 510 g was cut perpendicular to the skin from the entire specimen. This was homogenized in a Turmix blender and extracted with chloroform:methanol (2:1). The specimen yielded 410 g of odorless, pale yellow oil and 80 g fibrous remains. The black, tough skin that had been cut away separately weighed 12 g. Consequently ca. 80% of the wt of the melon consisted of oil. The consistency of the specimen before homogenization was hard and fibrous near the skin and flaccid far from the skin. Oil oozed spontaneously from the latter part of the specimen during work at room temperature.

Thin Layer Chromatography (TLC)

Silica Gel H (Merck, Rahway, NJ) 0.25 mm thick on 20 x 20 cm glass plates was used. The solvent used was light petroleum (40-60 C, redistilled):diethyl ether:acetic acid (80:20:1). Identification of lipid classes after staining of the spots with iodine vapors or sulfuric acid spray was possible by comparison with head oil lipids from *Globicephala* prepared earlier (2). For spot area determinations, the plate was photographed on color slides, and each slide was then projected and spots drawn on paper, cut out, and weighed in triplicate.

Radioisotope Determinations

After TLC, the spots were scraped out into filtration funnels. Lipids were extracted with chloroform, and the extracts were evaporated to dryness under nitrogen.

For determination of acyls, 11.7 μg of suberic acid (Fluka, Cleveland, OH) was added as an internal standard to each fraction. Then 0.1 ml of 1 N HCL in chloroform:methanol (3:1) containing 25 μCi of tritiated methanol (50 mCi/mM, New England Nuclear, Boston, MA) was added to each tube. The methanolysis proceeded for 4 hr on a boiling water bath in gas tight tubes with teflon faced screw caps. Then the fractions were neutralized by 0.1 ml of 1 N sodium hydroxide, and 0.5 ml of redistilled light petroleum (bp 40-60 C) was added to each tube, allowing the methyl esters (and dimethyl acetals) to be extracted into the petroleum phase. This took place in a well ventilated hood to avoid the radioactive vapors. The petroleum ether phase was sucked off with a piston pipette, transferred to another tube, and again taken to dryness. This procedure gives a total loss of volatile methyl esters, such as methyl isovalerate. However, if the evaporating conditions are not too hard, longer chain fatty acid methyl esters will stay in the tube.

The methyl esters were then separated on another Silica Gel H TLC plate with light petroleum (40-60 C):diethyl ether:acetic acid (97:3:1), which allowed the dimethyl suberate to migrate slowly and the monomethyl esters from the samples to migrate faster, i.e., close to the front. Spots could easily be detected without staining if ca. 0.1 mg of nonradioactive methyl palmitate and dimethyl suberate were allowed to migrate together with the radioactive samples. Staining with iodine vapors should be made with caution inasmuch as it has been noted that even short exposure before development of the plate results in additional spots. This is an indication of hydrolysis and could lead to losses of radioactive methanol from the plate. The spots were then scraped off directly into scintaillation counting vials. Scintillation counting was performed in Insta-Gel (Packard Instrument Co., Downers Grove, IL) using a Packard Tri-Carb Scintillation counter. In the present study, the losses of short chain methyl esters could be compensated for because the stoichiometric proportions of volatile fatty acids to nonvolatile fatty acids were defined from their behavior at TLC. Consequently, acyl values from monoisovalero TGs were multipled by 1.5 and diisovalero triglycerides by 3.

For determination of alkyls (hydroxyl groups), the same initial separation of lipid classes from oil specimens taken at different distances from the skin was performed. The wax and cholesteryl ester spots were collected together and extracted as described above. To each fraction was added 50 μg of batyl alcohol (Fluka) as an internal standard. Then, methanolysis in 0.1 ml of 1 N HCL in methanol was performed overnight at 80 C in gas tight tubes with teflon faced screw caps. After this, a two phase system was created by the addition of 0.5 ml light petroleum. The upper phase, which contained the radioactive acetates, was sucked off and taken to dryness. The samples were then acetylated with tritiated acetic acid anhydride according to a method described earlier (10). The monoacetates from the sample alkyls and the diacetates from the internal standard were then separated on TLC, using the same conditions as for acyl determination (see above). Radioactivity in the spots was assayed as indicated above.

Both in the acyl and alkyl determinations, the absolute amounts could be determined by dividing the counts from the sample with the counts from the internal standard and then multiplying by the number of μmol of internal standard that were put into each tube.

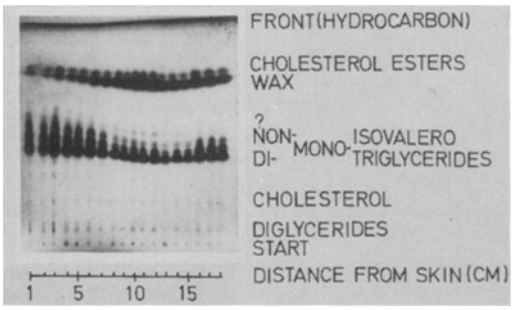

FIG. 2. Thin-layer chromatography of oil samples taken at different distances from the skin surface of pilot whale melon. 0.2 μl was spotted on the plate. 50% Sulphuric acid spray. Solvent: light petroleum: diethyl ether:acetic acid (80:20:1).

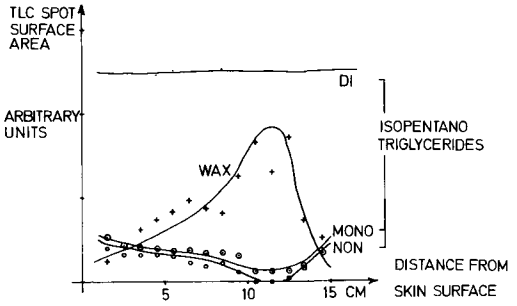

FIG. 3. Schematic plot of relative surface area of the spots on the thin layer chromatogram in Fig. 2 vs. distance from skin surface.

Sound Velocity Determinations

An ultrasonic echo scanning apparatus for medical use (Portascan PAS-64-2, Physionic Engineering, Longmont, CO), was used. The time required for sound with a frequency of 2 MHz to travel through the tissue piece between the surfaces of two ultrasonic probes was measured as the distance between start and arrival signals on an oscilloscope screen. The time equivalent to one division (10 mm) on the oscilloscope screen was calibrated by means of a pulse generator and a Hewlett Packard frequency counter. Under the conditions of the experiments, a pulse train consisting of 22 pulses with a repetition frequency of 0,395 MHz corresponded to 10 divisions. The time/division was consequently 5.57 μsec.

All distances were measured with a precision caliper, except for the distance from the point of measurement to the skin surface, which was measured with a ruler. The temperature of the tissue was 23 C.

RESULTS

TLC (Fig. 2) showed that, at 10-13 cm from the skin surface, waxes are more abundant.

The TLC spot area measurements gave approximate values for the relative abundances of the lipid classes (11). (Fig. 3). Unsaturated lipids were detected by exposing the TLC plates to iodine vapor for several days and then allowing excess iodine to evaporate. In the authors' experience, unsaturated lipids generally give black or dark brown spots after this treatment. Spots at a distance of 10-12 cm from the surface were faintly stained, indicating that these fractions had little unsaturation. Judging from the TLC, there seem to be variations in other lipid classes than waxes and TGs. Cholesteryl esters give a more intense staining with iodine

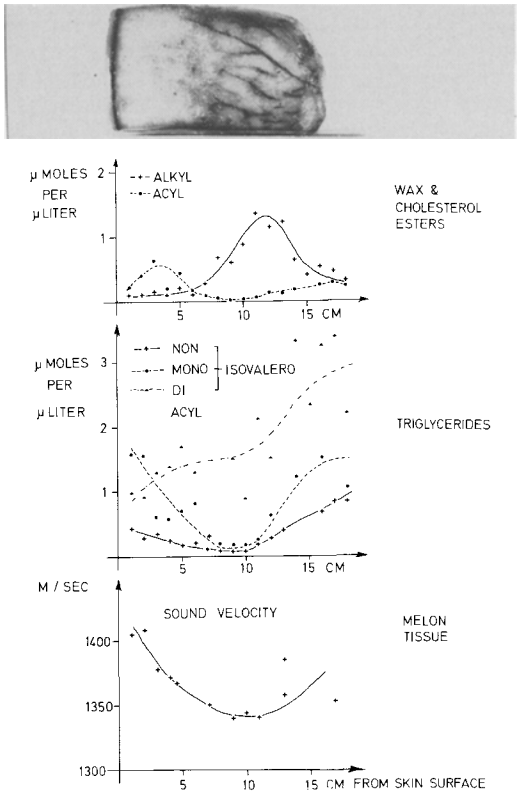

FIG. 4. Radioisotope and sound velocity determinations at different distances from the skin. From top to bottom: a. photograph of the sample on which the tests were performed; b. nonvolatile alkyls and acyls in the wax and cholesteryl ester fraction; c. nonvolatile acyls in the non-, mono-, and diisovalero triglyceride fractions; d. sound velocity of melon tissue.

near the surface. Cholesterol, on the other hand, gives a spot of ca. constant area throughout the melon. The absolute values of nonvolatile acyl and alkyl content of wax and cholesteryl esters, non-, mono-, and diisovalero TG are given by the radioisotope determina-

tions (Fig. 4, b & c). As indicated by the spot area determinations, the wax esters seem to have a peak concentration at 11-12 cm from the skin surface (Fig. 4b). Nonvolatile alkyls also show variations and are in excess of the acyls at 2-5 cm. This indicated either the presence of long chain alkyls esterified to short chain acyls (12) or some unexpected side reaction during the derivatization procedure, e.g., methyl uptake by decomposing cholesterol. The TGs also show marked variations in the radioisotope determinations (Fig. 4c).

Non- and monoisovalero TGs both show a minimum concentration at 8-10 cm, whereas the diisovalero TGs show a steady increase in concentration from the skin surface towards the center of the pilot whale head. The surface area and radioisotope methods generally show a surprisingly good correlation. A minimum of sound velocity at 9-11 cm from the skin surface is clearly indicated by the data shown in Figure 4d.

DISCUSSION

The occurrence of lipids containing high proportions of isovaleric acid and iso-branched longer fatty acids (2) in the melon of most toothed whales has intrigued several workers. A clue to the functional importance of these unique lipids can be derived from the literature on sound velocity in lipids. Branched and short chain lipids are known to have lower sound velocities than other lipids (13,14). Therefore, it can be expected that parts of the melon possessing a high concentration of these structures would have a lower sound velocity than the other parts. The present investigation shows that this is the case. A one dimensional study cannot, however, give definite answers to the question of the influence of the melon on the ultrasound emitted by the dolphin. The sonic data indicate that sound should be refracted both within the melon and at the melon/water interface. The data are not incompatible with a sonic lens model. In fact, they suggest a core of high sonic refractive index very much like that of the human optic lens. The data are compatible with those of Norris et al. (15).

Litchfield et al. (16) have performed two- and three-dimensional studies of lipid composition of the *Globicephala* melon. Their data indicate that the melon should be divided into at least two parts, outer and inner. This arrangement suggests a wave-guide model rather than a lens model. In any case, it is clear that some kind of sound refraction must occur, and it is quite probable that this phenomenon partly explains the narrowness of the ultrasonic beam emitted by the echolocating dolphin (17). The mean sound velocity calculated from the values presented in Figure 4d is 1366 m/sec (23 C). This compares well with an earlier determination (6) of 1380 m/sec at 23 C. The radioisotope methods of alkyl and acyl determination are simple to perform and can be made very sensitive. Similar methods have been described earlier (18,19).

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SHORT COMMUNICATIONS

γ-Linolenic Acid in *Acer* Seed Oils

ABSTRACT

The octadecatrienoic acids in *Acer negundo* L. (maple family) seed oil include both 9,12,15- (1%) and 6.9,12- (7%) isomers. The chief monoenoic acids identified were 9-octadecenoic (21%), 11-eicosenoic (7%), 13-docosenoic (15%), and 15-tetracosenoic (7%). Also present is a considerable amount of 9,12-octadecadienoic acid. Investigation of ten other Aceraceae revealed their seed oils to have a similar fatty acid composition.

INTRODUCTION

Hopkins and coworkers (1) reported fatty acid composition and positions of unsaturation in many acids from a series of Aceraceae seed oils. They indicated that these seed oils resemble those from Cruciferae species because they contain large amounts of docosenoic and tetracosenoic acids. However, the publication (1) does not mention the presence of C_{22} and C_{24} saturated acids or C_{20} and C_{22} dienoic acids; neither does it note the positions of unsaturation in the octadecatrienoic acids in the Aceraceae seed oils.

EXPERIMENTAL PROCEDURES

Seed oils were extracted and analyzed as previously described (2,3). Methyl esters of the fatty acids were prepared (4) and analyzed by both gas liquid chromatography (GLC) and thin layer chromatography (TLC) (2,5). Methyl esters of *Acer negundo* L. were separated according to degree of unsaturation by preparative TLC on plates coated with a 1 mm thick layer of Silica Gel G containing 20% silver nitrate. The plates were developed with benzene, which separated the esters into five fractions (I-V). Each fraction was subsequently analyzed by GLC. Olefinic bond positions were located either by GLC-ozonolysis (6) procedures or by combined GC-MS (mass spectrometry) of methoxy derivatives (7). Infrared (IR) and ultraviolet (UV) absorption of the oils were measured as previously described (8). Nuclear magnetic resonance (NMR) data of the trienoic esters from *A. negundo* were obtained with a Varian HA-100 spectrometer from a deuteriochloroform solution containing 1% tetramethylsilane as internal standard.

RESULTS AND DISCUSSION

Fraction I from $AgNO_3$-TLC of *A. negundo* methyl esters contained mostly even chain saturated compounds ranging from C_{14} to C_{20}. The monoenes in fraction II ranged from C_{18} to C_{24}, and fraction III was 98% C_{18} dienes. Fractions IV and V were essentially all trienes, fraction IV being rich in 9,12,15-18:3 and fraction V in 6,9,12-18:3. The NMR spectrum of fraction IV exhibited a well defined symmetrical triplet at δ 1.0 defining the terminal methyl protons as β to an olefinic bond, whereas fraction V showed an unsymmetrical triplet representing the terminal methyl protons at δ 0.9 and indicating those protons were remote to olefinic unsaturation. Mass spectra of the trienoic isomers were consistent with NMR data. Mass spectra of fractions IV and V were characteristic of 9,12,15-18:3 and 6,9,12-18:3, respectively, as compared to those reported by Holman et al. (9).

Positions of unsaturation in the trienoic isomers were confirmed by GLC of the reduced ozonides (6). The major ozonolysis product from fraction IV (9,12,15-:18:3) was the C_9 aldehyde-ester (9AE); C_3 aldehyde (3A) fragments expected from this isomer are not observed under these conditions (6). Fraction V yielded 6AE and 6A in a ratio of 1:1. These ozonolysis products firmly establish fraction V to be 6,9,12-18:3.

Double bond positions of the components in fractions II and III were identified by GC-MS of the methoxy derivatives (7). Mass spectra of the methoxylated monoenoic esters in fraction II all exhibit major ions at m/e 157 and at m/e 171 resulting from the fragment

$$^{+}\text{CH}(\text{OCH}_3)-(\text{CH}_2)_n-\text{CH}_3$$

(n = 7,8), respectively, indicative of $\omega 9$ unsaturation. In addition, the ions representing the corresponding fragments containing the ester moiety

$$\left(\text{O}=\text{C}(\text{OCH}_3)-(\text{CH}_2)_x-\overset{\oplus}{\text{C}}\text{H}(\text{OCH}_3)\right)$$

TABLE I

Composition of *Acer* Seed Oils

Species	Oil (% by wt)	Fatty acid (area percent by gas liquid chromatography)													HBr reactive[a] acids (%)
		16:0	18:0	18:1	18:2	18:3	18:3[b]	20:0	20:1	20:2	22:0	22:1	22:2	24:0	24:1	
A. buergerianum Miq.[c]	15.0	5.0	3.0	27.0	34.0	0.3	1.0	0.3	5.1	tr[d]	2.6	12.7	0.7	2.5	5.2	1.2
A. ginnala Maxim.	12.5	4.0	2.3	23.9	37.4	1.0	3.5	0.2	5.8	tr	0.7	14.0	---	0.3	6.0	2.2
A. heldreichii Orph. & Boiss	13.2	6.0	2.0	26.0	34.6	2.3	2.3	0.2	5.5	0.2	0.6	11.5	tr	0.2	4.4	3.9
A. hyrcanum Fisch. & Mey.[e]	16.0	7.0	2.0	26.0	35.0	0.2	1.5	0.1	5.6	tr	0.6	12.0	0.7	0.6	6.0	1.3
A. monspessulanum L.	34.7	5.0	3.0	29.0	34.0	0.5	1.5	0.2	7.0	0.2	0.7	13.0	---	0.5	5.3	1.4
A. negundo L.	9.0	4.0	1.0	21.0	34.0	1.0	7.0	0.3	7.0	0.2	0.9	15.0	tr	0.3	6.8	0.9
A. platanoides L.[c,e]	8.1	10.0	2.0	25.0	35.0	0.8	1.6	0.2	5.0	0.2	1.0	11.0	0.5	1.0	4.7	1.4
A. pseudoplantinus L.[e]	17.4	10.0	2.0	28.0	30.0	1.0	1.4	0.2	4.0	0.2	0.7	12.0	tr	0.6	6.4	3.6
A. saccharum Marsh[c,e]	17.0	6.4	3.2	28.2	36.6	0.5	1.8	0.6	6.6	tr	0.7	10.3	0.3	tr	3.8	2.0
A. tataricum L.	21.3	3.0	2.0	18.0	35.0	0.8	6.0	0.2	4.6	0.2	0.8	18.0	0.5	0.6	10.3	1.1
A. truncatum Bunge	18.0	5.0	3.0	27.0	38.0	0.4	1.0	0.2	7.5	0.2	0.9	11.8	0.6	0.8	3.6	0.5

[a]Material reacting to hydrogen bromide and calculated as epoxy oleic acid.

[b]γ-Linolenic.

[c]Also found 0.5% hexadecenoic.

[d]tr = Trace, <0.1.

[e]Also found 0.1-0.3% heptadecanoic.

from each monoene were observed. The spectrum of the methoxylated diene in fraction III was dominated by intense ions at m/e 129, 215, 115, and 201. These ions represent the methoxy derivatives of methyl 9,12-octadecadienoate.

The fatty acid composition of the 10 other Aceraceae seed oils is given in Table I. The percentages are based on GLC of the mixed esters. Although not analyzed as rigorously as *A. negundo*, all these oils appear to contain γ-linolenic acid as indicated by equivalent chain lengths (5) of 17.2 (Apiezon L. column) and 19.3 (Silar 5CP column).

In addition to the usual long chain fatty acids, the seed oils contain small quantities of material reacting to hydrogen bromide (Table I). They vary from 0.5 to 3.9% when calculated as epoxyoleic acid (2,3).

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[Received October 28, 1975]

Effect of Transplanted Human Ovarian Cancer Tissue on Liver Lipid Metabolism of Nude Mice

ABSTRACT

The changes in the lipids of liver tissues of nude mice with and without transplanted human cancerous tissues were studied to clarify the effect of transplanted human tumor tissues on host liver lipid metabolism. The total lipid was extracted and separated into phospholipid, triglyceride, and other fractions by thin layer chromatography. The amounts of methyl esters of fatty acids of each lipid fraction were measured by quantitative gas liquid chromatography after each lipid fraction had been subjected to methanolysis by 5% HCl-methanol. The phospholipid content of liver tissues of six tumor bearing nude mice was increased and the triglyceride content decreased in comparison with these fractions in three control nude mice. The ratio of the phospholipid fatty acid content to the triglyceride fatty acid content (phospholipid:triglyceride[PL:TG]) of six tumor bearing nude mice was distributed between 7.6 and 33.5, whereas PL:TG ratios of three control nude mice were distributed between 1.7 and 3.8. This result was similar to that reported for human liver tissues of patients with malignant neoplastic disease, indicating that nude mice with transplanted human cancer may be useful for clarifying the mechanisms of the lipid-chemical changes of liver tissues of patients with malignancies.

INTRODUCTION

In 1971, lipid-chemical changes of biopsied human lvier tissues, which might be useful for the diagnosis of gastroenterological and other cancers, including early cases of gastric cancer, were reported by Nakazawa and Yamagata (1).

Recently, reports by Flanagan (2) and Pantelouris (3) indicated that human malignant tumors are transplantable into the nude mouse.

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In addition to the usual long chain fatty acids, the seed oils contain small quantities of material reacting to hydrogen bromide (Table I). They vary from 0.5 to 3.9% when calculated as epoxyoleic acid (2,3).

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In 1971, lipid-chemical changes of biopsied human lvier tissues, which might be useful for the diagnosis of gastroenterological and other cancers, including early cases of gastric cancer, were reported by Nakazawa and Yamagata (1).

Recently, reports by Flanagan (2) and Pantelouris (3) indicated that human malignant tumors are transplantable into the nude mouse.

TABLE I

Phospholipid and Triglyceride Fatty Acid Contents and Ratios in Livers of Control and Tumor Bearing Nude Mice

	Phospholipid (mg/g liver)	Triglyceride (mg/g liver)	PL:TG ratio
Control liver			
1	9.8	2.6	3.8
2	7.0	2.2	3.2
3	10.9	6.4	1.7
Tumor bearing liver			
1	12.3	1.6	7.7
2	14.8	1.1	13.4
3	14.4	0.9	16.4
4	14.0	0.4	33.5
5	9.6	0.8	12.4
6	16.7	2.0	8.4

Since these reports, several authors (4-6) have reported success in repeating these experiments. It was hoped that these mice bearing transplanted human tumors might be useful for the study of the mechanism of lipid-chemical changes seen in liver tissues of cancerous patients. This study deals with the effect of transplanted human ovarian cancer on liver lipid metabolism of nude mice.

MATERIALS AND METHODS

Treatment of Animals

Cells from a human ovarian adenocarcinoma removed at surgery from one patient were transplanted into female BALB-C nude mice aged 8 weeks. After 7 months, tumor specimens grown in nude mice were transplanted into other nude mice under the same conditions as before. After a further 2 months, six nude mice in which retransplantation had been successful were sacrificed and their liver tissues collected. As controls, three BALB-C female nude mice of the same age that had rejected transplants were sacrificed and their liver tissues collected. All liver tissues were frozen in CO_2 and delivered to this laboratory from the laboratory of G. Sato (Dept. of Biology, University of California, San Diego).

Chemicals

All organic solvents except ethyl ether were redistilled. Ethyl ether was used directly from a freshly opened can of reagent grade. Other reagents used were also of reagent grade. Standards for gas liquid chromatography were obtained from Nu-Chek-Prep (Elysian, MN) and Applied Science Laboratory (State College, PA).

Analyses

The liver specimens were weighed and thawed. The total lipid of each liver was extracted by the method of Folch et al. (7) and separated into phospholipid, triglyceride, and other fractions by thin layer chromtography on Silica Gel G using as developing solvent petroleum ether:ethyl ether:acetic acid (85:15:1 v/v). Lipid fractions were identified by exposure to iodine vapors and were marked and scraped off the plate. Each lipid fraction adsorbed on silica was subjected to methanolysis by heating 1 hr at 85 C with 5% HCl-methanol. The methyl esters of fatty acids thus obtained were extracted twice with 10% ethyl ether in pentane.

Quantitation of the lipids was performed by addition of a known amount of methyl margarate to the methyl esters of the fatty acids of each lipid fraction obtained as described above, followed by chromatography using a Beckman Gas Chromatograph GC-M with a column packed with 10% diethyleneglycol succinate on Chromosorb W. The phospholipid fatty acid content and the triglyceride fatty acid content of the liver tissues are presented in Table I as mg of methyl esters of fatty acids per wet wt of liver tissue.

RESULTS AND DISCUSSION

As shown in Table I, the phospholipid fatty acid content (as methyl ester) of livers of nude mice bearing transplanted human ovarian cancerous tissues averaged 13.7 mg/g liver, definitely greater than that of control mice (9.2 mg/g liver, average). Conversely, the triglyceride fatty acid content of liver tissues of tumor bearing mice averaged 1.1 mg/g liver, significantly ($P<0.05$) lower than that of control nude mice (3.7 mg/g liver, average). When the PL:TG ratio of liver lipids was calculated in each case, it was found to distribute between 7.6 and 33.5 in tumor bearing nude mice and between 1.7 and 3.8 in controls.

In this study, the human ovarian adenocar-

cinoma cells used were from the second passage in the nude mice. However, there were no marked histological changes between the first and second transplantations and mucin producing ability was retained.

From these very preliminary studies, it appears that the nude mouse with transplanted human cancerous tissues may represent a useful model for the study of themechanism of the lipid changes seen in liver tissues of human patients.

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Inhibition of Cholesterol Absorption by (-)N-[α-Phenyl-β-(p-tolyl) ethyl] Linoleamide in Rats

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ABSTRACT

The effect of (-)N-[α-phenyl-β-(p-tolyl)ethyl] linoleamide (PTLA) on intestinal absorption of cholesterol was studied in rats. Oral administration of 15 mg PTLA to rats resulted in a significant ($P<0.05$) decrease in the radioactivity in serum and liver 4 hr after administration of labeled cholesterol. The effect of PTLA was greater on the absorption of cholesteryl oleate as compared with free cholesterol. The rate of hydrolysis of cholesteryl oleate in mucosal homogenates of rat intestine was decreased with PTLA, suggesting that the inhibition of cholesterol absorption by PTLA is related to its effect on cholesteryl ester hydrolysis in the intestine.

INTRODUCTION

(-)N-[α-Phenyl-β-(p-tolyl)ethyl] linoleamide (PTLA) has been reported to have the most potent lipid-lowering activity in cholesterol fed animals among the compounds of linoleamide series (1,2). Linoleamides are thought to affect lipid metabolism by a different mechanism from that of linoleic acid and other natural oils (3,4). Previous investigations on the mode of action of linoleamides have dealt almost exclusively with inhibition of lymphatic absorption of cholesterol in thoracic duct fistula rats (5,6). The present investigation was designed to confirm that PTLA inhibits cholesterol absorption in intact rats and to further study how PTLA interferes with cholesterol absorption.

MATERIALS AND METHODS

Compounds

Cholesterol-4-^{14}C, cholesterol-1-2-^{3}H, and cholesteryl oleate-^{14}C (oleic acid-1-^{14}C) were obtained from Daiichi Pure Chemicals (Tokyo, Japan) and checked for their radiochemical purity by thin layer chromatography before use. Cholesteryl oleate-^{3}H (cholesterol-1-2-^{3}H) was prepared enzymatically according to the method of Swell and Treadwell (7) and purified by silicic acid column chromatography. PTLA was prepared in this Research and Development Center (Sumitomo Chemical Co. Ltd.).

Effect of PTLA on Cholesterol Absorption

Male Wistar rats, weighing 200-250 g, were used after fasting overnight. Test emulsions as described in footnotes to the tables were prepared in a Virtis type 45 homogenizer just before administration. The emulsion was given to rats by stomach tube. PTLA was suspended in water with gelatine and administered by stomach tube (15 mg/animal) immediately after the test emulsion was given. A control group

TABLE I

Effect of PTLA[a] on Levels of Radioactivity in Serum, Liver, and Intestine and Its Contents in Rats Given Cholesterol-1-2-^{3}H[b]

	Number of animals	Radioactivity[c] Serum (x10^{3}dpm/ml)	Liver (x10^{4}dpm/g)	Intestine and its contents (x10^{6}dpm)
Control	8	5.55 ± 1.00[d]	4.10 ± 0.39	2.52 ± 0.19
PTLA (15 mg/animal)	8	1.78 ± 0.15	1.61 ± 0.15	2.85 ± 0.14
P		0.01	0.01	NS[e]

[a]PTLA = (-)N-[α-phenyl-β-(p-tolyl)ethyl] linoleamide.

[b]The emulsion for administration contained 5 mg cholesterol-1-2-^{3}H, 14 mg oleic acid, 68 mg sodium taurocholate, and 10 mg bovine serum albumin in 1 ml of 0.9% saline.

[c]4 hr after administration.

[d]Values are means ± standard errors.

[e]Statistically not significant.

TABLE II

Effect of PTLA[a] on Levels of Radioactivity in Serum and Liver of Rats Given Cholesterol-4-^{14}C and Cholesteryl Oleate (Cholesterol-1-2-^{3}H)[b]

	Number of animals	Radioactivity[c] Serum (x10^{3}dpm/ml) ^{14}C	^{3}H	^{3}H:^{14}C	Liver (x10^{4}dpm/g) ^{14}C	^{3}H	^{3}H:^{14}C
Control	6	2.37 ± 0.45[d]	9.10 ± 2.19	3.84	2.08 ± 0.43	7.95 ± 1.97	3.82
PTLA (15 mg/animal)	7	1.27 ± 0.19	2.50 ± 0.42	1.97	0.57 ± 0.05	1.31 ± 0.18	2.30
P		0.05	0.01		0.01	0.01	

[a]PTLA = (-)N-[α-phenyl-β-(p-tolyl)ethyl] linoleamide.

[b]The emulsion for administration contained 5 mg cholesterol-4-^{14}C, 9 mg cholesteryl oleate (cholesterol-1-2-^{3}H), 14 mg oleic acid, 68 mg sodium taurocholate, and 10 mg bovine serum albumin in 1 ml of 0.9% saline.

[c]4 hr after administration.

[d]Values are means ± standard errors.

received a gelatine solution instead. The rats were killed 4 hr after administration by venipuncture under ether anesthesia, and the liver was excised.

Cholesterol Esterase Studies

Mucosa of rat intestine was scraped off with a razor blade and 4 vol ice cold 0.9% saline was added. It was then homogenized in a Teflon-on-glass homogenizer and centrifuged at 4 C (9000 x g, 15 min). The supernatant was used for both esterification and hydrolysis studies.

Reaction mixtures for esterification studies contained 131 mg oleic acid, 75-600 mg sodium taurocholate, 54 mg ammonium chloride, and 15 mg bovine serum albumin in 15 ml of 0.154 M phosphate buffer, pH 6.2, according to the method of Borja et al. (8). Cholesterol-4-^{14}C (1 μc, 10 mg) was dissolved in 1.0 ml acetone with or without PTLA (10 mg), and 0.05 ml aliquots were added to 0.5 ml of the reaction mixtures. To each reaction mixture, 0.5 ml of the supernatant of the mucosal homogenates was added. Incubations were done for 1 hr at 37 C and stopped by addition of 20 ml of acetone:ethanol, 1:1. The mixture was then heated 5 min in a water bath (90 C) and filtered. The extract was evaporated and applied to a thin layer silicic acid plate (Silica Gel H, E. Merck, Darmstadt, West Germany) for separation of free and esterified cholesterol with a solvent system of petroleum ether:ethyl ether, 4:1.

For hydrolysis studies, the reaction mixture contained 17 mg sodium taurocholate and 18 mg lecithin in 6 ml of 0.154 M phosphate buffer, pH 6.6. Cholesteryl oleate-^{14}C (1 μc, 18 mg) and PTLA (10 mg) were added to the reaction mixture as in the esterification studies. Incubations were done for 5, 10, and 15 min at 37 C. Other procedures were the same as in the esterification studies.

Determination of Radioactivity

Radioactivity in serum was extracted with 20 vol acetone:ethanol, 1:1, by heating 5 min at 90 C. Fresh liver (5 g) was excised and ground with 25-30 g anhydrous sodium sulfate and extracted with chloroform. Aliquots of the extracts were assayed for radioactivity. Radioactive spots on the thin layer plates were scraped and put into counting vials, and a scintillator solution (4 g of 2,5-diphenyloxazol and 0.2 g of 1,4-bis[2-(5-phenyloxazolyl)]-benzene per liter of toluene:ethanol, 7:3) was added. A Packard liquid scintillation spectrometer (Model 3375) was used for determination of radioactivity, and quench correction was made by external standard method.

RESULTS

Effect of PTLA on Cholesterol Absorption

Rats were given emulsions containing cholesterol-1-2-^{3}H by stomach tube, and the influence of PTLA was studied. As shown in Table I, coadministration of PTLA resulted in a fall of serum and liver radioactivity to ca. one-third of the control. To examine the effect of PTLA on the absorption of esterified cholesterol, the following experiment was performed. Rats were given emulsions containing both cholesterol-^{14}C and cholesteryl oleate-^{3}H (cholesterol-1-2-^{3}H), and the radioactivity of ^{14}C and ^{3}H in serum and liver was determined (Table II). The ^{3}H:^{14}C ratio in the test emulsion was 5.0. In the control group, ^{3}H:^{14}C ratios in both serum and liver decreased to 3.8, indicating that the rate of ^{3}H absorption was ca. 75% that of ^{14}C. Administration of PTLA reduced both levels of ^{3}H and ^{14}C, and ^{3}H:^{14}C ratios were further reduced to ca. 40% of the ratio in the test emul-

sion. The effect of PTLA appeared to be more remarkable on the absorption of esterified cholesterol than free cholesterol.

Effect on Cholesterol Esterase

The effect of PTLA on esterification of cholesterol at various concentrations of taurocholate is shown in Figure 1. Maximal activity for esterification was observed at the taurocholate concentration of 7.5 mg/ml, and esterifying activity was lowered at concentrations >7.5 mg/ml. PTLA appeared to stimulate esterification of cholesterol, and the percentage of stimulation increased with the concentration of taurocholate. The apparent stimulation of esterification by PTLA increased by ca. 120% at a taurocholate concentration of 20 mg/ml. On the other hand, PTLA showed inhibition of hydrolysis of cholesteryl oleate (Fig. 2), suggesting that the apparent stimulation of esterification was caused by inhibition of hydrolysis of the ester.

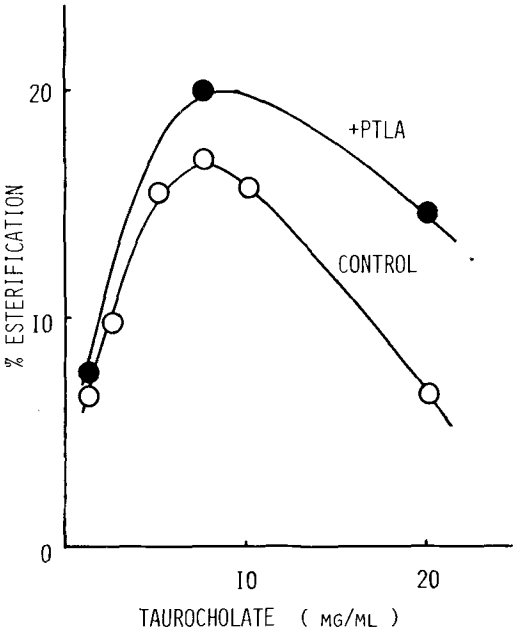

FIG. 1. Effect of (-)N-[α-phenyl-β-(p-tolyl)ethyl] linoleamide (PTLA) on esterification of cholesterol. Cholesterol-14C (0.5 mg) and PTLA (0.5 mg) were added to 0.5 ml of the reaction mixture and incubated 1 hr at 37 C.

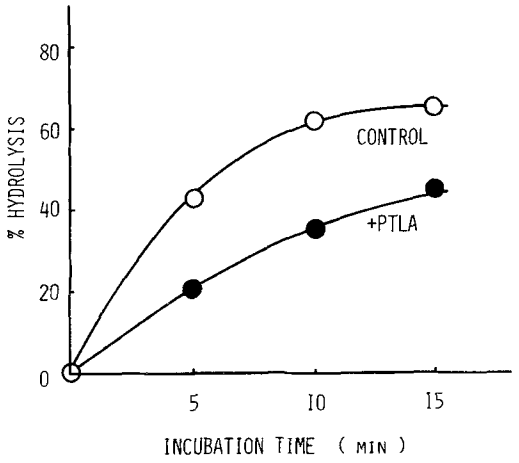

FIG. 2. Effect of (-)N-[α-phenyl-β-(p-tolyl)ethyl] linoleamide (PTLA) on hydrolysis of cholesteryl oleate. Cholesteryl oleate (oleic acid-14C) (0.5 mg) and PTLA (0.5 mg) were added to 0.5 ml of the reaction mixture and incubated at 37 C.

DISCUSSION

The results obtained in this study indicate that PTLA inhibits absorption of cholesterol from intestine and that the inhibition is related to the effect of PTLA on esterification and hydrolysis of cholesteryl esters. It is generally approved that a large portion of cholesterol which is taken up into mucosa as free cholesterol is esterified just before entering intestinal lymph. The finding that PTLA was more effective on the absorption of esterified cholesterol as compared with free cholesterol suggested that PTLA inhibited hydrolysis of cholesteryl oleate in the intestinal lumen. Inhibition of hydrolysis in the lumen, however, might not be the main mechanism of action, because PTLA also affects absorption of free cholesterol. A possible mechanism of action of PTLA that the compound acts on the esterification that occurs in the final step before cholesterol enters the intestinal lymph might not be acceptable, because PTLA is stimulative to the ester formation which seems to be favorable to cholesterol absorption. It has been reported by David et al. (9) that the intestinal mucosal cell of rats has a cholesterol esterifying enzyme on the inside of the brush border. If we assume that PTLA inhibits cholesterol absorption by affecting cholesterol esterification or hydrolysis of cholesteryl esters, there remains one more possibility that another step of esterification and hydrolysis is included in the transfer of cholesterol from lumen to lymph.

On the other hand, it is possible to interpret the present results from the point of view of chemical structures. PTLA is a relatively bulky molecule with a long chain fatty acid, and its conformation is rather similar to long chain fatty acid esters of cholesterol. It may be reasonable to consider that PTLA acts as a competitor with cholesteryl esters when the esters

are incorporated into lipoproteins. In this case, the effect of PTLA on the cholesterol esterase is rather secondary and is considered to support the view that PTLA competes with cholesteryl esters as a structural analog.

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Effect of (-)N-[α-Phenyl-β-(p-tolyl)ethyl] Linoleamide on Lipid Levels in Serum and Liver in Cholesterol-fed Rats

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ABSTRACT

The effect of (-)N-[α-phenyl-β-(p-tolyl) ethyl] linoleamide (PTLA) on lipid levels in serum and liver was compared with that of sitosterols in rats maintained on a diet supplemented with 1% of cholesterol, 0.5% of ox bile extracts, and 10% of hydrogenated coconut oil for 8 weeks. When PTLA was added to the diet at a level of 0.1%, the mean liver cholesterol level of male rats was reduced to 41% of the control and that of female rats was reduced to 19% of the control. In female rats, which showed higher cholesterol levels in serum and liver than male rats after cholesterol feeding, PTLA lowered the liver cholesterol level even at 0.0008% in the diet. Serum cholesterol was lowered by PTLA but not so markedly as liver cholesterol. The inhibition of cholesterol deposition in the liver suggests that the interference with cholesterol absorption is one of the main actions of PTLA. Sitosterols showed a similar pattern of lipid-lowering action, but the potency was far less than that of PTLA.

INTRODUCTION

We have reported that N-(α-methylbenzyl) linoleamide (MBLA) and N-cyclohexyl linoleamide (CHLA) showed remarkable lipid lowering effects in cholesterol fed rats, and the estimated potency of MBLA was about 6.5-fold of that of CHLA with respect to plasma-liver cholesterol pools (1). (-)N-[α-Phenyl-β-(p-tolyl)-ethyl] linoleamide (PTLA) was shown to more effectively reduce serum cholesterol levels and severity of atherosclerosis in rabbits maintained on a cholesterol diet than MBLA (2). In the present study, the influences of PTLA on lipid levels in serum and liver of cholesterol fed rats were investigated and compared with that of sitosterols.

MATERIALS AND METHODS

Test Compounds

PTLA was prepared in this Research and Development Center. The physical constants and analytical data were the same as in a previous report (2). Microcapsules of PTLA coated with gelatine were used in this experiment. Sitosterols (National Formulary) were obtained from Eli Lilly and Co. (Indianapolis, IN).

Animals and Feeding

Male and female SPF rats of Sprague-Dawley strain, aged ca. 8 weeks, were used. Body wt at the beginning of the experiment was ca. 270 g in the male rats and ca. 210 g in the female rats.

The composition of a high cholesterol diet was as follows (g/100 g): powdered commercial diet (CA-1, CLEA, Japan) 88.5 g; cholesterol, 1.0 g; ox bile extract, 0.5 g; and hydrogenated coconut oil, 10.0 g. Test compounds were added to the high cholesterol diet at the expense of the powdered diet.

Rats were maintained on a commercial diet for 4 weeks before the experiment, and those showing a normal increase in body wts were used. They were divided into six groups so that the distribution of body wts was comparable among the groups. Each group consisted of 8 male rats and 8 female rats. Another group of rats consisting of 8 males and 8 females was maintained on the normal commercial diet and served as normal control. The experiment was designed as follows: a control group of the cholesterol diet; groups treated with PTLA at 0.0008, 0.004, 0.02, and 0.1% in the cholesterol diet; a group treated with sitosterols at 1% in the cholesterol diet; and a normal diet group. The diet and water were supplied ad libitum. Body wts of the animals were measured once a week. At the 4th week of the experimental period, blood samples were taken from the tail vein through a heparinized syringe for analysis of plasma cholesterol. After 8 weeks the rats were killed by venipuncture and the livers were excised.

Lipid Analysis

Total cholesterol and triglycerides in plasma or serum were determined by the use of an Auto Analyzer (Technicon) (3,4). Lipid phosphorus was determined by Kings' molybdenum blue method (5). Phospholipid values are expressed as 25 times lipid phosphorus. Liver lipids were extracted as follows: 5 g of fresh liver were ground with 25 g of anhydrous sodium sulfate and extracted with chloroform.

TABLE I

Body Wt, Wt Gain, Liver Wt, and Liver:Body Wt Ratio[a] in Rats Fed a High Cholesterol Diet Supplemented with PTLA[b] or Sitosterols

Sex	Treatment	Final body wt (g)	Total wt gain (g/8 wk)	Liver wt (g)	Liver wt:body wt (%)
Male	Cholesterol control	545 ± 11	284 ± 9	23.0 ± 1.2	4.22 ± 0.16
	PTLA 0.0008%	548 ± 19	281 ± 16	23.2 ± 1.4	4.22 ± 0.16
	PTLA 0.004%	552 ± 14	273 ± 12	21.7 ± 1.0	3.93 ± 0.12
	PTLA 0.02%	547 ± 20	280 ± 18	19.4 ± 1.0[d]	3.55 ± 0.13[d]
	PTLA 0.1%	520 ± 20	255 ± 16	18.1 ± 0.7[d]	3.50 ± 0.11[d]
	Sitosterols 1%	540 ± 16	276 ± 13	20.9 ± 0.9	3.86 ± 0.13
	Normal diet	490 ± 16	[c]	17.5 ± 0.4[d]	3.59 ± 0.04[d]
Female	Cholesterol control	331 ± 13	118 ± 6	14.0 ± 0.7	4.23 ± 0.16
	PTLA 0.0008%	339 ± 13	128 ± 8	13.9 ± 1.0	4.09 ± 0.12
	PTLA 0.004%	323 ± 18	106 ± 11	12.3 ± 0.8	3.82 ± 0.10[d]
	PTLA 0.02%	309 ± 9	98 ± 5[d]	10.8 ± 0.4[d]	3.51 ± 0.07[d]
	PTLA 0.1%	311 ± 5	99 ± 5[d]	10.2 ± 0.3[d]	3.29 ± 0.13[d]
	Sitosterols 1%	327 ± 11	120 ± 7	11.9 ± 0.5[d]	3.63 ± 0.09[d]
	Normal diet	308 ± 7	[c]	10.6 ± 0.3[d]	3.46 ± 0.13[d]

[a]Values are means of 8 rats ± standard error.

[b]PTLA = (-)N-[α-phenyl-β-(p-tolyl)ethyl] linoleamide

[c]Not determined.

[d]Significantly different from cholesterol control ($P<0.05$).

Aliquots of the chloroform extract were used for lipid analysis. Total cholesterol was determined by the method of Herrman (6), free cholesterol by the method of Brown et al. (7), and esterified cholesterol was calculated from the amount of total cholesterol and free cholesterol. Triglycerides were determined by the method of Van Handel (8). Total lipids in the liver were estimated gravimetrically.

RESULTS

Body wts, body wt gain, and liver wts at the end of the experimental period are shown in Table I. The body wt gain of the female rats was depressed with 0.02 and 0.1% PTLA. The cholesterol fed rats showed an increase in the liver wt to body wt ratio compared with rats fed the normal diet. This increase was prevented by adding PTLA to the cholesterol diet.

TABLE II

Plasma Cholesterol Level[a] in Rats at the 4th Week of the Experimental Period[b]

Treatment	Plasma cholesterol (mg/100 ml)	
	Male	Female
Cholesterol control	121 ± 6	193 ± 28
PTLA[c] 0.0008%	133 ± 9	193 ± 17
PTLA 0.004%	128 ± 7	148 ± 16
PTLA 0.02%	100 ± 7[d]	110 ± 8[d]
PTLA 0.1%	105 ± 11	105 ± 6[d]
Sitosterols 1%	112 ± 8	122 ± 5[d]
Normal diet	57 ± 4[d]	82 ± 7[d]

[a]Values are means of 8 rats ± standard error.

[b]Rats were fed on a high cholesterol diet supplemented with PTLA or sitosterols.

[c]PTLA = (-)N-[α-phenyl-β-(p-tolyl)ethyl] linoleamide.

[d]Significantly different from cholesterol control ($P<0.05$).

TABLE III

Serum Lipid Levels[a] of Male Rats Fed a High Cholesterol Diet Supplemented with PTLA[b] or Sitosterols

Treatment	Cholesterol		Triglycerides (mg/100 ml)	Phospholipids (mg/100 ml)[c]
	Total (mg/100 ml)	Free (% of total)		
Cholesterol control	100.0 ± 6.9	11.6 ± 1.2	131 ± 16	119 ± 6
PTLA 0.0008 %	97.0 ± 6.2	13.5 ± 1.0	116 ± 14	119 ± 7
PTLA 0.004%	97.6 ± 4.7	12.8 ± 1.4	156 ± 9	129 ± 6
PTLA 0.02%	96.0 ± 7.1	11.4 ± 1.4	151 ± 7	120 ± 6
PTLA 0.1%	84.4 ± 6.2	11.1 ± 1.1	148 ± 13	115 ± 7
Sitosterols 1%	84.0 ± 6.5	13.2 ± 1.0	134 ± 12	135 ± 11
Normal diet	59.8 ± 3.2[d]	10.2 ± 1.1	153 ± 7	128 ± 6

[a]Values are means of 8 rats ± standard error.

[b]PTLA = (-)N-[α-phenyl-β-(p-tolyl)ethyl] linoleamide.

[c]Phospholipids values are given in lipid P x 25.

[d]Significantly different from cholesterol control ($P<0.05$).

TABLE IV

Liver Lipid Levels[a] of Male Rats Fed a High Cholesterol Diet Supplemented with PTLA[b] or Sitosterols

Treatment	Cholesterol		Triglycerides (g/100g)	Phospholipids (g/100g)[c]	Total lipids (g/100g)
	Total (g/100g)	Free (% of total)			
Cholesterol control	4.58 ± 0.20	8.1 ± 0.6	4.90 ± 0.25	2.87 ± 0.08	19.9 ± 1.0
PTLA 0.0008%	4.70 ± 0.23	7.7 ± 0.5	4.35 ± 0.28	2.72 ± 0.06	21.1 ± 1.3
PTLA 0.004%	4.00 ± 0.05[d]	7.3 ± 0.2	4.38 ± 0.06	2.90 ± 0.07	16.8 ± 0.7[d]
PTLA 0.02%	2.53 ± 0.22[d]	10.0 ± 1.2	3.58 ± 0.23[d]	3.10 ± 0.06[d]	11.7 ± 0.6
PTLA 0.1%	1.90 ± 0.22[d]	11.5 ± 1.2[d]	3.10 ± 0.33[d]	3.19 ± 0.04[d]	10.3 ± 0.7[d]
Sitosterols 1%	2.84 ± 0.28[d]	9.5 ± 0.9	4.28 ± 0.50	2.96 ± 0.10	13.5 ± 1.0[d]
Normal diet	0.30 ± 0.08[d]	60.7 ± 2.2[d]	0.48 ± 0.05[d]	2.83 ± 0.03	4.4 ± 0.1[d]

[a]Values are means of 8 rats ± standard error.

[b]PTLA = (-)N-[α-phenyl-β-(p-tolyl)ethyl] linoleamide.

[c]Phospholipids values are given in lipid P x 25.

[d]Significantly different from cholesterol control ($P<0.05$).

TABLE V

Serum Lipid Levels[a] of Female Rats Fed a High Cholesterol Diet Supplemented with PTLA[b] or Sitosterols

Treatment	Cholesterol		Triglycerides (mg/100 ml)	Phospholipids (mg/100 ml)[c]
	Total (mg/100 ml)	Free (% of total)		
Cholesterol control	129.9 ± 15.1	16.8 ± 1.2	92 ± 35	167 ± 12
PTLA 0.0008%	151.6 ± 8.1	18.1 ± 2.1	149 ± 32	207 ± 12[d]
PTLA 0.004%	120.6 ± 5.1	15.7 ± 0.9	151 ± 52	186 ± 8
PTLA 0.02%	96.8 ± 4.4[d]	9.8 ± 0.7[d]	94 ± 16	158 ± 10
PTLA 0.1%	94.3 ± 4.8[d]	10.4 ± 0.8[d]	73 ± 6	151 ± 9
Sitosterols 1%	114.1 ± 5.8[d]	15.7 ± 1.3	157 ± 14	221 ± 12[d]
Normal diet	92.6 ± 8.6[d]	8.5 ± 1.2[d]	70 ± 4	171 ± 13

[a]Values are means of 8 rats ± standard error.

[b]PTLA = (-)N-[α-phenyl-β-(p-tolyl)ethyl] linoleamide.

[c]Phospholipids values are given in lipid P x 25.

[d]Significantly different from cholesterol control ($P<0.05$).

TABLE VI

Liver Lipid Levels[a] of Female Rats Fed a High Cholesterol Diet Supplemented with PTLA[b] or Sitosterols

Treatment	Cholesterol		Triglycerides (g/100g)	Phospholipids (g/100g)[c]	Total lipids (g/100g)
	Total (g/100g)	Free (% of total)			
Cholesterol control	5.88 ± 0.54	6.3 ± 0.4	4.35 ± 0.43	2.66 ± 0.05	19.5 ± 1.1
PTLA 0.0008%	4.23 ± 0.42[d]	6.7 ± 0.5	3.56 ± 0.30	2.64 ± 0.06	15.1 ± 0.7[d]
PTLA 0.004%	3.18 ± 0.39[d]	10.1 ± 1.4[d]	3.12 ± 0.68	2.84 ± 0.05[d]	13.1 ± 1.4[d]
PTLA 0.02%	1.73 ± 0.42[d]	18.2 ± 3.5[d]	2.32 ± 0.48[d]	2.97 ± 0.04[d]	11.2 ± 1.3[d]
PTLA 0.1%	0.92 ± 0.21[d]	26.8 ± 4.7[d]	1.70 ± 0.25[d]	2.98 ± 0.06[d]	7.6 ± 0.7[d]
Sitosterols 1%	0.92 ± 0.06[d]	22.9 ± 1.8[d]	2.44 ± 0.28[d]	2.97 ± 0.06[d]	9.2 ± 0.6[d]
Normal diet	0.22 ± 0.01[d]	92.4 ± 2.0[d]	0.38 ± 0.09[d]	2.80 ± 0.10	4.4 ± 0.2[d]

[a]Values are means of 8 rats ± standard error.

[b]PTLA = (-)N-[α-phenyl-β-(p-tolyl)ethyl] linoleamide.

[c]Phospholipids values are given in lipid P x 25.

[d]Significantly different from cholesterol control ($P<0.05$).

Plasma cholesterol levels at the 4th week of the experimental period are shown in Table II. The plasma cholesterol in female rats was reduced to ca. 55% of the control with 0.1% PTLA and to ca. 63% of the control with 1% sitosterols, whereas the plasma cholesterol in male rats was not affected.

Lipid levels in serum and liver of male rats at the end of the experimental peiod are summarized in Tables III and IV. Serum levels of cholesterol, triglycerides, and phospholipids were not affected, but liver levels of total lipids and total cholesterol were reduced with PTLA at 0.004, and 0.02 and 0.1%, and with sitosterols at 1% in the diet. The liver triglycerides were reduced with PTLA at 0.02 and 0.1% and with sitosterols at 1% in the diet. The liver levels of phospholipids were increased with 0.02 and 0.1% PTLA, but the total amount in the liver was rather decreased.

Tables V and VI show the lipid levels in serum and liver of female rats. The serum levels of total cholesterol and phospholipids were ca. 1.5 times higher in the females than in the males, both in the control group and in the normal diet group. The influence of the addition of PTLA or sitosterols to the diet was more evident in the female rats than in the male rats; e.g., serum cholesterol, which was not affected in the males, was reduced with PTLA at 0.02 and 0.1% and with sitosterols at 1% in the diet. Further remarkable effects were found on the liver lipid levels of the female rats. The total lipids and total cholesterol were reduced with PTLA at all levels tested and with sitosterols at 1% in the diet. The liver level of total cholesterol was reduced to ca. 16% of the control with 0.1% PTLA, and the reduction of liver cholesterol by PTLA was statistically significant ($P<0.05$) even at 0.0008% in the diet. The ratio

TABLE VII

Serum and Liver Cholesterol Pool Sizes[a] in Rats Fed a High Cholesterol Diet Supplemented with PTLA[b] or Sitosterols

Sex	Treatment	Cholesterol pool size		
		Serum (mg)	Liver (mg)	Serum-Liver (mg)
Male	Cholesterol control	21.8 ± 1.7[c]	1056 ± 72	1078 ± 74
	PTLA 0.0008%	21.4 ± 1.8	1095 ± 99	1116 ± 100
	PTLA 0.004%	21.6 ± 1.3	867 ± 39	889 ± 40[d]
	PTLA 0.02%	20.6 ± 1.7	465 ± 46[d]	486 ± 47[d]
	PTLA 0.1%	17.6 ± 1.6	348 ± 49[d]	365 ± 50[d]
	Sitosterols 1%	19.7 ± 2.2	605 ± 81[d]	625 ± 82[d]
	Normal diet	11.8 ± 0.9[d]	53 ± 2[d]	65 ± 3[d]
Female	Cholesterol control	19.7 ± 2.0	822 ± 97	842 ± 99
	PTLA 0.0008%	20.8 ± 2.0	598 ± 79[d]	619 ± 81
	PTLA 0.004%	17.0 ± 1.5	395 ± 55[d]	412 ± 56[d]
	PTLA 0.02%	11.9 ± 1.7[d]	185 ± 46[d]	197 ± 46[d]
	PTLA 0.1%	11.7 ± 0.6[d]	93 ± 21[d]	105 ± 22[d]
	Sitosterols 1%	14.9 ± 0.9[d]	108 ± 8[d]	123 ± 8[d]
	Normal diet	11.4 ± 1.2[d]	23 ± 1[d]	35 ± 2[d]

[a]Serum pool size = body wt x 0.07 x 0.57 x (mg/ml); liver pool size = liver wt x (mg/g).

[b]PTLA = (-)N-[α-phenyl-β-(p-tolyl)ethyl] linoleamide.

[c]Values are means of 8 rats ± standard error.

[d]Significantly different from cholesterol control ($P<0.05$).

of free cholesterol to total cholesterol was increased with 0.004, 0.02 and 0.1% PTLA, and with 1% sitosterols. The liver triglycerides were reduced with PTLA at 0.02 and 0.1%, and with sitosterols at 1% in the diet.

A cholesterol pool size in an animal was calculated according to the method of Kritchevsky (9) and listed in Table VII. A dose related response in cholesterol lowering was evident.

DISCUSSION

One of the features of the cholesterol lowering effect of sitosterols is to prevent accumulation of cholesterol in the liver, and that is thought to be characteristic to drugs that inhibit cholesterol absorption. The similar pattern of action of PTLA suggests that the main mechanism of action of PTLA is the inhibition of cholesterol absorption as with other linoleamides (10,11). The present study showed that the estimated potency of PTLA was ca. 100-fold of that of sitosterols in male rats and ca. 10-fold in female rats. Such a great difference indicates that there might be some differences in the fine mechanism of action between PTLA and sitosterols.

Significant ($P<0.05$) sex differences were found in the effect of PTLA and sitosterols, namely, female rats were more sensitive to PTLA and sitosterols than male rats. Vahouny et al. (12) reported that, when rats were dosed with puromycin to inhibit protein synthesis in vivo, the influence on the lipid absorption in female rats was more significant than in male rats. In this connection, it would be interesting to study how lipid absorption is different between males and females.

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Effects of Pentadecan-2-one on the Growth of Cells in Culture

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ABSTRACT

The effect of inclusion of trace amounts of pentadecan-2-one in the incubation medium on the growth of HeLa Cells was evaluated by measuring viable cell counts (cells excluding trypan blue) and incorporation of [^{14}C] leucine into acid precipitable protein. Evidence is presented to show that exposure of the cells to trace amounts of the methyl ketone, 36μg/ml, effectively inhibits cell growth. This inhibition is relieved by simultaneously incubating the cells with a long chain primary alcohol, hexadecan-1-ol, but not with the secondary alcohol, pentadecan-2-ol. The observation that the ethyl ketone, hexadecan-3-one, also inhibits cell growth but at higher concentrations than that observed with pentadecan-2-one and that pentadecan-2-ol at similar concentrations has no effect on cell growth indicates that, for optimal effect, the keto function must be at the 2-position. Inhibition of cell growth by pentadecan-2-one is not unique to HeLa cells, as suggested by the inhibitory effects of this lipid type on the growth of other malignant cell lines of human origin.

INTRODUCTION

Enzymes involved in the interconversion of free fatty acids, aldehydes, and alcohols have been recognized in several different tissues (1-7). Although long chain alcohols are present only in trace amounts in mammalian tissues, they play an important biological role as the direct precursors of the fatty chain of the alkoxy glycerols (8,9). That alkoxy glycerols are lipids of unique importance in normal and abnormal cell growth is suggested by several observations. First, the alkoxy lipids comprise a considerable fraction of the phosphoglycerides from a number of mammalian tissues (5,10-12). Second, in the central nervous system, alkoxy glycerol biosynthesis occurs most rapidly at the time of myelination (13). Third, free long chain alcohols and alkoxy glycerols are present in increased amounts in numerous, but not all, malignancies (5,8,14). Finally, in Morris Hepatomas of various growth potential, alkoxy glycerol content is proportional to the growth rate of the tumor (15).

Previous studies have suggested that analogs of naturally occurring lipids may be useful chemotherapeutic agents. Alkoxy glycerols of nonphysiological chain length and aliphatic aldehydes and their derivatives were all found to be effective cytotoxic agents (16,17). Addition of 7-ketocholesterol and 7α and 7β-hydroxycholesterol to a lipid depleted incubation medium was found to markedly inhibit the growth of cultured cells (18,19). In this last instance, 3-hydroxy-3-methylglutaryl-CoA reductase appeared to be the enzyme involved, inasmuch as inclusion of mevalonate or cholesterol in the incubation medium with the above steroids relieved the inhibition. Recent results obtained in this laboratory indicate that another class of lipids, long chain methyl ketones, are competitive inhibitors of an aldehyde reductase and act to prevent the reduction of long chain aldehydes to alcohols when trace amounts are added to in vitro systems (20).

The purpose of this study was to determine if long chain methyl ketones present in the culture medium would inhibit cell growth as measured by the rate of incorporation of radiolabeled leucine into cell protein and by viable cell counts. Primary alcohols were added to the medium to assess whether or not the long chain alcohol could relieve this inhibition. The effect of ethyl ketones and secondary alcohols was also evaluated to determine if the methyl ketone was unique as a metabolic inhibitor.

EXPERIMENTAL PROCEDURES

Chemicals

All solvents, reagent grade, were redistilled before use. Silicic acid, 100 mesh, from Mallinkrodt (St. Louis, MO) was used in the column chromatographic procedures. Hexadecan-1-ol, Silica Gel G, and the solid support and liquid phases used for gas liquid chromatography (GLC) were obtained from Applied Science Laboratories, (State College, PA). Pentadecan-2-one, hexadecan-3-one, and pentadecan-2-ol were purchased from Pfaltz and Bauer, Inc. (College Point, Causeway, Flushing, NY) and DL-[1-^{14}C] leucine, 20 μCi/μmol from New England Nuclear (Boston, MA).

Assay Procedures

The procedures employed in silicic acid column, thin layer chromatography, (TLC) and GLC have been published (11). Reduction of the acyl groups of the phosphoglycerides to fatty alcohols and of pentadecan-2-one to pentadecan-2-ol was achieved by treatment of the lipids with $LiAlH_4$ (5). Pentadecan-2-one content of the incubation medium was determined by GLC (21).

Purication of Pentadecan-2-one

Silicic acid columns (18 g) were loaded with 540 mg of pentadecan-2-one dissolved in a small vol of n-heptane. Any lipid residue not initially applied to the silicic acid was washed onto the column with three 2 ml aliquots of n-heptane. The total n-heptane vol used was< 25 ml.

Development of the column was initiated with 250 ml of 1% ethyl ether in n-heptane because this developing solvent has been shown to effectively elute long chain aldehydes (22). Following concentration of this fraction under vacuum in a rotary evaporator, 35 C, it was observed that the wt of the material present in the eluate represented at least 90% of that applied to the column.

TLC of an aliquot of this fraction in two solvent systems (hexane:chloroform:methanol 73.5:25:1.5 and hexane:chloroform 75:25 v/v) indicated the presence of only one component which migrated as the pentadecan-2-one standard. GLC of another aliquot from this fraction indicated the presence of one major component, 99.3% of the total peak area, and two smaller components. The major component had the same retention time as the pentadecan-2-one standard.

A separate aliquot of the column eluate was treated with $LiAlH_4$ and the reduction product analyzed by TLC. Aliquots were analyzed on two separate chromatograms, one developed with hexane:chloroform:methanol (73.5:25:1.5) and the other with hexane:diethyl ether:acetic acid (30:70:1 v/v). In each instance, the lipid had the same chromatographic mobility as pentadecan-2-ol.

Resolution of a second aliquot of this compound by GLC indicated the presence of one compound having the same relative retention time as pentadecan-2-ol. Based on these observations, the pentadecan-2-one used in this study was chemically pure.

Hexadecan-1-ol, hexadecan-3-one, and pentadecan-2-ol were not purified before use because one component was observed on TLC, and GLC of these lipids indicated that one compound accounted for at least 98% of the total components detected in each sample.

Solubilization of Pentadecan-2-one

The methyl ketone was solubilized in calf serum by sonication for 15 sec intervals at 80 watts with 60 sec cooling intervals at 3 C, with a total sonication time of 120 sec. The solution was then centrifuged 20 min at 10,000 x g in a Sorvall RC-2B ultracentrifuge and filtered through Whatman No. 42 paper. Aliquots were removed from the solution for determination of ketone content by GLC (2). Solutions not water clear at the end of these procedures were clarified by adding more protein and repeating the above procedure. The ketone solution prepared in this manner was stable for 3 days.

Cell Culture

HeLa and KB cells were initially purchased from Flow Laboratories (Rockville, MD) and maintained in our laboratory; HEP-2-cells were obtained from Dr. W. Milligan III, Southern Illinois University (Edwardsville, IL); and human gingival cells (670 passage) were obtained from Dr. Smulow, Tufts University (Boston, MA). The cell stock cultures were grown in 32 oz prescription bottles containing Earles's cell culture medium (30 ml) to which was added 10% calf serum, 0.085% sodium bicarbonate, and antibiotics (penicillin, 100 units/ml; streptomycin, 100 μcg/ml and fungizone, 0.25 μcg/ml). The stock cultures were trypsinized, and equal aliquots of cells were grown in 16 x 125 mm screw cap tubes containing 1 ml of Earle's medium at 37 C for the inhibition studies. For lipid determinations, 2×10^7 cells were added to 300 ml of culture medium and grown in spinner cultures with three changes of medium for 8 days. At the end of this period, cells were harvested by centrifugation and washed three times with isotonic saline. Aliquots were taken for cell counts, and remaining cells were extracted for lipid analysis. Lipid extracts were analyzed for lipid phosphorous and neutral and phospholipid alkoxy glycerides (5,11,23).

Cell Counts

Cell monolayers were drained of growth medium, washed with 0.5 ml of trypsin (0.25%), drained, and incubated with 0.5 ml of fresh trypsin (0.25%) at 37 C for 15 min. Following incubation, these cell-trypsin containing tubes were mixed on a vortex for 20 sec, and 0.5 ml of trypan blue (0.05%) was added to the cell suspension. The number of unstained viable cells were counted in a hemocytometer (Spencer Bright, Line, improved Neubauer).

TABLE I

Effect of Incorporation of Selected Concentrations of Pentadecan-2-one into the Culture Medium on [^{14}C]-Leucine Uptake and Cell Counts of HeLa Cells after 24 hr Incubation Intervals[a]

Pentadecan-2-one (μg/ml)	Leucine uptake (dpm)	Cell count[b] x 10^4
0.0	19,930 ± 1050	19.3 ± 2.7
4.5	16,436 ± 831	19.4 ± 3.5
9.0	18,407 ± 465	13.7 ± 2.2
18.0	16,537 ± 301	11.0 ± 1.9
36.0	9,537 ± 350	7.8 ± 0.9
72.0	571 ± 19	0.0
144.0	503 ± 20	0.0

[a]Trypsinized cells (ca. 180,000/ml) were suspended in 0.9 ml Earle's medium containing pentadecan-2-one (μg/ml as indicated) and 0.1 ml DL-[1-^{14}C] leucine (0.1 μCi/ml) contained in 16 x 12.5 mm screwcap tubes. These continuously labeled systems were incubated 24 hr at 37 C. The values for leucine uptake are the average of eight individual observations ± the SD. HeLa cell plating efficiency ranged from 60 to 70% thus, the cell population in the untreated cultures nearly doubled in the 24 hr incubation interval.

[b]To obtain actual cell counts, all values must be multiplied by 10^4.

Radioisotope Incorporation

Incorporation of CL[1-^{14}C] leucine into acid precipitable material was also used to estimate cell growth in these culture systems. Identical cell cultures containing 0.1 μCi radiolabeled leucine in the medium were incubated for the time intervals noted in the text and then processed as follows: tubes in each group were drained and cell sheets were washed three times with 5% trichloroacetic acid (2 ml), drained again, and solubilized in 0.5 ml hydroxide of hyamine and 10 ml of scintillation solution (2,5 diphenyloxazole, 4 g; 1,4-bis-2- [4-methyl-5-phenyloxazolyl]-benzene, 200 mg; toluene, 950 ml; and absolute ethanol, 50 ml) prior to liquid scintillation counting. Radioactivity per sample was measured in a Tri-carb liquid scintillation counter, model 3320. Counting efficiency for ^{14}C was 89%.

RESULTS

Alkoxy Glycerol Content of HeLa Cells

Previous in vitro studies have indicated that, in cardiac muscle pentadecan-2-one acts as a competitive inhibitor of the aldehyde reductase to prevent reduction of a long chain aldehyde to a long chain alcohol (20). If pentadecan-2-one has a metabolic effect in cell culture, one would predict that the cells concerned must contain alkoxy phospholipids, inasmuch as long chain alcohols are known to be uniquely involved in the biosynthesis of this lipid type (8,9). The alkoxy glycerol content of HeLa cells was first determined by chemical analysis of two separate groups of cells (0.6 and 1.5 x 10^9 cells) grown in spinner culture and harvested by centrifugation. The concentration of the alk-1-enyl acyl phosphoglycerides was 5.6 and 7.9 μmol per 100 μmol of lipid phosphorous and the alkyl acyl phosphoglycerides 11.0 and 10.5 μmol per 100 μmol lipid phosphorous. The total alkoxy glycerol content, alkyl and alk-1-enyl, was quantitated and found to be 1.5 and 2.1 μmol per 100 μmol of lipid phosphorous. No attempt was made to measure the free long chain aldehyde or long chain alcohol content of these cells with the small amount of lipid available. From the results of these analyses, it is apparent that the alkoxy lipids are not trace components, especially in the phosphoglyceride fraction from HeLa cells.

Effect of Pentadecan-2-one on Cell Growth

To evaluate the effect of a long chain methyl ketone on the growth of HeLa cells, selected concentrations of pentadecan-2-one ranging from 4.5 to 144 μg/ml were added to the culture medium. The results of these experiments are presented in Table I. Cell growth, as determined by measurement of [1-^{14}C] leucine incorporation or viable cell counts, showed a progressive decrease as the concentration of ketone increased. At low concentrations of ketone (≤18 μg/ml), however, the observed incorporation of [1-^{14}C] leucine did not consistently correspond with the decrease in viable cells. This type of discrepancy has been noted by others in studies with metabolic inhibitors (17). A ketone concentration of 36 μg/ml was chosen for future studies because at this level ca. 50% decrease was observed in either assay parameter.

Apparently, qualitatively similar results are obtained whether assessment of cell growth is

TABLE II

Effect of Pentadecan-2-one on Incorporation of [1-^{14}C] Leucine into Protein by Cultured HeLa Cells during Their Growth Cycle[a]

Incubation interval (hr)	Leucine uptake (dpm)	
	Control	Pentadecan-2-one
3	1,505 ± 127	1,460 ± 85
6	3,409 ± 148	2,233 ± 55
12	7,487 ± 333	3,631 ± 215
24	12,021 ± 660	4,456 ± 279
36	19,012 ± 831	7,510 ± 572
48	20,693 ± 1,294	5,800 ± 893
60	25,632 ± 544	3,444 ± 359
72	30,836 ± 8 820	1,538 ± 93

[a]Cells were synchronized by serum depletion, trypsinized, and suspended in 0.9 ml Earle's medium with and without pentadecan-2-one (36 μg/ml). [1-^{14}C] 0.1 ml, 0.1 μCi/ml) was also added to the 16 x 125 mm screwcap tubes (2.4 x 10^5 cells/tube). Samples were collected at selected times from 3 to 72 hr post incubation at 37 C and the amount of [1-^{14}C] leucine present determined by liquid scintillation counting. All values are the average of five determinations ± the standard deviation.

TABLE III

Effect of Pentadecan-2-one Removal on DL [1-^{14}C] Leucine Incorporation by HeLa Cells[a]

Hours[b]	Leucine uptake (dpm)	
	Control	Pentadecan-2-one
3	312 ± 25	147 ± 45
6	3,199 ± 620	721 ± 48
30	23,487 ± 695	2,379 ± 215
54	31,405 ± 1,101	750 ± 46

[a]Trypsinized HeLa Cells, 1.8 x 10^5 cells, were dispensed in 16 x 125 mm tubes containing pentadecan-2-one (36 μg/ml). Similar numbers of control cells were incubated in an analogous manner, except that the ketone was omitted from the medium. After 18 hr, the cultures were rinsed three times with fresh ketone free medium and then incubated with DL [1-^{14}C] leucine (0.1 μCi/ml). The culture tubes were processed 21, 24, 48, and 72 hr after initiation of the experiment. Radioactivity present was evaluated as described before. All values are the average of five determinations ± SD.

[b]Incubation interval in hr after ketone removal.

based on the measurement of viable cell counts or the incorporation of radiolabeled leucine into cell protein. Because it is mechanistically easier to measure the incorporation of radiolabeled leucine into cell protein, this method was used to assess cell growth in later experiments.

Cell plating efficiency (number of cells present 6 hr after plating) ranged from 60 to 70%; similar decreases have been previously noted (18,24). In the following 18 hr, cell population in the untreated cultures increased from an average of 11.7 x 10^4 to 19.3 x 10^4 cells (Table I). The results of a typical experiment in which cell growth was studied over a prolonged interval are presented in Table II. In the untreated cells, growth as estimated by leucine incorporation into protein was linear over the 72 hr incubation interval. In the ketone treated cells, leucine incorporation was linear for the first 36 hr of incubation but at the end of this period the amount of radioactivity present was only 37% of that observed with the untreated cells. During the final 36 hr of the 72 hr interval, there was a progressive decrease in the amount of leucine incorporated by the treated cells, indicating that cells previously present were dying.

Statistical analysis of this data using Scheffe's method of multiple contrasts (25) indicated that, for each time point after the 6 hr incubation interval, the mean value for the ketone treated group was significantly ($P<0.01$) different from that of the corresponding control.

The possibility that cells treated with pentadecan-2-one could recover from its inhibitory effects was evaluated by treating cells with the methyl ketone for 18 hr. At the end of this time interval, the cells were washed free of the ketone and incubated with fresh medium containing [1-^{14}C] leucine for an additional 54 hr to yield a total incubation interval of 72 hr.

TABLE IV

Comparison of the Effect of Long Chain Methyl Ketones, Fatty Alcohols, and a Combination of These Lipids on Utilization of [1-^{14}C] Leucine by HeLa Cells[a]

Lipid addition	Concentrations (μg/ml)	Leucine uptake (dpm)
None		20,377 ± 1,740
Pentadecan-2-one	18.0	11,633 ± 1,080
	36.0	1,745 ± 896
Pentadecan-2-ol	18.0	21,984 ± 1,214
	36.0	18,059 ± 1,058
Hexadecan-1-ol	18.0	18,510 ± 1,184
	36.0	16,239 ± 2,064
Pentadecan-2-one plus hexadecan-1-ol	18.0 18.0	18,237 ± 3,241
Hexadecan-3-one	18.0	16,271 ± 1,400
	36.0	10,447 ± 2,477

[a]Cells were cultured under conditions identical to those described in Table I with the same addition of CL [1-^{14}C] leucine. Individual lipids or combinations were added in Earle's medium at the concentrations noted below. The values for leucine uptake are the average of eight individual observations ± the SD.

Corresponding aliquots of control cells were incubated 18 hr and treated thereafter in the same fashion as the cells in the experimental group (Table III). Leucine was teken up at a rapid and essentially linear rate by the untreated cells over the 54 hr incubation interval. The cells treated with ketone also incorporated leucine into protein, but the extent of incorporation was considerably less.

The possibility that the effect of pentadecan-2-one on the utilization of leucine was unique to HeLa cells was evaluated by incubating other malignant cell types—KB, Hep-2, and gingival cells—with pentadecan-2-one, 36 μg/ml medium, for 18 hr at 37 C. At the end of this incubation interval, the cells were harvested and the effect of this treatment on cell growth assessed by viable cell counts and the capacity of the cells to utilize radiolabeled leucine. In three separate experiments, treatment with the methyl ketone resulted in an average decrease of 35-55% in the utilization of leucine [1-^{14}C] and 20-25% decrease in viable cell counts, depending on the individual cell types studied.

Effect of Hexadecan-1-ol, Pentadecan-2-one, and Related Lipids on Cell Growth

If the long chain methyl ketones act to inhibit cell growth by restricting the availability of long chain primary alcohols and thus affecting the biosynthesis of the alkoxy acyl phosphoglycerides of cells, one would predict that inclusion of a long chain primary alcohol in the incubation medium along with the methyl ketone would relieve the inhibition of cell growth seen in the presence of the methyl ketone alone. Table IV presents the results of studies concerning the effect of inclusion of pentadecan-2-one, hexadecan-1-ol, and a combination of the methyl ketone and primary alcohol in the incuabation medium on the growth of cultured HeLa cells. Analysis of this data by Scheffe's method of multiple contracts (25) indicated that addition of pentadecan-2-one to the culture medium at concentrations of 18-36 μg/ml significantly inhibits the utilization of [1-^{14}C]leucine ($P<0.01$ for each concentration). Addition of hexadecan-1-ol resulted in no statistically significant differences in the values observed for the utilization of [1-^{14}C] leucine as compared to the control (25). However, when hexadecan-1-ol was added to the incubation medium in combination with equal amounts of pentadecan-2-one, the inhibitory effect of the ketone of leucine utilization was reversed. This result is what would be expected if the methyl ketone is acting to inhibit the biosynthesis of long chain alcohols in the cells.

Evidence that pentadecan-2-one or hexadecan-1-ol did not undergo chemical modification during the incubation interval was obtained by the analysis of equal vols of treated and untreated medium after a 24 hr incubation period. In two instances, lipid extracts of the treated medium contained two additional components, which were identified as pentadecan-2-one and hexadecan-1-ol by TLC and GLC.

To determine that the methyl ketone was the active species, the expected biological reduction product, pentadecan-2-ol, was added to the medium. HeLa cells were incubated under the same conditions as before and the

effect of this lipid type on the utilization of [1-^{14}C- leucine evaluated (Table IV). No significant difference was observed in the utilization of the radiolabeled amino acid by the cells treated with secondary alcohol as compared to the untreated controls.

The effect of the ethyl ketone on the utilization of [1-^{14}C] leucine by cultured cells was evaluated by the addition of hexadecan-3-one to the incubation medium. In this instance, hexadecan-3-one was not as effective an inhibitor of leucine utilization as pentadecan-2-one. An ethyl ketone concentration of 36μg/ml was required to produce the same effect on leucine utilization as that observed with the methyl ketone at a concentration of 18μg/ml. The degree of inhibition of leucine utilization at an ethyl ketone concentration of 36.0 μg/ml was significantly ($P<0.01$) different from that observed with the untreated cells. Evidence of this type indicates that, for optimal biological activity, the metabolic inhibitor must contain a keto function at the 2-position of the fatty chain.

DISCUSSION

Recent in vitro studies have indicated that pentadecan-2-one is an effective competitive inhibitor of the long chain aldehyde reductase of cardiac muscle (20). Although we have not demonstrated that a similar inhibition occurs here upon exposure of HeLa cells to the methyl ketone, it has been shown that inclusion of trace amounts of this lipid type, 36 μg/ml in the incubation medium, produces a marked inhibition of cell growth. This inhibitory effect is apparent whether growth is assessed by viable cell counts (cells excluding trypan blue) or the ability of cells to utilize radiolabeled leucine. The effect is considered irreversible because, after exposure to the methyl ketone for 18 hr, incubation of the exposed cells in a fresh, ketone free medium did not relieve the inability of the cells to utilize radiolabeled leucine.

Although we have not demonstrated that the site of action of the methyl ketone is intracellular, this possibility seems likely inasmuch as the inhibitory effect on growth persisted in the exposed cells 54 hr after removal of the methyl ketone from the culture medium. Another observation in support of this conclusion is that inhibition of cell growth, by ketone treated cells, could be relieved by the simultaneous addition of a long chain primary alcohol to the incubation medium. Previous studies have shown that long chain alcohols passively diffuse across cell membranes (26). This inhibitory effect is not unique to HeLa cells. Similar results were obtained when other cell lines–KB, Hep-2, and gingival cells–were exposed to this lipid during incubation.

The biological mechanism by which the methyl ketone is acting to inhibit cell growth has not been defined in this study. However, we propose that, if the mechanism of biosynthesis of the long chain alcohol is by reduction of the free fatty acid or long chain acyl CoA through a long chain aldehyde to the corresponding alcohol, inhibition of the aldehyde reductase could lead to an abolition of the long chain alcohol pool. Because previous studies have demonstrated that long chain alcohols are one of the substrates involved in the biosynthesis of the alkoxy glycerols (8,9), inhibition of fatty alcohol biosynthesis could have pronounced physiological effects if alkoxy glycerols are of physiological importance in the cell.

Depletion of the alkoxy glycerol contant could be fatal to the cell because alkoxy acylphosphoglycerides account for a major proportion of the total phospholipids of the mitochondria and microsomes of cardiac muscle (12), as well as some isolated mitochondrial enzymes (10).

Accumulation of long chain aldehydes within the cell can also inhibit cell growth. Aldehydes are effective in vitro cytotoxic agents at concentrations ca. 10 times greater than those employed here for the methyl ketone (16). In this instance, it was demonstrated that the aldehyde condenses in vivo with the amino acid cysteine to yield a thiazolidine carboxylic acid. It was proposed that this effect resulted in a change in protein synthesis due to an imbalance of the amino acid pool within the cell (16). Use of methyl ketones as inhibitors of the aldehyde reductase of cells should also lead to intracellular accumulation of aldehydes and formation of a condensation product analogous to that mentioned above.

In this study, we have used the incorporation of radiolabeled leucine into acid precipitable proteins as a measure of cell growth. We wish to emphasize that we are not proposing that long chain methyl ketones have a direct effect on protein synthesis. We are suggesting that exposure of the cell to trace amounts of methyl ketone causes an error in lipid metabolism which results in a decrease in the number of cell present. It is the decrease in the number of viable cells that accounts for the change observed in the incorporation of radio-leucine into protein.

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Rat Testicular Lipids and Dietary Isomeric Fatty Acids in Essential Fatty Acid Deficiency

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ABSTRACT

Weanling rats were fed essential fatty acid-deficient diets, either completely fat-free, or with partially hydrogenated fish oil (PHFO, 28 wt %), or with fractions derived from PHFO containing primarily positional isomers of *trans*-eicosenoate (20:1, 3 wt %) or *trans*-docosenoate (22:1, 3 wt %). Control animals were fed a peanut oil-containing diet (28 wt %). After 5 or 15 weeks on the diet, the content of neutral and phosphorus-containing lipids in the testes was determined. The fatty acid distribution in major lipid classes was analyzed for animals fed the diets for 15 weeks. The testicular stage of maturation or degeneration was assessed by histology. The group fed PHFO exhibited signs of complete testicular degeneration, or lack of maturation, already after 5 weeks, whereas the animals on the diets with the very long chain monoenoic acids suffered severe degenerations only after 15 weeks. In the PHFO-fed rats, a sharp decline in the concentration of testicular triacylglycerols was observed. In all of the essential fatty acid-deficient groups, an increase in testicular sphingomyelin was observed. Cholesterol levels were fairly similar among all dietary groups. The total testicular fatty acids of the PHFO-fed animals contained somewhat more eicosadienoic acid than found in the other groups, and somewhat less (n-9)-acids. In all EFA-deficient groups, (n-6)-acids were lowered, in particular in triacylglycerols and phosphatidyl cholines. The PHFO group did not show a lower (n-6)-concentration than the other deficient groups, in spite of the more severe symptoms of deficiency. There was no evidence of a major accumulation of long chain isomeric fatty acids in the degenerated testes of the PHFO-, 20:1-, and 22:1-fed groups.

INTRODUCTION

Dietary partially hydrogenated oils exert a stressing effect on the symptoms of essential fatty acid (EFA)-deficiency in the rat (1-3). Recently, partially hydrogenated soybean oil, peanut oil, and herring oil were examined in this respect (4-6). Of these oils, the partially hydrogenated herring and peanut oils were found to enhance the severity of the degeneration of spermatogenic tissue when compared to the effect of a fat-free diet. This enhancement was most pronounced when the partially hydrogenated herring oil was fed. The composition of this oil was characterized in particular by a large amount of *trans*-isomers and by a large amount of monoenoic acids with chain lengths of 20 or 22 carbon atoms (5). The partially hydrogenated soybean oil, with a high content of unchanged linoleic acyls, kept the animals normal in all respects studied. In the present study, the effects were investigated of those fractions of a partially hydrogenated fish oil which clearly offset its fatty acid composition from that of other hydrogenated oils. Two major fractions containing mostly *trans*-eicosenoic and *trans*-docosenoic acids, respectively, were fed to male rats as a supplement to a fat-free diet. The effects of the dietary lipids were followed through wt and dermal scores, and finally by lipid analysis and histology of the testes. The effects were compared to those of the fat-free diet and of the unfractionated, partially hydrogenated fish oil.

EXPERIMENTAL PROCEDURES

Animals

Male weanling rats (21 days old) of the Wistar strain (K.Møllegaard-Hansens Avlslab., A/S, Ll.Skensved, Denmark) were started on the experimental diets in groups of 10 rats, divided into two subgroups of 5 rats, which were sacrificed after 5 and 15 weeks, respectively. The small size of the groups was necessitated by the limited availability of the fish oil fractions described in "Diets". The rats were housed individually in wire mesh cages kept at 25 C at a relative humidity of 35±5%. The animals had access to tap water ad libitum. The diets were given at 10 g per day for the first 4 weeks of the experiment, at 12 g per day for the 5th week, and at 15 g per day through the remaining 6-15th weeks. Diet leftovers and spills were weighed daily, and diet intake calculated. The animals in the two groups given fractions of partially hydrogenated fish oil

TABLE I

Fatty Acid Composition of Dietary Oils (Gas Liquid Chromatography – Area %)

Fatty acid	Peanut oil	Partially hydrogenated fish oil (PHFO)	20:1 fraction of PHFO	22:1 fraction of PHFO
14:0	--	7.1	0.3	5.0
16:0	11.2	19.0	0.4	7.6
:1	--	8.3	0.2	1.8
18:0	2.8	5.6	--	0.8
:1	36.4	17.3	--	2.1
:2	42.8	2.6	--	--
20:0	--	2.2	8.9	0.8
:1	--	11.6	75.1	--
:2	--	4.1	10.6	--
22:0	--	3.0	--	3.7
:1	--	12.3	--	71.1
:2	--	1.1	--	5.5
Others	6.8	4.1	4.7	1.7
Trans double bonds (mol %, by IR spectrophotometry	0	75	99	81
Conjugated dienes (wt %, by UV spectrophotometry)	--	--	0.6	0.4

consumed all of the available food from the 5th through the 15th week, whereas all the other animals in general had sufficient food in the daily allotments. The rats were handled weekly for weighing and inspection of dermal symptoms. The animals were killed with an overdose of chloroform vapor, and the organs for investigation were immediately removed, weighed, and quickly frozen on dry ice (for lipid analyses) or fixed in 4% formaldehyde (for histology).

Diets

The diets were based on a fat-free diet which contained (by wt): Vitamin Test Casein (Genatosan Ltd., Loughborough, England), 20%; sucrose, 74%; vitamin mixture (4), 0.5%; choline chloride, 0.5%; salt mixture, 5.0% [100 g of the salt mixture contained: NaCl, 6 g; $Mg(HCO_3)_2$, 5 g; KCl, 7 g; K_2HPO_4, 17 g; $NaH_2PO_4 \cdot 2\,H_2O$, 10 g; $Ca(H_2PO_4)_2 \cdot 2\,H_2O$, 17 g; Ca-lactate $\cdot 5\,H_2O$, 36 g; ferric citrate, 2 g. The following trace elements were added per 100 g of salt mixture: $Ca(IO_3)_2 \cdot 6\,H_2O$, 5.81 mg; zinc carbonate, basic (56% Zn), 72 mg; $MnCO_3$ (47.8% Mn), 42 mg; Cu-carbonate, basic (53% Cu), 19 mg; NaF, 40 mg; $NaMoO_4 \cdot 2\,H_2O$, 5 mg; $Cr_2(SO_4)_3 \cdot 15\,H_2O$, 1.3 mg; SeO_2, 0.32 mg. In total: 185.43 mg per 100 g of salt mixture]. In the fat-containing diets, dietary fat replaced a corresponding amount of sucrose by wt. Five different diets were fed: the basic fat-free diet (FF), 28% peanut oil (PO), 28% partially hydrogenated fish oil (PHFO), 3.0% 20:1 fraction derived from the PHFO (20:1), and 3.0% 22:1 fraction derived from the PHFO (22:1). The fatty acids

TABLE II

Positionally Isomeric Fatty Acids in Fractions from Partially Hydrogenated Fish Oil (Gas Liquid Chromatography – Area %)

Double-bond position	20:1 fraction	22:1 fraction
Total monoene	75.1	71.1
Δ7	4.1	2.1
Δ8	5.6	4.3
Δ9	9.0	4.6
Δ10	11.6	11.7
Δ11	23.3	29.2
Δ12	10.2	11.4
Δ13	8.3	5.0
Δ14	4.1	1.8
Δ15	n.d.	0.7

of the latter two fractions were fed as the ethyl esters. The fatty acid compositions of the dietary fats are given in Table I. It should be noted that the oils used as dietary fats in this study are not the same oils described in the work by Hølmer and Aaes-Jørgensen (5). The partially hydrogenated fish oil used in the present work was the source of the monoene fractions. These fractions were prepared by distillation of the ethyl esters of the oil. Fractions No. 10 and No. 15 of a total of 20 fractions consisted primarily of C_{20} and C_{22} acids, respectively, and were chosen for the feeding experiment. The distilled fractions were protected against oxidation with 0.01% butylated hydroxytoluene. All of the dietary lipid material was generously supplied by Aarhus Oliefabrik, Inc. (Aarhus, Denmark).

The position of the double bond in the monoenoic acids of the 20:1 and 22:1 fractions was determined by argentation chromatography

and ozonolysis-gas chromatography of the methyl esters of these acids. The methyl esters were prepared from the ethyl esters by transesterification with 5% (wt) hydrogen chloride in methanol, in an ampoule heated 1 hr at 95 C (7). Chromatography was performed on plates with 0.5 mm $AgNO_3$:Silica Gel G (3:97, w/w). The plates were developed twice in diethyl-ether:petroleum ether (bp 60-70 C), 10:90 by vol. The extreme ends of the separated bands were visualized with 2′,7′-dichlorofluoresceinspray, and the unsprayed part was scraped off the plate and extracted with diethyl ether. The methyl esters from the monoene band (a relatively wide band, probably because of the large variation in double bond positions) were converted to the ozonides by treatment at -60 C with ozone in pentane (8). The ozonides were analyzed by pyrolysis-gas chromatography (9), and the quantitative distribution of the different positional isomers in the original monoene fractions was calculated from the amounts of aldehydes as well as of aldehyde esters formed by the pyrolysis procedure. The two methods of calculation agreed within 2% (10). The distributions of positional isomers in the monoene fractions are given in Table II.

Tissue Lipid Analysis

After removal of the tunica albuginea, the testes were homogenized and extracted in a mechanical homogenizer (M.S.E. Ltd., London, England) using 20 vol chloroform:methanol (2:1, v/v). The extract was filtered on a Büchner funnel through solvent-washed filter paper. Filter paper and residue were rehomogenized and extracted with chloroform:methanol (2:1) using half of the vol applied in the initial extraction. The filtration and reextraction procedures were repeated once more, this time using chloroform:methanol (1:2, v/v) (11). The solvent composition of the combined extracts was adjusted, by addition of chloroform and distilled water, to the chloroform:methanol:water ratio of 16:8:1 obtained when using the extraction procedure of Folch et al. (12). The extract was washed with 0.9% aqueous sodium chloride and with "upper phase" (12). The organic and watery layers were finally separated after centrifuging at low temperature (2,000 rpm; 5 C). The washed extracts were concentrated in a rotary evaporator and the residue dissolved in 2.0 ml chloroform:methanol (2:1). At all stages, except during the extractions, the vessels containing lipid material were flushed with nitrogen.

The amounts of each of the neutral lipid classes were determined by thin-layer chromatography (TLC) in combination with charring-densitometry as described by Blank et al. (13). Prewashed Silica Gel G plates were used. On each plate, the sample was applied in quadruplicate, alternating with the standard neutral lipid mixture which was applied in a total of five standard applications of different concentrations. The plates were developed in 70:30:2 (by vol) petroleum ether:diethyl ether:acetic acid for analysis of free fatty acids and cholesterol; in 83:17:1 petroleum ether:diethyl ether:acetic acid for analysis of triacylglycerols, alkyl-diacyl-glycerols, methyl esters, and cholesterol esters. The plates were sprayed with concentrated sulfuric acid saturated with potassium dichromate and charred on a hot plate (180-200 C) for 45 min. Densitometry was performed on a Photovolt densitometer equipped with a supplementary stage for 20 cm x 20 cm plates. Peak areas were determined by cutting and weighing. Calibration curves were constructed for each plate. In some cases, it was necessary to analyze a lipid extract in two concentrations in order to place unknown concentrations within the range of the standard concentrations. Generally, the mass of the individual neutral lipids was calculated from standard curves based on the same lipid class. An exception was alkyl-diacyl-glycerols, which were calculated as triacylglycerols. The standards used in this procedure were cholesteryl oleate, methyl oleate, trioleoylglycerol, oleic acid, and cholesterol, purchased from the Lipids Preparation Laboratory of The Hormel Institute (Austin, MN). The phospholipids were separated by two-dimensional TLC according to Rouser et al. (14) and quantified by colorimetry. After localization of the spots by charring, the spots as well as blanks were scraped directly into reaction vials. To some of the blanks, aliquots of a standard solution of diphenylphosphate were added. All scrapings were digested and analyzed according to Bartlett's modification (15) of the Fiske-Subbarow reaction.

Fatty acid compositions were determined for total lipids as well as for triacylglycerols, phosphatidyl ethanolamines, (PE), and phosphatidyl cholines (PC). The glycerolipids were isolated by preparative TLC, separating first the triacylglycerols in a petroleum ether:diethyl ether (80:20) solvent system, then the phospholipids within the lower half of the plate, using chloroform:methanol:water (65:25:4) as developing solvent. The positions of the bands were determined by exposing the plates briefly to iodine vapors. After evaporation of iodine from the plate, the bands were scraped off and the lipids extracted with diethyl ether, saturated with water. The fatty acids of the ex-

TABLE III

Fat Consumption, Body Wt, and Testis Wt and Histology

Dietary fat	5 wk on experimental diet						15 wk on experimental diet					
	Cumulative fat intake						Cumulative fat intake					
	Total fat (g)	20:1[a] (g)	22:1[b] (g)	Body wt (g)	Right testis wt (g)	Left testis histological score[c]	Total fat (g)	20:1[a] (g)	22:1[b] (g)	Body wt (g)	Right testis wt (g)	*Left testis* histological score[c]
None	0 ± [d]	0	0	142 ± 4[e]	0.78 ± 0.09[f]	0.6[m]	0	0	0	246 ± 11	0.99 ± 0.15[g]	2.4
Peanut oil	74 ± 6	0	0	202 ± 7	1.10 ± 0.02	0.0	342 ± 6	0	0	438 ± 14	1.62 ± 0.03	0.0
Partially hydrogenated fish oil	56 ± 4	6.5	6.9	146 ± 7	0.52 ± 0.04[f,h,i]	5.0	288 ± 8	33	35	220 ± 12[j,k]	0.55 ± 0.01[g,l]	4.2
20:1 fraction	8.0 ± 0.3	6.0	0	161 ± 6[e]	0.91 ± 0.07[h]	0 - 1[n]	39.2 ± 0.2	27	0	269 ± 6[j]	0.86 ± 0.13[l]	2.8
22:1 fraction	7.6 ± 0.3	0	5.4	153 ± 3	0.74 ± 0.07[i]	0 - 1[m]	38.2 ± 0.5	0	30	264 ± 13[k]	0.82 ± 0.13	4.3

[a]Ethyl esters of fatty acids; mainly positional isomers of *trans*-eicosenoate (Tables I & II).

[b]Ethyl esters of fatty acids; mainly positional isomers of *trans*-docosenoate (Tables I & II).

[c]Assessed on a scale from 0 (no degeneration) to 5 (total degeneration) (Ref. 1).

[d]Mean ± standard error; five determinations.

[e-l]Values with same superscript are significantly different ($P<0.05$) (Student's t-test). Peanut oil controls are significantly different from all other groups.

[m]Delayed maturation of the spermatogenic tissue in three out of five rats.

[n]Delayed maturation of the spermatogenic tissue in all of five rats.

tracted glycerolipids were trans-esterified as described for the fish oil fractions. Completion of the methanolysis was verified by TLC. The methyl esters were extracted from the reaction mixture with petroleum ether and analyzed by gas liquid chromatography using a Beckman GC-M chromatograph with flame ionization detector and equipped with a 225 cm x 0.32 cm (outside diameter) stainless steel colum packed with 15% diethyl glycol succinate on Chromosorb W (AW), 80/100 mesh (Applied Science Lab., Inc., State College, PA). The chromatography was carrie l out at 180 C with a helium carrier gas flow of 30 ml/min. Retention times were compared with those of reference mixtures of methyl esters. For very long chain fatty acids, further verification of their chain lengths was obtained by equivalent chain length comparisons and by gas chromatography of completely hydrogenated samples. Quantification was made on the basis of the direct proportionalities of the peak areas.

RESULTS

The partially hydrogenated fish oil used in the present study exhibited a remarkably high content of *trans* double bonds (Table I). The unfractionated fish oil contained *trans* double bonds at a level three times higher than that used by Hølmer and Aaes-Jørgensen (5). The 20:1 and 22:1 fractions derived from the present oil were virtually free of *cis* double bonds. The lower *trans* percentage for the 22:1 fraction has been shown to be due to contamination by the 14- and 16-carbon fatty acids as seen in Table I. The positional distributions of the double bond in the monoene fractions (Table II) displayed an even migration of the double bond to both sides of the Δ11(-12)-double bond, as has also been found in other preparations, e.g., by Lambertsen et al. (16) and Scholfield et al. (17).

In Table III, it is shown that the supply of 20:1 and 22:1 isomers was similar between the PHFO-fed animals and the respective monoene-fed groups. However, the intake of total fat—with 75% trans double-bonds—was, of course, much greater in the PHFO-fed group than in the monoene-fed groups. The growth of animals fed on the fat-free diet, peanut oil-containing, or PHFO-containing diets followed closely the previously reported results (4). Compared to the rats reared on the fat-free diet, there were signs of an increased growth of animals fed the diet with 3% monoenes, whereas the PHFO diet had no effect or caused a slower growth. These trends were consistent over the experimental period, but generally they were not statistically significant.

The macroscopic and microscopic effects of the dietary regimens on the testes are also described in Table III. The earliest and most severe degeneration of the spermatogenic tissue was seen in the group fed 28% PHFO, where a total absence of spermatids was observed at 5 weeks. In the other three essential fatty acid deficient groups, testicular degeneration was well in progress at 15 weeks on the diets. After 5 weeks on the diets, the monoene fractions had caused no delay in maturation in comparison with the totally fat-free diet. After 15 weeks, the degeneration had progressed further in the 22:1-fed animals than in the other groups, as judged by histology. The testis size, however, in the two monoene-fed groups was not different, whereas the high degree of degeneration in the PHFO-fed rats was associated with a very low testis wt. It is worth noting that after 15 weeks a tendency was appearing of smaller testes in the monoene-fed groups than in the fat-free group, whereas the body wts of the former groups tended to be higher than those of the fat-free rats. Even though these tendencies were not significantly different at the 95% level of confidence, the corresponding differences between the relative wt of testes (% of body wt) were significant.

The effect of the various dietary regimens on the lipid class composition of the testes is illustrated quantitatively in Table IV, in which the different EFA-deficient groups after 15 weeks of experiment are compared with the control groups fed on peanut oil (PO). Testes from all dietary groups were analyzed after 5 and after 15 weeks on the diets, but differences between lipid content at these two stages were generally insignificant, except for a high concentration of PC in the testes of the FF- and 22:1-fed rats after 5 weeks (8.06±0.79 and 8.53±1.20 mg/g, respectively). Testes from these two groups showed no clear signs of degeneration, in contrast to those from rats fed on PHFO for 5 weeks. In Table IV, data for both 5 and 15 week analyses are given only for the PHFO dietary groups. The most striking differences among all the groups were found in the data for the PHFO groups. Already at 5 weeks, the testes of this group contained less than half the amount of triacylglycerol, i.e., 0.63 mg per g of testis, found in all other groups. The decrease in neutral lipid content of the PHFO testes apparently can be ascribed to the reduction in triacylglycerol level alone. For the phospholipids, the only consistent trend in concentration changes was found for the sphingomyelins. The concentration of these was generally low in the normal or in the slightly degenerated testes, whereas higher values were found in testes with a high histological score of degeneration. The total amount of cholesterol varied relatively little, and the variations did not correlate with diet composition nor with length of experimental period.

The fatty acid compositions of total testicular lipids are illustrated in Table V. These data indicate a significantly higher ($P<0.01$) level of eicosadienoic acids in the rats fed the PHFO than in the FF- and 20:1-fed rats. In addition, the PHFO-fed rats displayed, when compared to the other EFA-deficient groups, a relatively lower level of eicosatrienoic acid derived from oleic acid. In none of the three groups fed the very long chain, isomeric fatty acids was there any significant increase in the content of the very long chain monoenoic acids when compared to the EFA-deficient group (FF) not receiving isomeric fatty acids. Aside from the noted changes in the PHFO group, the shift in fatty acid composition of total lipids (Table V) was parallel for all four EFA-deficient groups when compared with the control group fed peanut oil. The usual decrease was observed for the (n-6)-acids, accompanied by an increase in the (n-9)-acids. These changes progressed from the 5th to the 15th week for the PHFO-fed animals, where the testicular degeneration (or lack of development) was total already after 5 weeks in experiment. The same progression was found in the other deficient groups. For the sake of brevity, these data are not tabulated. Concluding the analyses of the present experiment, a comparison was made of the fatty acid composition of triacylglycerols, PC, and PE. Here it was necessary to pool all lipid from each age-diet-group, thus excluding an assessment of the biological variation within each group. For the 15 week groups, it was found that the characteristic high content of docosapentaenoic acid in testicular triacylglycerols (18,19) was reduced from 9.2% in the control group (PO) to 3.1% in the fat-free group and to levels between 0.6 and 1.5% for the other deficient groups. Similarly, marked decreases in the levels of (n-6)-polyunsaturates were found in PE and, especially, in PC. There was no correlation of these changes, either with the supplied fish oil derivatives or with the degree of testicular degeneration. This series of analyses gave no indication of a deposition of dietary 20:1 or 22:1 acids in any of the fish oil supplemented groups.

DISCUSSION

The present experiments have shown that fractions of PHFO containing the docosenoic or eicosenoic fatty acid isomers characteristic for

TABLE IV

Lipid Class Composition of Testicular Lipids (mg lipid per g tissue wet wt)

Dietary fat	Fat-free	Peanut oil	Partially hydrogenated fish oil		20:1 fraction	22:1 fraction
Duration of feeding (wk)	15	15	5[a]	15	15	15
Cholesterol	1.86 ± 0.16[b]	1.79 ± 0.16	2.04 ± 0.14	1.60 ± 0.05	1.68 ± 0.22	1.73 ± 0.05
Cholesterol esters	0.49 ± 0.36	0.13 ± 0.05	0.39 ± 0.13	0.22 ± 0.08	0.26 ± 0.09	0.11 ± 0.02
Alkyl-diacyl-glycerols	0.46 ± 0.18	0.08 ± 0.04	0.18 ± 0.05	0.03 ± 0.01	0.23 ± 0.02	0.18 ± 0.03
Triacylglycerols	1.84 ± 0.23	1.67 ± 0.16	0.63 ± 0.13	0.51 ± 0.13	1.43 ± 0.18	1.13 ± 0.25
Free fatty acids	0.35 ± 0.04	0.40 ± 0.07	0.32 ± 0.04	0.34 ± 0.03	0.35 ± 0.04	0.29 ± 0.02
Total "neutral lipids"	5.0 ± 0.5	4.1 ± 0.3	3.6 ± 0.2	2.7 ± 0.2	4.0 ± 0.3	3.4 ± 0.3
P.[c] ethanolamines	3.06 ± 0.43	2.79 ± 0.32	3.40 ± 0.24	3.65 ± 0.66	3.48 ± 0.05	2.29 ± 1.04
P. cholines	5.96 ± 0.11	6.00 ± 0.57	6.58 ± 0.28	6.81 ± 0.90	6.25 ± 0.26	5.67 ± 0.57
P. serines	0.83 ± 0.13	0.95 ± 0.20	0.80 ± 0.09	0.54 ± 0.05	1.37 ± 0.07	1.57 ± 0.10
P. inositols	1.12 ± 0.04	0.36 ± 0.10	0.90 ± 0.20	0.56 ± 0.05	0.33 ± 0.28	
Sphingomyelins	1.60 ± 0.74	0.97 ± 0.20	1.65 ± 0.59	1.18 ± 0.17	1.56 ± 0.37	1.47 ± 0.57
Total phospholipids	12.6 ± 0.9	11.1 ± 0.7	13.3 ± 0.7	12.7 ± 1.1	13.0 ± 0.5	11.0 ± 1.3
Total of analyzed lipids[d]	18.1 ± 1.0	15.6 ± 0.8	18.2 ± 0.8	16.2 ± 1.2	17.3 ± 0.7	14.8 ± 1.4

[a]Data for 5-wk results from the other dietary groups are available from the author.

[b]Mean ± standard error; five determinations.

[c]Phosphatidyl.

[d]Includes minor amounts of diacylglycerols, methyl esters, "hydrocarbons," cardiolipin, and an unidentified polar lipid.

TABLE V

Fatty Acid Composition of Total Testicular Lipids (peak area %)

Dietary fat	Fat-free	Peanut oil	Partially hydrogenated fish oil		20:1 fraction	22:1 fraction
Duration of feeding (wk)	15	15	5[a]	15	15	15
Fatty acid						
16:0	28.4 ± 1.9[b]	31.0 ± 0.9	27.4 ± 1.2	27.5 ± 1.3	29.6 ± 0.6	28.7 ± 0.8
:1	2.0 ± 0.3	1.0 ± 0.2	3.1 ± 0.3	2.6 ± 0.1	2.8 ± 0.1	2.6 ± 0.1
18:0	7.1 ± 0.5	6.5 ± 0.2	7.2 ± 0.2	8.1 ± 0.4	7.6 ± 0.1	7.8 ± 0.6
:1 (n-9)	19.0 ± 0.4	10.1 ± 0.6	24.2 ± 1.5	22.2 ± 1.1	18.5 ± 1.1	18.8 ± 1.0
:2 (n-6)	0.8 ± 0.2	4.6 ± 0.5	2.1 ± 0.4	1.9 ± 0.1	1.1 ± 0.2	0.7 ± 0.2
20:1	0.6 ± 0.2	0.2 ± 0.1	2.8 ± 1.7	0.9 ± 0.1	0.8 ± 0.1	1.0 ± 0.3
:2	0.9 ± 0.1[c]	n.d.	1.6 ± 0.2	1.7 ± 0.2[c,d]	1.0 ± 0.1[d]	2.5 ± 1.7
:3 (n-9)	13.2 ± 0.9[f]	n.d.	5.2 ± 0.2	10.6 ± 0.3[e,f,g]	12.5 ± 0.3[e]	12.8 ± 0.8[g]
:3 (n-6)	0.7 ± 0.2	1.3 ± 0.1	1.4 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
:4 (n-6)	5.3 ± 0.8	15.3 ± 0.6	7.7 ± 0.5	4.7 ± 0.2	5.0 ± 0.7	4.8 ± 0.8
22:1	0.6 ± 0.1	0.6 ± 0.1	1.3 ± 0.2	1.2 ± 0.3	0.6 ± 0.2	0.6 ± 0.1
:3	3.4 ± 0.5	n.d.	1.8 ± 0.4	1.8 ± 0.2	2.5 ± 0.2	2.8 ± 0.2
:4	3.9 ± 0.8	2.4 ± 0.1	0.9 ± 0.3	1.4 ± 0.3	3.7 ± 0.5	2.9 ± 1.1
:5 (n-6)	6.4 ± 0.8	18.6 ± 0.7	3.9 ± 0.6	5.5 ± 0.8	6.7 ± 0.1	6.8 ± 0.2
Other fatty acids	7.2	7.8	7.6	7.2	5.9	4.3

[a]Data for 5-wk results from the other dietary groups are available from the author.

[b]Mean ± standard error; five determinations for 5-wk results; three determinations (on one to three testes, pooled) for 15 wk results.

[c-g]Values with same superscript are significantly different; for c,d,e: $P<0.01$; for f & g: $P<0.05$ (Student's t-test).

PHFO do not confer the same augmentation of EFA-deficiency symptoms in the rat as does the whole PHFO. It should be noted, though, that whereas the experimental design provided comparable amounts of the very long chain isomeric fatty acids to the groups fed the 20:1 and 22:1 fractions of PHFO and to the group fed the whole PHFO, the latter group did consume considerably more fat and more isomeric fatty acids than did the groups given the fractions. Actually, rat growth was somewhat improved when feeding these fractions, whereas PHFO fed over 15 weeks resulted in slightly poorer growth. A comparable observation was made by Holman and Aaes-Jørgensen (20), who found that elaidate-supplementation to fat-deficient rats was growth-promoting, wheras supplementation of more complex isomers (*cis-trans* and *trans-trans* isomers of linoleate) was growth-retarding. The growth retardation observed in the present experiment could be presumed in part to be due to a lower digestibility of the PHFO, having a melting point of ca. 36 C. However, Njaa et al. (21) have shown that partially hydrogenated marine fats with melting points of 40 C are well digested, with the experimental rats losing only 8-9% of the calories as fat in the feces in comparison with a loss of 4-5% of the calories in control groups. The effect of the fractions of PHFO on the testes did not parallel the effect on body wt. After 15 weeks, both testis wt and histological evaluation of the degree of spermatogenesis suggested an adverse effect of the monoene fractions, especially of the docosenoic isomers.

One of the purposes of the present experiment was an investigation of a possible deposition of dietary isomeric fatty acids in the testicular lipids. If such deposition occurred, the resulting changes in the physico-chemical characteristics of lipid components of membranes (22) could well induce changes in testicular function. Incorporation of *trans* fatty acids into rat tissue lipids has been reported by several authors, e.g., in liver lipids (23,24) and in depot fat (25). However, Sinclair (24) found that, in contrast to other tissues examined (except brain), dietary elaidate was not incorporated into testicular lipids. Walker (26) found very long chain monoenoic acids to be deposited in testicular lipids at levels of 2-5% following rapeseed oil or erucate feeding, whereas Coniglio et al. (27) found only 0.2-0.4% docosenoic acid in testis lipids after feeding rapeseed oil. Brockerhoff et al. (28) found eicosenoic and docosenoic fatty acids of marine origin to be incorporated in depot fat triacylglycerols, but not (or to a small extent only) in liver phospholipids. These results would indicate little chance of finding very long chain *trans*-isomers in structurally important lipids in the testis. However, all of the cited studies, with the exception of that of Sgoutas et al. (23) have involved diets containing linoleate. Noting that the degeneration effects of PHFO observed by Aaes-Jørgensen et al. (2,4) had an EFA-deficiency as a prerequisite, it appeared possible that incorporation of the isomeric dietary fatty acids could occur in the testicular lipids of our EFA-deficient rats.

As demonstrated by the fatty acid analyses of total lipids (Table V) and of major lipid classes, there was no evidence of a major accumulation of dietary eicosenoic or docosenoic acids in the testicular lipids of the rats fed PHFO or fractions thereof. This was true not only for the structurally important PCs and PEs but also for the triacylglycerols. In view of Sinclair's results (24), this could be due to the extensive *trans*-isomerization in our fish oil preparations. Whether or not there is a deposition of conversion products of the dietary very long chain *trans*-isomeric fatty acids has not been determined. The gas chromatographic data displayed no evidence of an increased level of possible elongated or further desaturated products of the eicosenoic and docosenoic acids. However, the double bond structure in the very long chain polyunsaturated acids was not determined for comparison with that of the dietary fatty acids.

The present experiment also supplies information about possible secondary effects of the diet on the tissue fatty acid composition. Even if not deposited themselves in major amounts, low levels of dietary fatty acids might influence the biosynthesis of endogenous fatty acids. Egwim and Sgoutas (29) found that high levels (5 or 10 wt % of the diet) of *trans* fatty acids caused a decrease in liver arachidonic acid beyond that observed on a fat-free diet. The *trans* fatty acid supplement also produced an increase in eicosadienoic acids. These authors suggested a hindrance of the interconversion of unsaturated fatty acids as being responsible for the accumulation of the eicosadienoic acids. Guo and Alexander (30,31) fed ethyl elaidate (8-9 wt % of diet) and observed liver fatty acid changes indicative of a decreased conversion of oleic to eicosatrienoic acid. Simultaneously, an elaidate metabolite, 5-*cis*,9-*trans* octadecadienoate, first described by Lemarchal and Munsch (32), was found in liver phospholipids. Supplementation of marginal amounts of linoleate reduced the concentration of this acid, as well as the level of the eicosadienoic acid discussed above (29). Our results demonstrated a similar influence of feeding 28% PHFO on the

fatty acid composition in testicular lipids. Eicosatrienoate levels were reduced when compared with levels in animals on the fat-free diet. Further, there was a slight but obvious increase in the amount of eicosadienoic acids. The effect of whole PHFO could be ascribed to the higher level of dietary fat, including a higher level of total *trans* fatty acids in diet, inasmuch as the eicosenoic or docosenoic *trans* fatty acids of the PHFO did not, when given alone, cause any significant change in the fatty acid patterns. It is noteworthy that, in spite of the generally poorer condition of the rats, the testicular lipids of the PHFO-fed rats contained as high concentration of (n-6)-acids as did the other deficient groups.

Another secondary effect of dietary fatty acids is the effect on lipid class composition. Earlier studies by Privett et al. (33) showed that *trans*-isomerism of fatty acids influenced their incorporation into liver glycerolipids in a manner agreeing with the "saturated" physicochemical nature of the *trans*-isomers. An influence of fatty acid structure upon enzymatic activities in acyl transferase reactions has been demonstrated in liver phosphatidyl choline synthesis by Lands et al. (34) and in liver cholesteryl ester synthesis by Sgoutas (35). Guo and Alexander (30) studied the effect of elaidate on liver lipid class composition and noted a substantial increase in the level of PEs while the level of PCs was reduced. Hølmer and Aaes-Jørgensen (6) studied dietary influences on testicular lipid classes and found that the distribution of classes within the neutral lipids showed an increasing proportion of triacylglycerols and a decrease in free cholesterol when degeneration proceeded. Total phospholipid concentrations decreased by 30-40% as degeneration became complete. This decrease was accompanied by a changed distribution of phospholipid classes.

The present experiments confirm the data on the relative amounts of the major phospholipids (PE, PC, and sphingomyelins) as reported by Hølmer and Aaes-Jørgensen (6). We found an increase in sphingomyelin levels in the EFA-deficient animals, though this increase was not well correlated with the histological observations. However, the individual variations within some of the deficient groups was considerable. In contrast to the results of Hølmer and Aaes-Jørgensen, the present results showed no general trend, either of a lowering of the cholesterol concentration or of an increase in triacylglycerols as degeneration progressed. Quite the opposite result was demonstrated in PHFO-fed animals, in which the triacylglycerol concentration already after 5 weeks was down to 40% of the control value. For these animals, histology revealed a total absence of spermatids. Hence, the situation is probably more correctly described as a delayed or hindered maturation and not as a state of degeneration (4). It is likely that there is a relationship between the facts that these testes never matured (4), that the content of docosapentaenoic acid parallels the maturation of the testes (18,19), and that in normal, mature rats a large proportion of the docosapentaenoic acid occurs in the triacylglycerols. The discrepancy in cholesterol levels compared to the results of Hølmer and Aaes-Jørgensen (6) is unexplained, but comparison with neutral lipid values is obscured by the high levels of free fatty acids found by these authors, possibly caused by hydrolysis during storage of tissue. The values reported for cholesterol and triacylglycerols, and the relative amounts of neutral and phospholipids found in control rats in the present study, are in agreement with the values reported by Davis et al. (18) and by Oshima and Carpenter (19).

As has been pointed out by Lee and Barnes (36), comparison between lipid patterns in control and degenerated testes are difficult to interpret because of the changes of cellular composition inherent in degenerations. In normal testes, the contribution of spermatids and spermatozoa to the total lipid of the testes is considerable. Changes observed in the lipid composition of degenerated testes will, to a large extent, be due to the disappearance of spermatozoa and spermatogenetic tissue. Interpretations are also difficult when speculating on the mode of action of the dietary fatty acids. Greenberg and Ershoff (37) demonstrated a wt recovery of EFA-deficient rat testes when administering chorionic gonadotropin, and Roland and Edwards (38) showed that, for EFA-deficient cockerels, the microscopic picture of the testes was restored almost to normal when luteinizing hormone was given. This change was not accompanied by a change in fatty acid pattern. These results would indicate at least a partial influence of the EFA-deficiency via the pituitary gland or via the hypothalamus, governing the release of the luteinizing hormone (38). Although Ahluwalia et al. (39) have found that the EFA-deficiency in rats does not reduce the level of androgens, there is still in these results evidence for a decreased responsiveness of testes and ventral prostate gland to circulating androgens. On this basis, Ahluwalia et al. (39) suggested a direct role of EFA in providing vital nutrients to the testicular epithelium.

Considering the extensive interplay between the testicular functions (production of sperma-

tozoa and of hormones), circulating hormones and lipids, and superior glands, conclusions from the data presented here cannot be carried too far. This study has demonstrated that the deleterious effect of high levels of dietary partially hydrogenated fish oil is not due to a deposition of the long chain isomeric fatty acids in the testes. Also, the effect cannot be ascribed alone to the presence of these fatty acids in the diet as demonstrated by the results of feeding the eicosenoic or docosenoic fractions to the rat. However, a combination of one of these dietary fractions and a high dietary lipid load has not been tested. Further investigations along this line will require experiments where the dietary lipid level is the same for all groups. Changes in fatty acid patterns when feeding the partially hydrogenated oil or the fractions have indicated an effect of the diet on fatty acid conversions in vivo. The possible effect of dietary lipids on testicular fatty acid metabolism is the subject of studies in progress.

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Positional Analysis of Isovaleroyl Triglycerides Using Proton Magnetic Resonance with $Eu(fod)_3$ and $Pr(fod)_3$ Shift Reagents: II. Cetacean Triglycerides

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ABSTRACT

The positional distribution of isovaleric acid in natural cetacean triglycerides has been studied using proton magnetic resonance with lanthanide shift reagents. Twenty-nine samples of melon, jaw, and blubber triglycerides from 13 genera in the Delphinidae, Phocoenidae, and Monodontidae families were examined. Isovaleric acid was found esterified only at the 1,3-positions in the monoisovaleroyl and diisovaleroyl triglycerides of these animals.

INTRODUCTION

The unusual isovaleroyl triglycerides (TGs) occurring in the head and blubber fats of many toothed Cetaceans have interested many biochemists (1-7). They have recently been characterized as mixed acid TGs containing one or two isovaleroyl chains in combination with longer C_{12}-C_{22} fatty acids.

Structural studies on the isomeric forms of these unique isovaleroyl TGs have been limited. Methods employing pancreatic lipase are inappropriate because the β-methyl branch on the isovaleroyl chain makes the ester linkage resistant to lipolysis (8-10). Blomberg (11,12) and Varanasi et al. (13) have used mass spectrometry (MS) to characterize purified TG fractions, demonstrating the presence of 2-isopentadecano-1,3-diisovalerin in *Globicephala* melon fat and 2-(long chain)-1,3-diisovalerin in *Tursiops* mandibular blubber fat. However, there has been no systematic study of both mono- and diisovaleroyl TGs from different fatty tissues in a wide number of cetacean genera.

Earlier we reported (14) a new technique for determining the positional distribution of isovaleric acid in mixed acid TGs using proton magnetic resonance (PMR) with lanthanide shift reagents. This method employs $Eu(fod)_3$ or $Pr(fod)_3$ to produce different γ-methyl proton signals for the 1,3- and 2-isovaleroyl chains. Longer acyl groups in the same molecule create no interfering signals, so analyses can be run on total TG mixtures with little or no prior subfractionation.

This paper reports the application of the new PMR positional analysis technique to 29 different cetacean fats representing the majority of cetacean genera containing high levels of isovaleric acid.

EXPERIMENTAL PROCEDURES

Materials

Cetacean fat sample used in this study are the same as described previously (2,3). When possible, the fat samples were extracted from center melon, total mandibular fat body (jaw), and dorsal blubber tissues.

Pure mixed acid TGs of isovaleric and palmitic acids were synthesized as already described (14). Their structural formulas and the specialized nomenclature used in this report appear in Figure 1.

The lanthanide shift reagents employed were the deuterated "fod" (1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octadione) derivatives of europium III and praseodymium III. These $Eu(fod)_3d_{30}$ (Norell Chemical Co., Landisville, NJ) and $Pr(fod)_3d_{27}$ (Merck, Sharp, and Dohme Ltd., Montreal, Canada) reagents were stored under reduced pressure over P_2O_5 and used as received. Residue free solvents (Burdick & Jackson, Muskegon, MI) were used throughout.

Methods

Highly unsaturated fat samples, mainly blubber fats, were first hydrogenated (15) to improve thin layer chromatography (TLC) separations of TG subclasses and to eliminate the effects of the double bonds on subsequent PMR spectra.

When samples contained other lipid classes, the TGs were isolated by preparative TLC on 200 x 200 x 1.0 mm layers of silicic acid impregnated with 0.04% rhodamine 6G and predeveloped in diethyl ether to remove any impurities. After sample application, the plates were developed in petroleum ether (bp 30-60 C):diethyl ether 90:10. The TG band was

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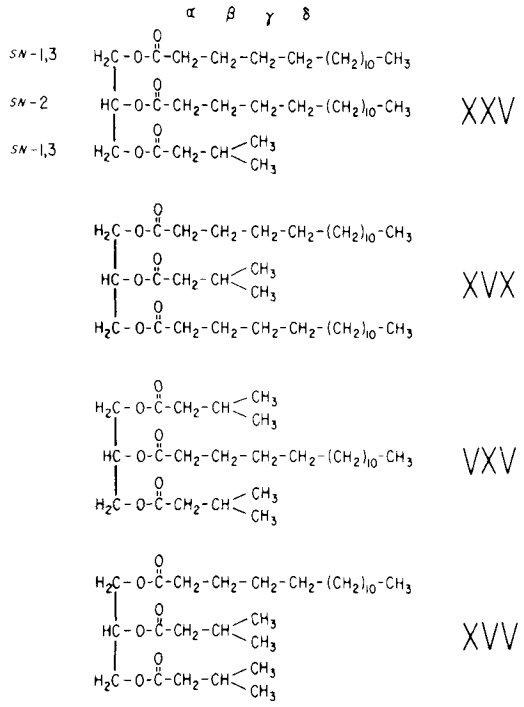

FIG. 1. Structural formulas and nomenclature for the monoisovaleroyl (XVX and XXV) and diisovaleroyl (VXV and XVV) triglyceride positional isomers studied in this investigation. X = any long chain acyl group, V = isovaleroyl chain. The usual numerical nomenclature is employed for positions on the glycerol moiety, whereas the Greek letters α, β, γ, and δ designate the carbon positions on the fatty acid chains. The XXV and XVV structures can exist in two enantiomorphic forms, but only one isomer has been drawn here.

located under UV light, scraped off the plates, placed in a small chromatography column, and the lipids were recovered by elution with diethyl ether.

Where the isolation of individual TG subclasses (XXV or VXV) was required, two to three developments with 92:8 petroleum ether: diethyl ether and low sample loadings were required to achieve satisfactory separations. Most blubber fat samples studied contained large amounts of XXX nonisovaleroyl TGs, making it difficult to resolve clean isovaleroyl signals in the PMR spectra. In such cases, samples of enriched isovaleric acid content were obtained using preparative TLC and recovering just the lower part of the TG band where the XXV and VXV subclasses are known to occur (6).

All PMR spectra were obtained at 60 MHz employing a Varian T-60 PMR spectrometer at room temperature in the usual manner. Each TG sample (33-120 mg) was dissolved in 0.4 ml CCl_4 containing 0.5-1.0% tetramethylsilane as

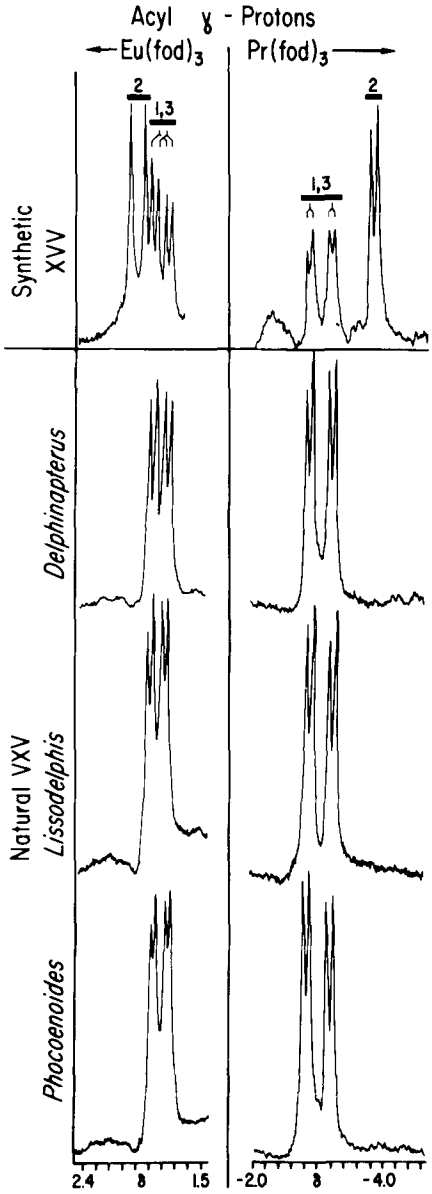

FIG. 2. Chemical shifts of γ-proton resonance signals obtained by adding Eu(fod)3 or Pr(fod)3 to diisovaleroyl triglycerides (TG). No 2-γ-isovaleroyl proton resonance is present in the cetacean VXV spectra. Mole ratio Eu:TG ≈ 1.0-1.5, Pr:TG ≈ 1.5-2.0. Sample size = 39-67 mg TG in CCl_4. Sweep width = 250 Hz with Eu(fod)3, 500 Hz with Pr(fod)3.

an internal standard (δ = 0). The shift reagent was added in 25-50 mg increments, and the PMR spectrum was recorded after each addition until satisfactory isovaleroyl PMR signals were obtained (1.0-2.2 shift reagent: TG molar ratio). Signals were identified as previously described (14).

RESULTS

Cetacean TGs from *Delphinapterus leucas* melon fat, *Lissodelphis borealis* jaw fat, and *Phocoenoides dalli* jaw fat were subjected to detailed analysis. These animals represent the three cetacean families (Monodontidae, Delphinidae, and Phocoenidae, respectively) known to possess appreciable amounts of isovaleroyl lipids (2,3). The VXV and XXV TG subclasses were isolated from each of the three samples by preparative TLC. Each purified subclass was then studied with PMR in the presence of Eu(fod)$_3$ and Pr(fod)$_3$ to define the resonance signals of the acyl-γ-protons.

The PMR spectra for the diisovaleroyl TGs are shown in Figure 2. With Eu(fod)$_3$, the synthetic XVV (*rac*-1-palmitoyl-2,3-diisovalerin) exhibits markedly different shifts for the γ-methyl protons of the 2- and 1,3-isovaleroyl chains. The prominent 2-γ-methyl doublet is shifted further downfield than the two overlapping 1,3-γ-methyl doublets. With Pr(fod)$_3$, similar upfield shifts again separate the 2-γ-methyl and 1,3-γ-methyl signals of the isovaleroyl chains, but with considerably greater resolution.

These two spectra for synthetic XVV were then compared with the γ-methyl PMR signals for *Delphinapterus, Lissodelphis,* and *Phocoenoides* diisovaleroyl TGs (Fig. 2). In each case and with either shift reagent, there was a complete absence of any 2-γ-methyl isovaleroyl doublet, whereas prominent 1,3-γ-methyl isovaleroyl resonances were always observed. Therefore, the isovaleroyl moieties in the natural VXV TGs must be esterified only at the 1,3-positions of the glycerol.

The PMR spectra for the monoisovaleroyl TGs are presented in Figure 3. With Eu(fod)$_3$, a 90:10 mixture of *rac*-1,2-dipalmito-3-isovalerin and 1,3-dipalmito-2-isovalerin produced a distinctive 2-γ-methyl doublet superimposed on the palmitoyl 2-γ-methylene multiplet and partially resolved from the two 1,3-isovaleroyl γ-methyl doublets. With the upfield shift reagent Pr(fod)$_3$, the same model mixture provided a distinctive 2-γ-methyl isovaleroyl doublet clearly resolved from the 1,3-γmethyl signals.

The two spectra for the synthetic monoisovaleroyl mixture were then compared with the γ-methyl signals for *Delphinapterus, Lissodelphis,* and *Phocoenoides* XXV TGs (Fig. 3). Isovaleroyl 2-γ-methyl doublets were not observed in any of the three natural XXV fractions using either Eu(fod)$_3$ or Pr(fod)$_3$. This clearly indicates exclusive esterification of isovaleric acid at the 1,3-positions in the monoisovaleroyl TGs.

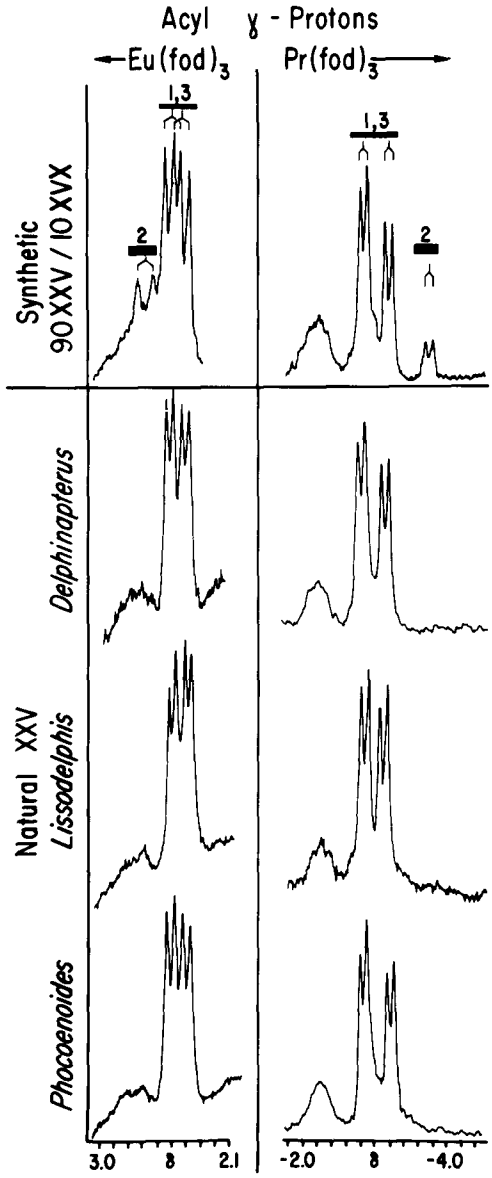

FIG. 3. Chemical shifts of γ-proton resonance signals obtained by adding Eu(fod)3 or Pr(fod)3 to monoisovaleroyl triglycerides (TG). No 2-γ-isovaleroyl proton resonance is observed in the cetacean XXV spectra. Mole ratio Eu:TG ≃ 1.0-1.5, Pr:TG ≃ 1.5-2.0. Sample size = 33-120 mg TG in CCl_4. Sweep width = 250 Hz with Eu(fod)3, 500 Hz with Pr(fod)3.

Because no 2-isovaleroyl isomers had been found in the first three natural samples examined, all additional analyses were run on unfractionated TG samples or, for the XXX-rich blubber fats, on enriched XXV/VXV mixtures. Because of the better signal resolution

TABLE I

Position of Isovaleroyl Chains in Cetacean Triglycerides Determined by Proton Magnetic Resonance with Lanthanide Shift Reagents

Family Genus species	Position of isovaleroyl chains		
	Melon	Jaw	Blubber
Delphinidae			
Delphinus delphis	1,3		1,3
Globicephala melaena	1,3		
Grampus griseus	1,3	1,3	
Lagenorhynchus albirostris	1,3		1,3
Lissodelphis borealis		1,3[a]	
Pseudorca crassidens	1,3	1,3	
Stenella plagiodon	1,3	1,3	1,3
Sotalia fluviatilis	1,3	1,3	1,3
Tursiops truncatus	1,3	1,3	1,3
Phocoenidae			
Phocoena phocoena	1,3	1,3	1,3
Phocoenoides dalli	1,3	1,3[a]	1,3
Monodontidae			
Delphinapterus leucas	1,3[a]		1,3
Monodon monoceros	1,3	1,3	

[a]Pure XXV and VXV fractions were isolated from this fat, and each fraction was studied with both $Eu(fod)_3$ and $Pr(fod)_3$.

afforded with $Pr(fod)_3$, this shift reagent was used exclusively for the remaining samples.

Eleven more cetacean melon fat samples, eight additional jaw fat samples, and eight blubber fat samples rich in isovaleric acid (2,3) were tested in this manner. Results are reported in Table I. In all 29 cases examined, no signals for any isovaleroyl moiety at the 2-position were observed. Hence, only 1,3-isovaleroyl esters occur in these natural cetacean TG molecules.

DISCUSSION

Positional Analysis of Triglycerides

The positional analysis of isovaleric acid in natural TGs can be readily accomplished by PMR with lanthanide shift reagents as demonstrated here and in our previous study (14). The resonance frequencies of the γ-protons in the 2-isovaleroyl, 1,3-isovaleroyl, and longer acyl chains are sufficiently distinctive that both qualitative and quantitative data on the isomers present can be obtained. The heterogeneity of the long chain fatty acids found in natural TGs does not affect the analysis, inasmuch as their γ-proton resonances are essentially identical for each position in the TG under the operating conditions used. Note, for example, the definitive isovaleroyl γ-proton spectra obtained for *Delphinapterus* melon TGs (Figs. 2 & 3) even though C_9 through C_{22} acids are present (6). Natural TG mixtures can thus be analyzed directly, unlike MS techniques (11-13) where purified TG subclasses or individual molecular species must be isolated before analysis.

The same approach should also prove useful for the positional analysis of any short chain acid in synthetic or natural TG mixtures. For example, we have found (14) that tributyrin shows distinctive 2- and 1,3-butyroyl γ-methyl proton resonance frequencies when $Pr(fod)_3$ is present. Hence, this method could be used to define the position of 4:0 esterification in butterfat TGs. Similarly, the α-methyl proton signals of the acetate in the acetotriglycerides of seed fats (16,17) could be used to characterize positional isomers.

The presence of additional functional groups near the ester linkage could complicate analyses, however, either enhancing or masking isomeric distinctions. The presence of a double bond in the C_2-C_7 region of the long chain acids might make the resonance spectra undesirably complex (18,19), but the ethylenic linkage could be easily removed by hydrogenation. On the other hand, a double bond in the short chain acid, as in sorbic acid TGs (20), would greatly facilitate isomer analysis.

Cetacean Isovaleroyl Triglycerides

The 29 samples analyzed here represent a majority of the genera within the three cetacean families known to possess high levels of isovaleroyl TGs in their fats (2,3). Melon, jaw, and blubber fats from 9 of the 16 genera in

the family Delphinidae, 2 of the 3 genera in the family Phocoenidae, and all of the genera in the family Monodontidae were examined.

Our results clearly show that isovaleric acid is present only at the 1,3-positions of the TGs of these animals. These findings agree with earlier reports of 2-isopentadecano-1,3-diisovalerin in *Globicephala* melon fat (11,12) and of 2-(long chain)-1,3-diisovalerin in *Tursiops* mandibular blubber fat (13) based on MS studies of purified TG fractions. We can now conclude that this same positional specificity of isovalerate chains is found in melon, jaw, and blubber fats throughout the Delphinidae, Phocoenidae, and Monodontidae families and in both monoisovaleroyl and diisovaleroyl TGs.

Although the PMR technique employed here does not discriminate between the *sn*-1 and *sn*-3 positions, one would expect the isovaleroyl chains to appear mainly at the *sn*-3 position in cetacean monoisovalveroyl TGs. Such tendencies for unusual short chain fatty acids to occupy the *sn*-3 position have been reported for natural TGs containing acetic (16,17), butyric (21), and hexanoic (21) acids. The isovalerate in diacyl glyceryl ethers from rabbit harderian gland also appears only at the *sn*-3-position (22). Apparently the *sn*-3-acyl transferase enzyme can accommodate irregular acyl chain structures better than the enzymes responsible for acylation at the *sn*-1 or *sn*-2-positions of glycerol.

The large amounts of diisovaleroyl TGs (up to 83% by wt) and isovaleroyl wax esters found in some cetacean head lipids (2,3) have been correlated with the postulated role of the melon and jaw fat bodies in dolphin echolocation (23,24). In this hypothesis, the lower mol wt isovaleroyl lipids produce lower sound velocities that aid sound beam refraction. It is uncertain whether the 1,3-isovaleroyl specificity found represents some acoustical advantage for the animal or merely reflects the enzyme specificities of TG biosynthesis. Pure TG positional isomers of long chain acids exhibit practically identical sound velocities (25), but whether this is also true for the isovaleroyl-TG/wax ester mixtures found in dolphin acoustical tissues (26) remains to be seen.

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Influence of Diet on Conversion of $^{14}C_1$-Linolenic Acid to Docosahexaenoic Acid in the Rat

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ABSTRACT

$^{14}C_1$-Linolenic acid was incorporated into lipids of hearts, livers, and carcasses of male rats. We studied the influence of diet composition on extent and distribution of radioactivity. A CHOW diet, a purified, essential fatty acid (EFA)-deficient diet, a purified control diet, and EFA-deficient diets with four fatty acid supplements were used. Supplements of 18:2n-6, 20:4n-6, 18:3n-3, and 22:6n-3 were given as single doses. Radioactivities in liver phosphatidyl ethanolamines (PE), phosphatidyl cholines, and neutral lipids were measured. The distribution of radioactivity among the fatty acids in liver phospholipids was determined. Rats on CHOW diet incorporated far less radioactivity than any other group into lipids of hearts and livers. Most of the activity in livers was recovered as 20:5n-3 and 22:6n-3 in all rats. In EFA-deficient rats, the radioactivity in 22:6n-3 of liver PE was still increasing 36 hr after $^{14}C_1$-linolenic acid had been administered. The n-6 supplements (18:2n-6 and 20:4n-6) seemed to reduce the conversion of 20:4n-3 to 20:5n-3 (desaturation), whereas the n-3 supplements (18:3n-3 and 22:6n-3) reduced the conversion of 20:5n-3 to 22:5n-3 (elongation). Formation of 22:6n-3 may be controlled by 22:6n-3 itself at the elongation of 20:5n-3 to 22:5n-3.

INTRODUCTION

No biological function has yet been assigned to docosahexaenoic acid (22:6n-3) or, in fact, to any member of the n-3 (α-linolenate) series of fatty acids, either in mammals or in birds. [In the abbreviations used, e.g., 22:6n-3, the first number is the chain length, second number is number of double bonds, and final number is position of first double bond counted from methyl end of chain.] In contrast, rainbow trout appear to require dietary linolenic acid or its homologs, whereas members of the linoleate family (n-6) appear to have no specific function (1-4).

Nevertheless, the metabolic behavior of n-3 fatty acids strongly suggests that these fatty acids do have specific functions in warm-blooded animals. Members of the linolenate family are absorbed from the diet, incorporated into tissue lipids, elongated and desaturated efficiently, and, finally, are located specifically within certain lipid classes in particular organelles. For example, 22:6n-3 is the major unsaturated fatty acid in retinal rods of the dog, pig, sheep, bovine, rabbit, and human, and it is located especially in the phosphatidyl ethanolamine (PE) molecule (5). Large proportions of 22:6n-3 are also found in PE and phosphatidyl serines of many other tissues, such as rat liver, heart, rat brain synaptosomes, grey matter of the human brain, etc. This specific location of 22:6n-3 in particular phospholipids (PL) in specialized cells or organelles implies that 22:6n-3 should have some special function.

Previously, we attempted to produce symptoms of linolenate deficiency in rats by maintaining rats for three generations on a diet devoid of n-3 fatty acids. Although the levels of n-3 fatty acids in tissues became very low, small amounts of 22:6n-3 still remained, and the rats showed no abnormality in growth, reproduction, or appearance (6).

This report describes the incorporation of radioactive linolenic acid into the rat, and the rates at which it is converted to longer chain homologs. In addition, we studied the effects of several dietary manipulations on these parameters.

EXPERIMENTAL PROCEDURES

Experimental Design

To determine the extent and distribution of labeling in lipids in normal rats, $^{14}C_1$-linolenic acid was administered to rats which were sacrificed after 1, 3, 4, 6, 8, 12, 16, and 24 hr of incorporation. These rats had been maintained on Purina Laboratory Chow. Radioactivity in the total lipids of livers, hearts, and carcasses was measured. Liver lipids were fractionated, and the distribution of radioactivity among neutral lipids (NL), phosphatidylcholines (PC) and PE was determined. Fatty acids from liver PC and PE were converted to methyl esters, and the amounts and radioactivities of each of these were measured.

The extent and distribution of labeling was measured also in rats that had been fed a purified diet, deficient in essential fatty acids (EFA). Labeled linolenic acid was administered to rats that were sacrificed after 4, 6, 8, 12, 16, 24, and 36 hr of incorporation. A group of control rats was maintained on the EFA-deficient diet supplemented with linoleic acid. These were sacrificed 4 hr after administration of labeled linolenic acid.

To determine the effect of a lipid load on the metabolic behavior of linolenic acid, groups of EFA-deficient rats were tube-fed a single dose (50 mg/100 g body wt) of linoleic, arachidonic, linolenic, or docosahexaenoic acids. After 24 hr, labeled linolenic acid was administered, and the rats were sacrificed 4 hr later.

Animals and Diets

Male Sprague-Dawley rats (Horton Laboratories, Oakland, CA) were used throughout these studies. Three different diets were used: Purina Laboratory Chow (CHOW; Purina Co., Davenport, IA); an EFA-deficient diet (EFAD; Table I); and a control diet (CONTROL), which had the same composition as the EFAD diet, except that 1% of hydrogenated coconut oil was replaced by 1% of methyl linoleate. CHOW diet had 3.8% fatty acids (as measured by gas liquid chromatographic (GLC) analysis of a chloroform:methanol, 2:1, extract) of which ca. 47% was linoleic acid (18:2n-6), 5% α-linolenic (18:3n-3), 2% eicosapentaenoic (20:5n-3), and 2% was 22:6n-3. To produce the EFA-deficiency, mother rats were given the EFAD diet when litters were 1 week old to reduce the supply of 18:2n-6 and 18:3n-3 in the mothers' milk. This procedure produced an EFA deficiency, as shown by the liver PE fatty acid composition (Table II), in about 6 weeks. There was no mortality.

CHOW-fed rats were used when their wts were ca. 200 g. EFAD rats were used when they weighed ca. 200 g, at which time they were 7 weeks old. CONTROL rats, also ca. 200 g, were ca. 8 weeks old.

SUPPLEMENTED rats (ca. 200 g) were EFAD rats which had been tube-fed a single dose (50 mg/100 g body wt) of 18:2n-6, 18:3n-3, 20:4n-6 arachidonic acid, or 22:6n-3 fatty acids. After 24 hr, $^{14}C_1$-linolenic acid was administered and allowed to incorporate for 4 hr, after which the rats were sacrificed.

Food and water were available to the rats at all times. Livers averaged 4.5-5.8% of body wt, and hearts were 0.30 to 0.38% of body wt.

Administration of $^{14}C_1$-Linolenic Acid

$^{14}C_1$-Linolenic acid (58 mCi/mmol) in benzene solution was purchased from Amersham/Searle Corporation (Arlington Heights, IL). Radiopurity was 97-99%, as shown by gas chromatographic (GC) analysis. The benzene was removed under a jet of N_2, and to the residue was added 20% rat serum (rat fasted overnight) in isotonic saline. The solution was stirred 1 hr at 25 C. A rat was restrained in a cage, and the labeled solution (0.4 ml) was injected into the tail vein over a period of 1 min. The dose was 8.7 to 10.1 x 10^6 dpm/100 g rat.

TABLE I

Composition of Essential Fatty Acid-Deficient Diet

Major components	Parts by wt
Vitamin-free casein[a]	20.0
Powdered sucrose	66.5
Salt mix,[b] ICB-IRb	3.5
Choline chloride	0.28
Hydrogenated coconut oil[c]	10.0
Vitamins	Amount per 100 g diet
Vitamin A powder[d]	1690 units
Vitamin D_3 powder[d]	104 units
Vitamin E[d]	7 units
Thiamine hydrochloride[d]	0.50 mg
Riboflavine[e]	0.50 mg
Folic acid[a]	0.020 mg
Pyridoxine hydrochloride[d]	0.25 mg
Calcium pantothenate[e]	2.0 mg
Nicotinic acid[d]	2.5 mg
Biotin[a]	0.010 mg
Inositol, meso[d]	10.0 mg
Vitamin B_{12}[a]	0.0020 mg
Vitamin K (menadione)[d]	0.050 mg

[a]General Biochemicals, Chagrin Falls, OH.
[b]Ref. 7.
[c]Plastin IC, Durkee Co., Berkeley, CA.
[d]Nutritional Biochemicals, Cleveland, OH.
[e]American Cyanamid Corp., Wayne, NJ.

Lipid Extraction and Analysis

Animals were sacrificed by decapitation and their blood was collected. Livers and hearts were removed, rinsed with distilled water and isotonic saline solution, blotted with filter paper, weighed, and frozen on solid CO_2. Frozen livers and hearts were lyophilized and pulverized, and lipids were extracted as described earlier (6). The rest of the animal, including blood, was termed "carcass." Carcasses were digested in a saturated solution of KOH in 95% ethanol. Aliquots of the alkaline digest were acidified (H_2SO_4), and the liberated fatty acids were extracted into petroleum ether (br 30-55 C). All chemicals and solvents were reagent grade.

Liver lipids were fractionated according to the procedure of Skipski et al. (8). Bands were

TABLE II

Fatty Acids in Liver Phosphatidyl Ethanolamines from Male Rats[a] Fed Different Diets: Percent by Wt of Total Methyl Esters

	Diets[b]						
Fatty acid	CHOW	EFAD	CONTROL	18:2n-6 SUPP	20:4n-6 SUPP	18:3n-3 SUPP	22:6n-3 SUPP
16:0	16.6 ± 0.48[c]	17.2 ± 0.40[c]	16.2	17.1	15.7	16.4	16.7
16:1	0.57 ± 0.05	2.00 ± 0.11	0.60	1.96	1.13	1.43	0.62
18:0	21.6 ± 0.39	22.8 ± 0.43	24.8	21.3	23.4	25.7	24.6
18:1n-9	5.22 ± 0.19	13.6 ± 0.22	6.50	13.6	9.20	10.6	6.50
18:2n-6	9.03 ± 0.30	1.00 ± 0.07	4.80	2.00	0.20	0.41	0.30
18:3n-3	0.31 ± 0.02	0.31 ± 0.01	0.20	0.30	0.20	0.18	0.10
20:3n-9	---	20.0 ± 0.56	---	19.5	12.0	14.2	9.90
20:3n-6	1.12 ± 0.05	---	0.80	---	---	---	---
20:4n-6	22.4 ± 0.34	13.3 ± 0.32	31.5	17.3	29.0	10.1	9.30
20:5n-3	3.11 ± 0.16	0.68 ± 0.03	0.10	0.07	0.70	5.51	1.40
22:5n-6	0.22 ± 0.02	1.69 ± 0.09	9.60	1.70	2.40	1.08	0.70
22:5n-3	3.30 ± 0.13	0.10 ± 0.02	0.10	0.10	0.10	1.42	0.20
22:6n-3	15.6 ± 0.57	5.97 ± 0.43	2.60	4.80	4.20	12.0	28.4

[a]CHOW diet, averages of 16 individual measurements; EFAD (essential fatty acid-deficient) diet, averages of 14 individual measurements; CONTROL and SUPPLEMENTED diets, averages of three individual measurements.

[b]Supplements (50 mg/100 g body wt) were tube-fed 28 hr before rats were sacrificed.

[c]Mean ± standard error.

TABLE III

Incorporation of Radioactivity from Linolenic Acid into Heart, Liver, and Carcass Lipids of Rats Fed Different Diets[a]

Diet	Supplement[b]	Time after injection (hr)	Radioactivity in Heart (% of dose)	Liver (% of dose)	Carcass (% of dose)
CHOW	none	1	0.048	5.73	81.5
		3	0.056	7.71	79.6
		4	0.046	5.51	73.8
		6	0.059	5.72	73.2
		8	0.038	3.91	68.5
		12	0.030	3.06	69.8
		16	0.029	2.37	66.5
		24	0.025	2.19	63.3
EFAD	none	4	0.19	21.8	71.7
		6	0.16	17.6	68.4
		8	0.14	16.9	68.4
		12	0.16	15.2	68.5
		16	0.16	15.4	68.1
		24	0.19	16.7	56.5
		36	0.17	15.2	42.3
CONTROL	none	4	0.08	15.2	76.5
SUPPLEMENTED	18:2n-6	4	0.14	17.8	62.7
	20:4n-6	4	0.10	15.3	66.6
	18:3n-3	4	0.16	14.0	71.1
	22:6n-3	4	0.08	9.2	74.0

[a]CHOW and EFAD (essential fatty acid-deficient) diets, two rats/interval; SUPPLEMENTED and CONTROL diets, three rats/group.

bSupplements (50 mg/100 g body wt) were tube-fed 24 hr before administration of radioactivity.

scraped directly from thin-layer chromatography (TLC) plates into scintillation vials and counted with dioxane scintillation mixture. For analysis of radioactivity in individual fatty acids, PE and PC were eluted from the silica gel (9) and converted to methyl esters for analysis by GC (10).

Gas Chromatography

GC analyses were performed on an F & M Chromatograph (Model 810) equipped with a Packard fraction collector (Model 830). The instrument was calibrated with a mixture of methyl esters of 16:0, 18:0, 18:1n-9, 18:2n-6, 18:3n-3, 20:5n-3 and 22:6n-3. Other components (20:3n-9, 20:4n-3, 22:5n-6, and 22:5n-3) were identified by comparison with published relative retention times and by log plots (11). Individual methyl esters were collected in glass tubes containing glass wool, which were placed in scintillation vials and counted with toluene scintillation mixture. An internal standard, ^{14}C-heptadecanoic acid of known specific activity (Nuclear Chicago Co., Des Plaines, IL), was used to determine both the total mass and the total radioactivity in each methyl ester. All fatty acids or methyl esters used in the diets and in the GC standard were obtained from The Hormel Institute (Austin, MN) and were $\geqslant$ 99% pure by GC analysis.

Radioactivity Measurements

Radioactivities were measured with a Beckman LS-100 liquid scintillation counter. Toluene mixture (0.5% 2,5-diphenyloxazole in toluene) gave 81% counting efficiency, and dioxane mixture (0.6% 2,5-diphenyloxazole plus 10% naphthalene in dioxane, to which is added 0.1 vol H_2O) had 77% efficiency. Lipids in solution were evaporated to dryness and counted in toluene scintillation mixture.

Precision of Radioactivity Data

Most of the values for percentage incorporation of label (Tables III and IV) are averages of data that agreed within 10% or less for replicate animals, but a few values represent a range of up to 25%.

RESULTS

Table III shows the distribution of radioactivity in lipids of hearts, livers, and carcasses. Hearts incorporated very little activity under any conditions and were not analyzed further. Most of the activity was recovered in carcasses,

TABLE IV

Incorporation of Radioactivity from Linolenic Acid into Liver Lipids from Rats Fed Different Diets[a]

Diet	Supplement[b]	Time after injection (hr)	Radioactivity in[c] PC (% of dose)	PE (% of dose)	NL (% of dose)
CHOW	none	1	1.1	0.95	3.4
		3	2.1	1.3	2.5
		4	1.3	0.96	2.8
		6	1.2	1.0	2.6
		8	1.2	0.80	1.5
		12	1.0	0.56	1.1
		16	0.64	0.47	1.0
		24	0.80	0.53	0.63
EFAD	none	4	8.3	5.5	2.6
		6	6.9	6.8	2.1
		8	5.4	5.7	1.5
		12	5.5	4.2	1.7
		16	6.0	6.1	1.3
		24	4.8	9.4	1.7
		36	4.2	8.8	0.96
CONTROL	none	4	4.6	6.2	2.1
SUPPLEMENTED	18:2n-6	4	7.5	7.8	1.4
	20:4n-6	4	7.7	5.2	1.7
	18:3n-3	4	7.5	3.4	1.9
	22:6n-3	4	4.8	1.6	2.2

[a]CHOW and EFAD (essential fatty acid-deficient) diets, two rats/interval; SUPPLEMENTED and CONTROL diets, three rats/group.

[b]Supplements (50 mg/100 g body wt) were tube-fed 24 hr before administration of radioactivity.

[c]PC = phosphatidyl cholines; PE = phosphatidyl ethanolamines; NL = neutral lipids.

nearly all as unchanged linolenic acid (data not presented). Maximum incorporation into liver lipids was observed at 3 hr in CHOW rats, and by 4 hr in EFAD rats.

Incorporations into heart and liver lipids were much higher (2- to 5-fold) in the rats fed purified diets than in those fed CHOW (Table III). Carcass incorporations were ca. equal in CHOW and EFAD rats, so that more activity was recovered from EFAD rats than from the CHOW-Fed.

Rats on CONTROL or SUPPLEMENTED diets incorporated slightly less activity into hearts and livers than was found in EFAD rats. In particular, the CONTROL diet and the 22:6n-3 SUPPLEMENT reduced incorporation into hearts and livers in comparison with EFAD rats (4 hr) and diverted the label into the carcass.

Distribution of radioactivity among liver lipid classes is shown in Table IV. In CHOW rats, radioactivity was incorporated fastest and to the greatest extent into NL). PC possessed the next highest proportion and PE contained almost as much. Much smaller amounts were recovered in other lipids (data not shown). In sharp contrast, EFAD rats incorporated most of the activity into PC and PE, at levels 5-fold or more higher than in the CHOW rats. Neutral lipids, however, had amounts of radioactivity much like those found in CHOW rats.

The changes in activity with time in PE and PC of EFAD rats also contrasted with those in CHOW rats. In CHOW rats, all lipids gradually lost activity after 4 hr, but in EFAD rats the PE began to regain activity at 16-24 hr.

The CONTROL diet reduced incorporation into PC but not into PE, in comparison with EFAD rats. However, a single large dose of 18:2n-6, 20:4n-6, or 18:3n-3 had little effect on incorporation of radioactivity into PE or PC. In contrast, 22:6n-3 greatly reduced incorporation into both PE and PC.

Fatty acid patterns in liver PE are shown in Table II. PE from rats given CHOW diet reflected the dietary fatty acids in that there were considerable proportions of 20:5n-3, 22:5n-3, and 22:6n-3. PE from CONTROL rats, whose diet contained no n-3 fatty acids, had levels of n-3 fatty acids even lower than PE from EFAD rats. PE from CONTROL rats also contained an unusually high proportion of 22:5n-6, a phenomenon we have seen before (6). EFAD diet produced the well-known increase in

20:3n-9 and, despite the deficiency, the PE still retained considerable 22:6n-3.

Supplementation with a single dose of fatty acid produced various degrees of response in fatty acid patterns of liver PE. Supplementation with 18:2n-6 had little effect; only 20:4n-6 was increased a few percent above the proportion in unsupplemented EFAD rats, with very minor changes in other components. Supplementary 20:4n-6 produced greater changes, causing a reduction in 20:3n-9 and increasing 20:4n-6 to more than twice the EFAD value: 22:5n-6 was also increased. Supplementation with 18:3n-3 reduced the 20:3n-9 and increased the 20:5n-3 and 22:6n-3 greatly. Feeding 22:6n-3 produced the most dramatic effects by reducing 18:1n-9 and 20:3n-9 and by increasing 22:6n-3 almost 5-fold. There was a small increase in 20:5n-3, which suggests retroconversion of 22:6n-3 (12). Ca. 25% of the 22:6n-3 supplement was recovered in liver PE and PC as 22:6n-3.

Fatty acid patterns in liver PC (not shown) were similar to those in PE, and responses to the supplements paralleled those in PE.

Figure 1 shows the distribution of radioactivity among the important n-3 fatty acids in liver PC of EFAD rats as the conversion of 1-^{14}C-linolenic acid progressed with time. Very little activity was recovered as 18:3n-3 itself. The 20:5n-3 contained the highest percentage of the dose at 4 hr, and this activity declined with time. Its presumed precursor, 20:4n-3, had much less activity and maintained a steady level

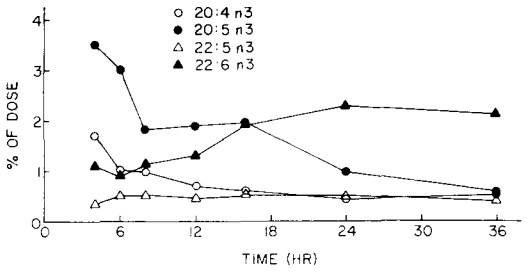

FIG. 1. Radioactivity in n-3 fatty acids of liver phosphatidyl cholines from essential fatty acid-deficient rats. Averages of data from two rats per time period.

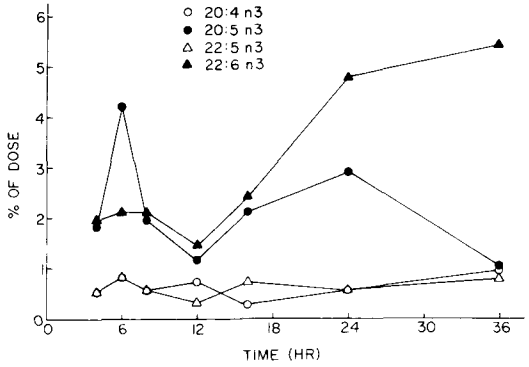

FIG. 2. Radioactivity in n-3 fatty acids of liver phosphatidyl ethanolamines from essential fatty acid-deficient rats. Averages of data from two rats per time period.

TABLE V

Distribution of Radioactivity Among n-3 Fatty Acids in Liver Phospholipids from Rats[a] Fed Different Diets: Percentage of Activity Recovered in n-3 Fatty Acids 4 hr after Administration of $^{14}C_1$-Linolenic Acid

	Diets						
				SUPPLEMENTED with[b]			
Fatty acid	CHOW	EFAD	CONTROL	18:2n-6	20:4n-6	18:3n-3	22:6n-3
Phosphatidyl cholines							
18:3n-3	17	3	3	3	2	4	5
20:4n-3	6	25	13	40	35	18	15
20:5n-3	67	51	24	33	37	63	64
22:5n-3	5	5	20	8	8	4	5
22:6n-3	5	16	40	16	18	10	12
Phosphatidyl ethanolamines							
18:3n-3	24	3	2	2	2	3	3
20:4n-3	3	11	5	33	14	6	6
20:5n-3	41	37	11	30	34	52	51
22:5n-3	18	11	25	13	18	14	8
22:6n-3	13	39	57	23	34	24	32

[a]There were three rats in the SUPPLEMENTED and CONTROL groups, and two rats each in CHOW and EFAD (essential fatty acid-deficient) groups.

[b]Supplements were fed 24 hr before administration of radioactivity.

of activity after 12 hr. The amount of activity in 22:5n-3 remained low throughout the experiment. By 4 hr, 22:6n-3 had already incorporated ca. 1% of the dose, and this fatty acid gained activity gradually until 24 hr when activity leveled off. In CHOW rats (data not presented), similar behavior was seen, although activities in each fatty acid were much lower.

Figure 2 shows the incorporation of activity from linolenic acid into fatty acids of liver PE from EFAD rats. This lipid class, although only half as large by wt as PC, incorpoated much more of the administered radioactivity. As in PC, 20:4n-3 and 22:5n-3 maintained a low steady level of activity throughout the experiment. The activity in 20:5n-3 fluctuated with apparent maxima at 6 and 24 hr. 22:6n-3 gained activity with time and appeared to be still increasing its activity even at 36 hr.

The effects of dietary differences on distribution of radioactivity among n-3 fatty acids of liver PL are shown in Table V. CHOW rats incorporated very little activity into PC and PE, and the largest proportions were recovered in 20:5n-3 and 18:3n-3. CONTROL rats, which might be expected to resemble the CHOW rats, instead incorporated the greatest proporations of activity into 20:5n-3 and 22:6n-3 of PC and into 22:5n-3 and 22:6n-3 in PE. Rats fed single supplements of 18:2n-6 and 20:4n-6 produced very similar distribution patterns of activity in PC; most activity was in 20:4n-3 plus 20:5n-3. A similar but less pronounced effect was produced in PE. Animals fed supplements of 18:3n-3 and 22:6n-3 incorporated the greatest proportion of activity into 20:5n-3 in both PE and PC.

DISCUSSION

The phenomena visible in the liver lipids are the result of many concurrent processes in the rat. These include uptake of the label from the circulation by tissues, incorporation of the linolenic acid molecule into lipids, acyl CoA derivatives or other products, and, of course, elongation and desaturation of the fatty acyl chains. Our data show the combined effects of all these processes.

Table III shows that EFAD rats incorporated into heart and liver lipids far more radioactivity than CHOW rats did. This effect can also be seen in CONTROL rats and rats supplemented with a single dose of fatty acid, which also incorporated much more activity than CHOW rats did. These differences can be attributed partly to elevated activities of lipogenic enzymes, which occur in rats given fat-free or EFAD diets. Dietary 18:2n-6, 20:4n-6, or 18:3n-3 reduce these activities to normal levels after 2-7 days (13,14). Probably our SUPPLEMENTED rats had insufficient time to reduce their enzyme activities to normal levels. However, CONTROL rats also incorporated more radioactivity than CHOW rats did. This suggests either that n-3 fatty acids (in the CHOW diet) can suppress enzyme activity more effectively than 18:2n-6 can, or that some other factor is responsible. The dietary carbohydrate can also influence fatty acid incorporation, inasmuch as Macdonald found higher levels of 18:2n-6 in liver lipids of rabbits fed sucrose in comparison with those given starch at the same level in the diet (15). Our purified diets contain sucrose, whereas CHOW diet contains polysaccharide. Thus, the sucrose may have increased the incorporation of linolenic acid and its homologs into the livers of all rats fed purified diets. The profound differences between CHOW-fed rats and those given purified diets illustrate dramatically the fallacy of comparing metabolic changes in CHOW-fed animals with those in animals fed well-defined diets (16).

The major path of chain elongation and desaturation of linolenic acid is as follows:

$$18{:}3n\text{-}3 \rightarrow 18{:}4n\text{-}3 \rightarrow 20{:}4n\text{-}3 \rightarrow 20{:}5n\text{-}3 \rightarrow$$

$$22{:}5n\text{-}3 \rightarrow 22{:}6n\text{-}3 \rightarrow ?$$

We found very little radioactivity in 18:3n-3 of liver PL even at the shortest periods of incorporation, although there already was activity in 22:6n-3 (Table V). This indicates that 18:3n-3 is not well incorporated into liver PL, although its metabolic products are. This observation is consistent with data obtained from feeding experiments. When rats were fed diets containing flaxseed oil (52% 18:3n-3), the amounts of 18:3n-3 in liver PE and PC were negligible, whereas the proportion of 20:5n-3 rose 10-fold (17).

Similarly, very little activity corresponding to either 18:4n-3 or 20:3n-3 was found. Thus, these intermediates, if they are formed, must be converted very rapidly to 20:4n-3.

There was little accumulation of radioactivity in 20:4n-3 of EFAD rats (Figs. 1 and 2). Its formation and removal must occur at equal rates because activity did not remain high in 20:4n-3, nor does its mass accumulate to measurable levels even when excess linolenic acid is fed in the diet (17).

In contrast, 20:5n-3 always contained a large proportion of the radioactivity (Figs. 1 and 2, Table V). Its rate of removal must have been lower than its rate of formation and incorporation. When flaxseed oil is fed, the amount of 20:5n-3 in rat liver PL rises ca. 10-fold (17). A

small quantity of this acid may also be formed by retroconversion of 22:6n-3 (Table II, 22:6n-3 SUPPLEMENTED) (12).

Figures 1 and 2 show that the radioactivity in 22:5n-3 remained low and constant, and its mass was low under various dietary conditions (Table II). Even in flaxseed oil-fed rats, 22:5n-3 does not rise above control levels in liver PE and PC (17). Its rates of formation and incorporation must be practically equal to its rate of removal from PL.

Radioactivity was present in 22:6n-3 at 4 hr and continued to increase up to 24 hr in PC, and was still rising in PE at 36 hr (Figs. 1 and 2). The fact that 22:6n-3 is usually the major n-3 fatty acid in PL suggests that its rate of removal must be slow.

Experiments with single-dose supplements were designed to show how dietary fatty acids would influence accumulation of intermediates in the elongation-desaturation sequence. Influences of the supplements can be separated into two effects, an effect on total incorporation of radioactivity into PL and another effect on distribution of radioactivity among the n-3 fatty acids. Table IV shows the effects of different diets on total incorporation into PL. The n-6 fatty acid supplements had little effect on early incorporation into PC and PE in comparison with EFAD rats, but 18:3n-3 reduced incorporation into PE and 22:6n-3 reduced incorporation into both PE and PC.

The distribution of radioactivity among the n-3 fatty acids probably reflects the effects of diet composition on chain elongation and desaturation (Table V). The many competitions between various fatty acid structures for desaturation, elongation, and incorporation, mostly observed in vitro, have been reviewed by Brenner (18). Usually, a fatty acid with more double bonds and greater chain length will be desaturated faster than a more saturated, shorter chain under equivalent conditions in vitro. In the living animal, concentrations of the reacting species are unknown, as are the contributions from side reactions. Table V shows that radioactivity from linolenic acid was often concentrated in 20:5n-3 but rarely in 22:5n-3. This suggests that conversion of 20:5n-3 to 22:5n-3 is the rate-limiting step in the formation of 22:6n-3, in vivo. This idea is supported by evidence from feeding experiments in which 20:5n-3, but not 22:5n-3, accumulated when high levels of 18:3n-3 were fed (17). Furthermore, the conversion of 20:5n-3 to 22:5n-3 appeared to be controlled by the amount of 22:6n-3 in liver PL (Table II). This is shown by the high proportions of radioactivity in 20:5n-3 found when the liver PL contain high levels of 22:6n-3, that is, in rats supplemented with a single dose of either 18:3n-3 or 22:6n-3 and in those on the CHOW diet (Table II). Likewise, when the liver PL contained extremely low amounts of 22:6n-3 (CONTROL diet, Table II), there was minimum accumulation of activity in 20:5n-3 (Table V). PL that contain 22:6n-3, therefore, seem capable of suppressing their own formation at the elongation of 20:5n-3 to 22:5n-3. These PL may inhibit this elongation by releasing 22:6n-3 (for example, through the action of phospholipase A_2), which is known to inhibit elongation of 18:3n-3 in vitro (19).

Desaturation of 20:4n-3 to 20:5n-3 appeared to be impaired in animals given supplements of 18:2n-6 or 20:4n-6 (Table V). This must have been a temporary effect caused by the sudden large dose of fatty acid because the CONTROL animals, fed small but adequate amounts of 18:2n-6 throughout the experiment, showed no suppression of this step.

Evidently, the rat has great ability to form 22:6n-3 from precursors and to incorporate large amounts of this acid into PL, especially PE. In addition, the amount of 22:6n-3 in liver PL of the rat appears to be controlled by 22:6n-3 itself for reasons as yet unknown. These elaborate systems for biosynthesis and incorporation of 22:6n-3 imply that this molecule should have some important function in the cell, but at present we have no specific suggestions.

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Physiological and Analytical Variation in Cholesterol and Triglycerides

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ABSTRACT

Plasma cholesterol and triglyceride levels were determined, on each of two AutoAnalyzer systems in 11 healthy subjects, weekly over a 10-week and monthly over a 12-month period. Analytical variation was 1-2% for cholesterol and 2-5% for triglyceride. Cholesterol and triglyceride values on frozen quality control serum pools were not indicative of absolute values on fresh plasma. Even though the two AutoAnalyzer systems averaged within 1-2 mg/dl for triglyceride and cholesterol on the serum quality control pools during the 12-month period, the two systems differed by 7-8 mg/dl on fresh or frozen plasma samples. The coefficient of physiological variation on the 10 weekly samples averaged 5% (range 3-10%) for plasma cholesterol and 18% (range 9-27%) for plasma triglyceride. Analysis of the monthly samples suggested significant ($P<0.05$) seasonal trends: cholesterol was highest in the winter months and lowest in October, whereas triglyceride was highest in January and February and lowest in May and December. We conclude that intra-individual variation can be an important source of error in attempting to make a genetic diagnosis of hyperlipidemia and/or in evaluating hypolipidemic regimens in a given subject.

INTRODUCTION

Total cholesterol and triglyceride levels are prognostic indices for coronary heart disease (1,2). As such, they are used to estimate the risk of such disease, assess the effectiveness of therapeutic regimens, and select subjects for coronary prevention trials. Furthermore, extensive family studies of the genetics of hyperlipidemia have been performed through analyses of plasma total cholesterol and triglyceride (3). These applications, particularly those which attempt to make a genetic diagnosis in a given hyperlipidemic subject, frequently are based on a single cholesterol or triglyceride determination. Because individual lipid variation, regardless of cause, limits the predictive value of such a single lipid determination, it is important to quantitatively estimate individual cholesterol and triglyceride variation in both normal and hyperlipidemic subjects. Furthermore, Groover et al. (4) have suggested that cholesterol variation may be more important than cholesterol concentration per se in predicting coronary disease.

Cholesterol and triglyceride levels are influenced by physiological and environmental factors such as diet, exercise, stress, and state of health, variations due to blood-drawing techniques, and analytical variation. Variations with season have been observed (5,6), though these may be due in part to changes in diet (6,7) or exercise pattern. In 1963 Cromie reported 7.6% mean coefficient of total variation ($CV = SD/\bar{x} \times 100$) in cholesterol and 26.2% in triglyceride in 88 males, but the time period of the study was not specified (8), nor were the analytical vs. the physiological components of this variation segregated. In a 3-month study of 28 adult male patients, Hollister et al. (9) reported a mean coefficient of total variation of 6.6% for cholesterol and 15.9% for triglyceride. The contribution of analytical to total variation was also not considered in these studies. Williams et al. (10,11) have estimated analytical variation ($CV = 3.9\%$) in cholesterol measurement by a direct Lieberman-Burchand method and cholesterol physiological variation ($CV = 6.4\%$) in 68 subjects over a 10-12 week period. More recently, a physiological variation of ca. 4% was reported for a group of nine males on 10 weekly samples (12). Variations within day and those caused by conditions prior to and during blood drawing, e.g., exercise, posture, and venous stasis, have also been studied (13-15).

We have attempted to quantify physiological and analytical variation in cholesterol and triglycerides in 11 healthy adults over a 10-week and a 12-month period. Sample collection and lipid analysis were performed according to Lipid Research Clinic (LRC) protocol (16).

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MATERIALS AND METHODS

Study Population

Eleven adult employees or associates of the Northwest Lipid Research Clinic participated in the 10-week phase of the study. Four were male; seven female. Six were in the third decade of life, three in the fourth, one in the fifth, and one in the sixth. All were Caucasians with the exception of one Oriental female. Two subjects were taking oral contraceptives, one a thyroid supplement, and one an anti-hypertensive medication. Diet and exercise were not strictly controlled, though participants were encouraged not to make major changes in diet or exercise level during the study.

Sample Collection

Plasma was obtained each week for 10 weeks between January 28 and April 14, 1974. Thereafter, samples were obtained the first week of each month through January, 1975. Subjects reported to the Clinic between 7:00 a.m. and 11:00 a.m. on the same day each week, after a 12-14 hr fast. Subjects who reported not fasting or who were not available for sampling were rescheduled the following day or the following week. Blood was collected under conditions standardized for the LRC program (16). The times of blood drawing and of the last previous food or drink consumption were recorded. Subjects were in the sitting position during blood drawing. A tourniquet, used to distend the vein, was removed after blood flow was obtained. Samples were collected through a 20 gauge needle into a 15 ml Vacutainer with a silicone lubricated stopper and containing 16 mg dry sodium ethylenediaminetetraacetic acid. Samples were mixed thoroughly by inverting and cooled immediately to 4 C. Cells were removed by centrifugation within 1 hr. Aliquots were stored at -20 C in sealed vials for analysis in one assay at the conclusion of the study. The remaining plasma, stored at 4 C, was used for routine cholesterol and triglyceride analysis. Each subject was assigned an identification number, and all samples were labeled by number and date of collection.

Analytical Procedures

Cholesterol and triglycerides were quantitated within 4 days of collection on the samples stored at 4 C. Measurements were performed on the two analytical systems, designated AA-I and AA-II, used in the LRC program (16). The preliminary extraction with isopropyl alcohol-zeolite mixture and the triglyceride procedure on both instruments are based on the method of Kessler and Lederer (17). Both procedures incorporate on-line mixing of base reagent (18). Blanks were determined and subtracted from total glycerides on all quality control pool samples and on other samples with glycerides >300 mg% (16). The AA-I cholesterol uses ferric chloride color reagent (19), and the AA-II uses Lieberman-Burchand reagent (16,20). Each system measures cholesterol and triglyceride simultaneously on the isopropyl alcohol extract. Calibration is by primary standards prepared by the Lipid Standardization Laboratory, Center for Disease Control, Atlanta, GA (CDC). The AA-II cholesterol calibration is adjusted downward ca. 10% with a reference serum (16) to obtain comparability to the manual Abell-Kendall method (21). To assess the importance of seasonal and between-assay analytical variation, samples were analyzed both soon after collection and on frozen aliquots at the conclusion of the study. Thus, for each plasma sample, cholesterol and triglyceride were each analyzed 4 times: on both the AA-I and AA-II, once immediately after blood collection, and again at the conclusion of the study. Samples measured immediately after collection were analyzed routinely in assays with other non-study plasma samples. Frozen samples measured at the conclusion of the study were thawed, mixed thoroughly, and analyzed in a single assay with samples from each subject in random order interspersed with quality control samples.

Quality Control

Twenty samples from each of three fresh plasma pools with low, normal, and elevated cholesterol and triglycerides, respectively, were randomly spaced in one assay to estimate the within-assay analytical variation. In addition, two frozen serum pools, one normal and one elevated, prepared by the Lipid Standardization Laboratory, CDC, were each analyzed 4 times per day. Reference values were established for cholesterol (21) and triglycerides (22) by the Lipid Standardization Laboratory. A frozen pool prepared from fresh donor plasma was analyzed once per day.

Definition of Terms

Analytical variation comprises the variation due to the extraction process and instrumental analysis. Physiological variation includes both true fluctuation within the individual and that produced by changes in such factors as diet, stress, and exercise. For this study, this variation includes a component referred to elsewhere (23) as pre-instrumental variation due to changes in blood collection, processing, and storage conditions.

TABLE 1

Analytical Variation of Cholesterol and Triglyceride Determinations on Autoanalyzers AA-I and AA-II

	Cholesterol CV (%)		Triglycerides CV (%)	
	AA-I	AA-II	AA-I	AA-II
Within-assay[a]	1.14	1.36	2.33	3.32
Total 10-week[b]	1.73	2.10	4.54	4.49
Total 12-month[b]	1.72	1.59	4.32	3.65

[a]Mean CV of low, normal, and elevated plasma pools, 20 determinations each in one assay.

[b]Weighted mean CV of normal and elevated serum pools, four determinations each per day, and one plasma pool, one determination per day.

Calculations

The mean value ($\bar{x}_T$) and standard deviation of the total variation (SD_T) were calculated for each of the four series of measurements on each individual during both the weekly and monthly periods. Analytical variation was estimated from results on the quality control pool samples. The CV ($SD/\bar{x} \times 100$) was computed for each pool for each series of measurements, and a mean coefficient of analytical variation (CV_A) calculated. An analytical standard deviation (SD_A) was calculated for each subject's level from the CV_A and his or her mean value. Physiological variation (SD_P) was calculated as the square root of the total variance minus the analytical variance ($SD_P = \sqrt{SD_T^2 - SD_A^2}$ [10]) from the four measurements of each individual's cholesterol and triglyceride.

RESULTS

Analytical Variation

Coefficients of analytical variation (CV_A) for within assay, 10-week, and 12-month assay periods on cholesterol and triglycerides by AA-I and AA-II methodologies are shown in Table I. The within-assay CV represented a mean CV obtained from a low, normal, and elevated plasma pool each analyzed 20 times. To simplify calculations, the CV_A was assumed to vary independently of lipid level even though CV_A varies slightly with triglyceride level (16). The 10-week and 12-month CV_A represented a weighted mean of the two serum pools analyzed 4 times each and the plasma pool analyzed once per assay. The analytical variation in lipid analysis for both AA-I and AA-II systems was quite small, only 1-2% for cholesterol and 2-5% for triglyceride. Accuracy on both instruments as assessed by the two CDC serum pools was within 1 mg/dl of the reference method for cholesterol and 2 mg/dl for triglycerides.

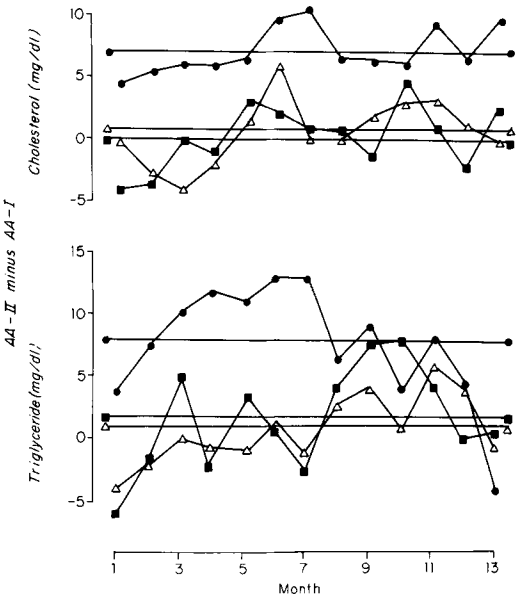

FIG. 1. Comparison of AA-I and AA-II cholesterol and triglyceride determinations, results expressed as AA-II minus AA-I value, with the fresh plasma represented by closed circles (●), the normal frozen serum pool by open triangles (△), and the elevated frozen serum pool by closed squares (■). The average of the overall difference between AA-I and AA-II is represented by a horizontal line.

Comparison of AA-I and AA-II

The mean monthly difference between AA-I and AA-II cholesterol and triglyceride values during the 12-month study is shown in Figure 1. For both cholesterol and triglyceride, the difference in accuracy on the quality control pool samples did not exactly predict the accuracy on fresh plasma samples (n=150). During the 12-month period of the study, the AA-I cholesterol on frozen serum pools averaged within 1 mg/dl of that on the AA-II, whereas on fresh plasma the AA-II was 7 mg/dl higher than the AA-I. Assuming a linear rela-

TABLE II

Physiological Variation of Plasma Cholesterol and Triglyceride

	Weekly variation			
	Cholesterol		Triglyceride	
Subject	Mean[a] (mg/dl)	CV_P[b] (%)	Mean[a] (mg/dl)	CV_P[b] (%)
10	208	4.85	62	14.26
11	224	5.58	202	17.66
20	188	7.20	59	19.77
21	193	6.02	72	9.20
30	157	10.15	54	16.99
31	155	4.13	116	26.64
40	170	4.85	45	18.70
41	159	3.05	66	18.71
51	258	4.03	141	17.91
60	184	6.11	80	21.16
70	208	3.60	59	14.27
	Group mean (mg/dl)	Group CV_P (%)	Group mean (mg/dl)	Group CV_P (%)
	191	5.42	87	17.75
	Monthly variation			
	Group[c] mean (mg/dl)	Group CV_P (%)	Group mean (mg/dl)	Gropp CV_P (%)
	194	6.22	81	18.55

[a]Mean of 10 weekly samples, four analyses per sample.

[b]CV_P (or coefficient of physiological variation) = $(SD_P/\bar{x} \times 100)$, where $SD_P = \sqrt{SD_T^2 - SD_A^2}$ and $SD_A = (CV_A \cdot \bar{x})/100$.

[c]Group mean of 12 monthly samples for each subject, four analyses per sample.

tionship between cholesterol values on fresh plasma on the two AutoAnalyzer systems, the two methods were related as follows: AA-II value (mg/dl) = 1.06 AA-I value (mg/dl) - 3.86 (mg/dl), r = 0.99.

For triglycerides, the two AutoAnalyzer systems were on the average within 1-2 mg% with the quality control pools, whereas with fresh plasma the AA-II was ca. 8 mg/dl higher than the AA-I. During June and July, the average difference between the two systems was 13 mg/dl. The relationship found between the two AutoAnalyzer methods for triglyceride analysis on fresh plasma was as follows: AA-II value (mg/dl) = AA-I value (mg/dl) + 8.16 (mg/dl), r = 0.99.

Physiological Variation

Table II presents the 10 weekly and 12 monthly physiological variation (CV_P) for cholesterol and triglyceride. Coefficients of physiological variation on weekly samples ranged from 3.6% to 10.2% for plasma cholesterol and from 9.2% to 26.6% for plasma triglyceride, with means of 5.4% and 17.8% for plasma cholesterol and triglyceride, respectively. The mean physiological variation on the monthly samples was only slightly greater than that obtained on the weekly samples, 6.2% for cholesterol and 18.6% for triglyceride. There was no correlation between an individual's CV_P and mean level for either cholesterol or triglyceride. Each individual CV was based on the mean of four analyses on each sample. The CV derived from each of the four analyses were in good agreement (within 4% on the average for cholesterol and within 8% for triglyceride).

Because the physiological variation included pre-instrumental variation, a separate experiment was performed in which 54 and 22 duplicate samples were obtained at venipuncture for cholesterol and triglyceride analyses, respectively, and analyzed in routine fashion. An estimate of pre-instrumental variation was obtained by subtracting the estimate of long-term analytical variance from the estimate of the variance obtained on the paired blood duplicates, where the latter variance includes the variance of the blood collection and processing procedures as well as that caused by instrumental error. These results suggested a pre-instrumental variation of 2.9% for cholesterol and 2.6% for triglyceride.

Seasonal Variation

To minimize day-to-day analytical variation and thereby obtain a more accurate estimate of seasonal variation, the analysis of monthly samples performed in one assay at the conclusion of the study was used to assess seasonal variation. Examination of the mean monthly cholesterol values for the 11 subjects showed a varying seasonal pattern (Fig. 2). Cholesterol was significantly higher ($P<0.05$) in the winter month of December and significantly lower ($P<0.05$) in October than the yearly mean cholesterol for the group. Furthermore, cholesterol was somewhat higher than the yearly mean cholesterol during the winter months of November and January. The pattern seen for the group was reflected in the pattern observed for individuals. In December, 91% of the group had cholesterol levels above their respective yearly mean and in October 91% had cholesterol levels below their yearly mean.

Triglyceride also had a variable pattern but did not follow the same pattern as cholesterol. Triglycerides for the group were significantly higher ($P<0.05$) in January and February and significantly lower ($P<0.05$) in May and December. In January and February, 100% and 64%, respectively, had triglyceride levels above their yearly means whereas, in May and December, 91% and 64%, respectively, had triglycerides below their yearly average.

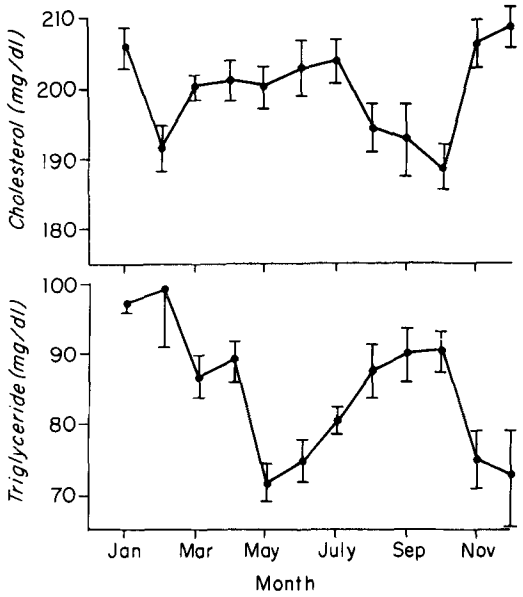

FIG. 2. Seasonal variation in cholesterol and triglyceride. Each monthly value represents the group mean of two analyses on each plasma sample performed at the conclusion of the study, once on the AA-I and once on the AA-II. Vertical bars represent the standard error of the mean group deviation of each individual from his own yearly mean.

DISCUSSION

This study has attempted to quantitate the analytical and physiological (including preinstrumental) components of the total variation in plasma cholesterol and triglyceride levels for a healthy adult working group for 10-week and 12-month periods. The coefficient of analytical variation for the cholesterol analysis over the 12-month period was 1.72% and 1.59% on the AA-I and AA-II, respectively. This analytical variation is ca. one-half of the 3-4% variation reported by previous authors (11,23). For triglycerides, a coefficient of variation of 4.32% and 3.65% for the AA-I and AA-II, respectively, was obtained. The cholesterol determination is, therefore, more precise than the triglyceride analysis, and the AA-II appears to be more precise than the AA-I for both cholesterol and triglyceride for the year.

The high correlations between the AA-I and AA-II for both cholesterol and triglyceride ($r = 0.99$) reflect the excellent precision obtained on these instruments during the course of this study. There were, however, consistent systematic differences between the two analytical systems. A fresh plasma sample which contained 200 mg/dl cholesterol as determined by the AA-I would contain 208 mg/dl on the average by the AA-II. Furthermore, the AA-II obtained triglyceride values which were ca. 8 mg/dl higher than the AA-I. This systematic bias between instruments on fresh plasma samples was not reflected with the serum quality control samples. The reason for the difference between fresh plasma and the serum pools has not been determined but may relate to the preparation of the quality control pools, e.g., age of pooled serum, addition of nonhuman plasma proteins. These results demonstrate the importance of using quality control samples which are as similar as possible to the test samples. No inferences should be extrapolated from this data concerning absolute accuracy of the methods on fresh plasma samples; results pertain only to the specific instruments and time period of study.

The physiological component of variation was ca. 4 times greater than the analytical variation obtained for cholesterol and triglyceride analysis. The magnitude of the physiological variation was such that, for example, 95% of replicate samples from a subject with a mean plasma cholesterol of 250 mg/dl would be in the range of 218-282 mg/dl, with 5% of the samples outside of this range, assuming the

mean CV_P of 6.4% for cholesterol. For triglyceride, the coefficient of total variation was ca. 19% (range 10-27%), almost 3 times the variation for cholesterol. This is not surprising inasmuch as triglyceride has a considerably faster metabolic turnover than cholesterol, and triglyceride levels are very susceptible to hormonal and lypolytic stress factors. A subject with a mean plasma triglyceride of 150 mg/dl would be expected to have values within the range of 93-207 mg/dl 95% of the time. Therefore, to assess a significant therapeutic effect on triglyceride levels, this subject would need to have a triglyceride level outside the maximum range which occurs due to normal variation, i.e., ca. 2 times the coefficient of variation or 38% of the subject's mean value. These results demonstrate that intra-individual variation of plasma lipid levels is an important potential source of error in making a genetic diagnosis or in the evaluation of a therapeutic regimen.

Even though the effect of the season of the year on cholesterol level was assessed on a small sample size [11], it is of interest that higher cholesterol levels were obtained during the winter months of November, December, and January, with the lowest level in October. A previous study on seasonal trends in cholesterol also found higher levels in the winter months and a minimum level in October (5). For triglycerides, the highest levels were obtained in January and February with the lowest levels obtained in May and December. An earlier study reported a highly significant fall in plasma triglyceride from summer and winter (6), whereas a more recent report suggested serum triglycerides tended to be higher during the winter months (24).

It should be stressed that these studies were performed in a normolipidemic employee group. Additional investigation will be required to assess the magnitude of physiological, including seasonal, variation in a hyperlipidemic and/or atherosclerotic subject population, inasmuch as variation per se might vary according to the genetic and/or environmental basis of hyperlipidemia and the size of tissue lipid storage pools.

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Mass Spectrometric Analysis of the Fatty Acids and Nonsaponifiable Lipids of *Eimeria tenella* Oocysts

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ABSTRACT

The fatty acids and nonsaponifiable lipids of *Eimeria tenella* oocysts were analyzed by gas liquid chromatography and combined gas liquid chromatography-mass spectrometry. The fatty acids detected were identified as $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, and $C_{18:2}$. Though the wt of the fatty acid fraction decreased during sporulation from 91 μg per 10^6 oocysts to 47 μg per 10^6 oocysts, the relative amounts of these fatty acids did not change appreciably. The nonsaponifiable lipids of *E. tenella* consisted of cholesterol and unbranched primary alcohols of 22, 24, 26, 28, 30, and 32 carbons. Mass fragmentography demonstrated that each species of alcohol consisted of saturated and monounsaturated derivatives. Trimethylsilyl ethers of fatty alcohols were found to offer several important advantages over free alcohols for mass spectrometric characterization. Before sporulation, most fatty alcohols were in the oocyst wall. During sporulation, the wt of the nonsaponifiable lipids increased from 16 μg per 10^6 oocysts to 44 μg per 10^6 oocysts due largely to synthesis of C_{24} and C_{26} alcohols. The newly synthesized fatty alcohols were not deposited in the oocyst wall.

INTRODUCTION

Eimeria tenella is a protozoan parasite which reproduces within the epithelial cells of the caeca of the domestic checken (1). After reproduction within this host, unsporulated oocysts are passed with the feces and begin to sporulate upon exposure to atmospheric oxygen. During sporulation, four sporocysts, each containing two infective sporozoites, are formed from the undifferentiated cytoplasm of the unsporulated oocyst. Because unsporulated oocysts can be purified from infected caeca (2), sporulation can be studied in vitro.

The lipids of *E. tenella* and their metabolism during sporulation have not been well characterized. Ryley (1) extracted lipid from the portion of the oocyst wall which remained after extensive sodium hypochlorite digestion and found that it contained what appeared to be waxy esters as well as some nitrogen and phosphorus. Previously, Strout et al. found a 25% decrease in total lipid during sporulation. Cholesterol was shown to be the major cytoplasmic sterol, but the remaining nonsaponifiable lipids were not identified (3).

For the related coccidial species *Eimeria acervulina*, Wilson and Fairbairn (4) found a 50% decrease in total lipid during the later stages of sporulation. They also found that the respiratory quotient decreased to less than unity at about the time lipid began to decrease and concluded that lipid was being oxidized to CO_2 to provide energy for the final stages of sporulation. More recently, van der Horst and Kouwenhoven (5), using gas-liquid chromatography (GLC), found five components among the nonsaponifiable lipids of sporulated *E. acervulina* oocysts and concluded from their retention times that three of these were cholesterol, squalene, and progesterone.

In the present communication, we report the identification of the major fatty acids and nonsaponifiable lipids of *E. tenella* oocysts and an investigation of their changes during sporulation. The distribution of nonsaponifiable lipids within oocysts before and after sporulation has also been characterized.

EXPERIMENTAL PROCEDURES

Lipid Standards

Cholesterol and hexacosanol were obtained from Analabs, Inc., (North Haven, CT). *cis*-11-Eicosen-1-ol (gondoyl alcohol), *cis*-5-eicosen-1-ol, and 1-eicosenol (arachidyl alcohol) were obtained from Applied Science Laboratories, Inc., (State College, PA).

E. tenella Oocysts

Unsporulated oocysts of *E. tenella* Merck Strain No. 18 were purified, stored, and sporulated by previously described procedures (2).

Preparation of Lipid Extracts

Lipid was extracted from *E. tenella* oocysts by a modification of the Folch procedure (6). Ten vol of methanol were added to a pellet containing 2 x 10^8 oocysts per ml, and the suspension was sonicated in an ice bath at 40-60 watts with a Sonifier Cell Disruptor Model 185

(Heat Systems – Ultrasonics, Inc., Plainview, NY). Cell disruption was monitored microscopically. Sonication of unsporulated oocysts was continued until <5% of the oocysts remained (ca. 10 min). Sonication of sporulated oocysts was continued until < 5% of the sporocysts remained (ca. 15 min). Sonication was necessary because oocysts were not readily disrupted by suspension in chloroform:methanol, 2:1, or by suspension in either of these solvents alone.

Two vol of chloroform and two vol of water:methanol, 1:1, containing 0.75% KCl (Folch upper phase) were added to the methanolic sonicate (6). The aqueous phase was removed and was back-extracted with chloroform:methanol, 2:1. The two organic phases were pooled and washed 3 times with Folch upper phase. Finally, the three aqueous phases were pooled and back-extracted once with chloroform:methanol. The two organic phases were then pooled and dried to constant wt at 70 C under a stream of nitrogen.

To determine recovery by this procedure, palmityl [1^{14}C] alcohol (DHOM Products, Ltd., North Hollywood, CA) was added to the methanolic sonicate, and the number of counts recovered in the final lipid preparation was determined. Recoveries in three replicates of this experiment were 62%, 73%, and 78%. All gravimetric data were corrected for the yield of that particular extraction.

Saponification

One ml of methanol and 1.0 ml of 10 *N* KOH were added to ca. 5 mg of lipid, and the mixture was heated at 90 C for 2 hr. The mixture was extracted 3 times with petroleum ether, and the pooled extracts were washed 3 times with water. Finally, the three aqueous phases were pooled and back-extracted once with petroleum ether. The two ether extracts were pooled and dried to constant wt as described above. Gravimetric data for the nonsaponifiable lipids were corrected for recovery as determined from the recovery of palmityl [1^{14}C] alcohol.

The aqueous phases were then pooled and, following acidification with HCl, the fatty acids were extracted as described above for the nonsaponifiable lipids. Recoveries, which were measured by adding ^{3}H-palmitate to the saponification mixture and then determining the counts recovered in the fatty acid preparation, were used to correct gravimetric data. The recovery was ca. 80%.

Oocyst Fractionation

To determine the distribution of the nonsaponifiable lipids, aqueous suspensions containing ca. 10^{8} oocysts per ml were sonicated as described above. The sonic extracts were then centrifuged at 10^{4} g for 20 min, and the supernatant was decanted.

The pellet was resuspended in 10 *N* KOH, and the supernatant was made 10 *N* by addition of solid KOH. Both samples were heated at 90 C for 10 min. Equal vol of methanol was then added, and the saponification and extraction of nonsaponifiables were performed as described above. Estimations of the wt of the different nonsaponifiable components were based on their peak area in GLC, determined by triangulation, compared with that of arachidyl alcohol, a known amount of which had been added to the saponification mixtures. Arachidyl alcohol is not a component of *E. tenella* oocysts.

Analytical Gas-Liquid Chromatography

GLC was performed on a Perkin-Elmer 900 gas chromatograph equipped with a flame ionization detector. Columns were of glass, 6 ft x 1/4 in. outside diameter, packed with 3% OV-1 on 60/80 mesh Gas-Chrom Q. Injector and detector temperatures were 300 C, and a flow rate of 70 ml/min (helium) was employed. The column temperature for analysis of fatty acid methyl esters was 185 C and that for analysis of nonsaponifiable lipids was 250 C.

TABLE I

Gravimetric Analysis of Lipids from *Eimeria tenella* Oocysts

Source	Total lipid	Fatty acids	Nonsaponifiable lipids
	(μg per 10^{6} oocysts)		
Unsporulated oocysts	128	91	16
Sporulated oocysts	130	47[a]	44[a]

[a]These data were corrected for the fact that 10% of the oocysts did not sporulate. The lipid content of these oocysts was assumed to remain constant.

Preparation of Fatty Acid Methyl Esters

Ca. 5 mg of fatty acids were dissolved in 1.0 ml of methanol containing 2% sulfuric acid, and the mixture was heated at 90 C for 30 min. Several ml of water were then added, and the methyl esters were extracted with petroleum ether.

Preparation of Trimethylsilyl (TMSi) and Deutero-Trimethylsilyl (TMSi-d_9) Ethers

One to five mg of nonsaponifiable lipids were dissolved in 1.0 ml of *bis*(trimethylsilyl)-acetamide or *bis*(trimethylsilyl)acetamide-d_9 in pyridine (2:1) and allowed to stand at room temperature for 20 min before use.

Combined Gas-Liquid Chromatography–Mass Spectrometry (GLC-MS)

GLC-MS was carried out with an LKB-9000 instrument. The columns were 1.5 m x 3 mm inside diameter spiral glass containing 3% OV-1 on 80/100 mesh, acid-washed and silanized Gas-Chrom P. Column temperatures were programmed at 5 C/min from 180 C, and helium (30 ml/min) was employed as carrier. The spectrometer conditions were as follows: 70 eV electron energy, 3.5 kV accelerating voltage, 60 μA trap current, and 270 C source temperature.

RESULTS

Gravimetric Analysis of Oocyst Lipid

The representative data from one experiment shown in Table I indicate that the total lipid remained ca. constant during sporulation at ca. 130 μg per 10^6 oocysts. Fatty acids decreased by almost 50%, but this decrease in wt was almost matched by the nearly 3-fold increase in nonsaponifiable lipids. Two replicates of this experiment yielded values which were within 15% of those in Table I.

Fatty Acids of *E. tenella* Oocysts

GLC analysis of methyl esters prepared from the fatty acids of either unsporulated or sporulated oocysts revealed seven major components. Combined GLC-MS demonstrated that these components possessed, in order of their retention times on an OV-1 column, molecular ions of m/e 242, m/e 268, m/e 270, m/e 294, m/e 296, and m/e 298. The possibility of branching cannot be definitively excluded from mass spectra (7). However, the molecular ions above are those from methyl esters of fatty acids with even numbers of carbons, and most naturally occurring branched fatty acids are of odd numbers of carbons (8). These considerations suggest that the fatty acids of *E. tenella* are unbranched and are, in order of their retention times on an OV-1 column, 14:0 (myristic acid), 16:1 (palmotoleic acid), 16:0 (palmitic acid), 18:2 (linoleic acid), 18:1 (oleic acid), and 18:0 (stearic acid). These assignments are supported by the observation that the retention times of the fatty acid methyl esters from the isolate matched those of the appropriate reference methyl esters. The relative amounts of these fatty acids did not change appreciably during sporulation (Table II), even though the total mass of the fatty acid fraction decreased considerably (Table I).

TABLE II

Fatty Acid Methyl Esters from *Eimeria tenella* Oocysts

Fatty acid	Unsporulated oocysts (%)[a]	Sporulated oocysts (%)[a]
14:0	1	2
16:1	1	3
16:0	12	8
18:2	Trace	Trace
18:1	75	68
18:0	10	17

[a]Percent was determined from the peak area of the component and the total peak area of all components.

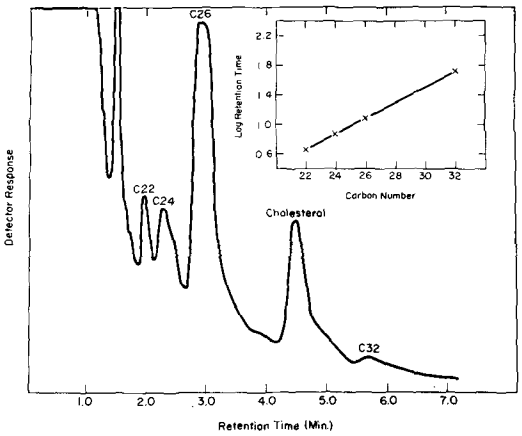

FIG. 1. Gas-liquid chromatogram of nonsaponifiable lipids from sporulated *Eimeria tenella* oocysts.

Nonsaponifiable Lipids of *E. tenella* Oocysts

GLC of the free nonsaponifiable lipids from sporulated oocysts revealed five components (Fig. 1). The TMSi ethers and TMSi-d_9 ethers of the nonsaponifiable lipids were prepared, and these, as well as the underivatized sample, were subjected to GLC-MS. Comparison of the retention times and mass spectra of the third peak with those from a reference sample demon-

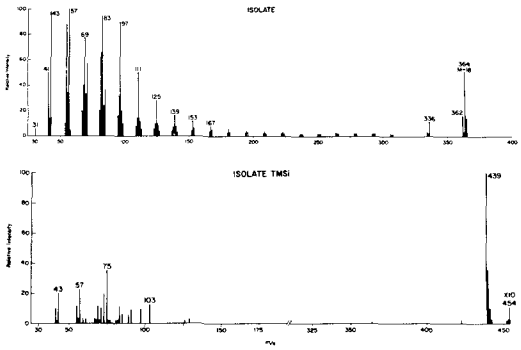

FIG. 2. Mass spectra of the free (upper and trimethylsilyl (TMSi) ether (lower) of the C_{26} component from the isolate.

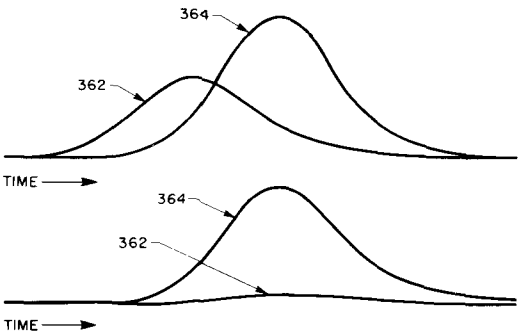

FIG. 3. Mass fragmentograms of chromatographic peak due to the free C_{26} component (upper) and peak due to standard hexacosanol (lower).

strated conclusively that this component was cholesterol. The remaining components yielded mass spectra which suggested they were mol wt homologues of the predominant component.

The mass spectrum for the underivatized C_{26} component taken from the middle of the peak on OV-1 (Fig. 2) agrees well with that expected for hexacosanol, an unbranched, saturated primary alcohol of 26 carbons (7,9). In particular, there was no molecular ion; fragments of highest mass occurred at m/e 364 and m/e 362, formally corresponding to loss of H_2O and loss of H_2O and H_2 from the molecular ion. A major fragment was also seen at m/e 336, corresponding to loss of water and C_2H_4 from the molecular ion. The remainder of the spectrum consists primarily of ions which can be described by the formula C_nH_{2n-1}. The fragment at m/e 31 is unique to primary alcohols and is presumably due to the fragment $[CH_2OH]^+$ (7,9).

Figure 2 also shows the mass spectrum of the TMSi ether of the C_{26} fatty alcohol taken from the center of the peak from an OV-1 column. A molecular ion of weak intensity can be seen at m/e 454, and a very intense M-15 signal occurred at m/e 439 due to loss of a TMSi methyl group from the molecular ion. The m/e 31 ion of the underivatized sample has been replaced by an ion of m/e 103 which corresponds to $[CH_2\text{-}OSi\ (CH_3)_3]^+$. This spectrum agrees well with the mass spectra reported for the TMSi ethers of 1-decyl alcohol and other medium chain alcohols (10).

The required mass shifts were observed with the deutero TMSi ether of the C_{26} component. The molecular ion appeared at m/e 463, and a very intense ion was observed at m/e 445 (loss of CD_3 from the molecular ion). An ion at m/e 112, corresponding to $[CH_2\text{-}O\text{-}Si\ (CD_3)_3]^+$, was also evident.

Comparison of the mass spectra and retention times of these three compounds with those from a reference sample of hexacosanol and from its TMSi and TMSi-d_9 ethers confirmed that the major C_{26} component was this fatty alcohol. The mass spectra of the remaining three components were quite similar to those of hexacosanol, and it was concluded that the major C_{22}, C_{24}, and C_{32} components were saturated, unbranched primary alcohols of 22, 24, and 32 carbons, respectively. As shown in Figure 1, the logs of the retention times of these four alcohols were a linear function of carbon number, as would be expected for members of an homologous series. When TMSi ethers of very concentrated lipid preparations were analyzed by GLC-MS, two additional, minor alcohols of 28 and 30 carbons were detected by their M-15 fragments.

Mass spectra taken across the chromatographic peak of the C_{26} component suggested that it was not homogenous and contained a small amount of the monounsaturated C_{26} alcohol hexacosenol which was incompletely resolved from the more abundant hexacosanol. Mass fragmentography (11) of the underivatized C_{26} component using an OV-1 column showed that the ratio of the m/e 362 and m/e 364 ions changed with time across the chromatographic peak, with the m/e 362 ion preceding the m/e 364 ion (Fig. 3). When the same experiment was performed with reference hexacosanol, the m/e 362 ion was much less intense and was coincident with the m/e 364 ion (Fig. 3). When a similar experiment was performed with a polar ECNSS column, from which an unsaturated compound elutes later than the analogous saturated compound, the intensity maximum of the m/e 364 ion of the C_{26} component preceded that of the m/e 362 ion. This demonstrated that the C_{26} peak was nonhomogeneous and that the species produc-

ing the m/e 362 fragment eluted at a different retention time from the species yielding the m/e 364 ion. Apparently, in the two component peak from the isolate, the m/e 362 fragment was largely due to loss of water from the monounsaturated alcohol rather than to loss of water and molecular hydrogen from the saturated alcohol. The mass fragmentography data in Figure 3 do not provide a direct measure of the amounts of hexacosanol and hexacosenol in this chromatogrphic peak, because experiments with reference compounds have shown that monounsaturated fatty alcohols produce M-18 ions which are considerably more intense than those produced by the homologous saturated alcohol. Experiments with reference hexacosanol demonstrated that the intensity of the m/e 362 ion (M-20) relative to that of the m/e 364 ion (M-18) was directly proportional to the temperature of the stainless steel molecular separator between the column and the spectrometer. When the reference compound was introduced by direct probe and volatilized at relatively low temperature, the m/e 362 ion was almost entirely eliminated.

The mass spectrum of the TMSi ether of the C_{26} component, taken from the leading edge of the chromatographic peak from an OV-1 column, confirmed that a portion of the C_{26} peak was due to the monounsaturated alcohol hexacosenol (Fig. 4). The expected molecular ion at m/e 452 and the M-15 ion at m/e 437 are evident. This spectrum differs in several additional ways from that of the TMSi ether of hexacosanol (Fig. 2). The molecular ion of hexacosanol is considerably less intense than its M-15 peak, whereas the corresponding ions of hexacosenol are at about equal intensity. Hexacosenol produced unique fragments at m/e 129, m/e 143, and m/e 185, as well as an intense M-90 ion at m/e 362. The TMSi ethers of commercially available 11-eicosen-1-ol and *cis*-5-eicosen-1-ol produced mass spectra which also possessed intense molecular ions and fragments at m/e 129, 143, 185, and at M-90. Apparently these effects are independent of double bond position and of chain length, at least for the TMSi ethers of monounsaturated fatty alcohols of 20-26 carbons. The TMSi ethers of monounsaturated alcohols seem similar in this regard to methyl esters of monounsaturated fatty acids in that most positional isomers yield very similar spectra (7). Accordingly, the position of the double bond in the hexacosenol from the isolate could not be assigned.

Similar experiments demonstrated that the C_{22}, C_{24}, C_{28}, C_{30}, and C_{32} components from both sporulated and unsporulated oocysts

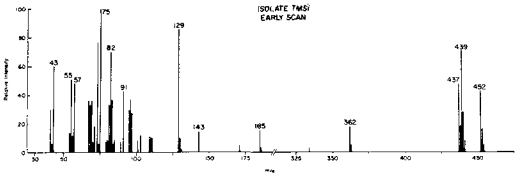

FIG. 4. Mass spectrum taken from the leading edge of the chromatographic peak due to the C_{26} component. TMSi = trimethylsilyl.

also consisted in part of a small amount of monounsaturated alcohol which was incompletely resolved chromatographically from the predominant, saturated alcohol.

Distribution of Nonsaponifiable Lipids in Oocysts

Microscopic examination of a 10^4 g pellet from a sonic extract of oocysts revealed that it contained amylopectin granules, the coccidial energy reserve (1,12), and oocyst wall fragments. GLC analysis of the nonsaponifiable lipids from the pellet showed that it contained very little cholesterol (Table III), suggesting that the wall fragments were not appreciably contaminated with cellular membranes. For unsporulated oocysts, most fatty alcohols were found in the pellet with the wall fragments. During sporulation, the total amount of fatty alcohol increased with the increase being especially dramatic for C_{22} and C_{24} alcohols which increased more than 10- and 4-fold, respectively. For all species of alcohol, the increase occurred in the 10^4 g supernatant fraction and, at the end of sporulation, this fraction contained about 3 times as much fatty alcohol as the oocyst wall fraction.

In this experiment, the percent C_{26} alcohol not esterified was determined from GLC peak area before and after saponification. Eighty seven percent of the C_{26} alcohol in the supernatant and 95% of that in the pellet from unsporulated oocysts was free. For sporulated oocysts, 78% in the supernatant and 80% of the pellet C_{26} alcohol was free. The C_{22} and C_{24} alcohols of both sporulated and unsporulated oocysts were also found to be amost entirely free.

DISCUSSION

Gravimetric analysis revealed that the total lipid content of *E. tenella* oocysts remained ca. constant during sporulation (Table I). Though the wt of the fatty acid fraction decreased by ca. 50% during sporulation, this decrease was almost matched by a ca. 3-fold increase in the wt of the nonsaponifiable lipids (Table I). These data suggest that lipids are not an im-

TABLE III

Distribution of Nonsaponifiable Lipids in *Eimeria tenella* Oocysts

Component	Unsporulated oocysts			Sporulated oocysts		
	Supernatant	Pellet	Total	Supernatant	Pellet	Total
	(μg per 10^6 oocysts)					
Cholesterol	9.2	0.2	9.4	10.1	0.3	10.4
Fatty alcohols						
C_{22}	1.2	1.8	3.0	2.8	1.7	4.5
C_{24}	0.2	0.3	0.5	4.2	1.3	5.5
C_{26}	1.4	3.8	5.2	17.1	4.1	23.2

portant energy source for *E. tenella* during sporulation.

GLC-MS demonstrated that the nonsaponifiable lipids of *E. tenella* oocysts consist of cholesterol and unbranched primary alcohols of 22, 24, 26, 28, 30, and 32 carbons. Each species of alcohol was represented by both saturated and monounsaturated derivatives, with the saturated derivative being predominant.

The identification of these alcohols and the observation that wall fragments are rich in fatty alcohols are consistent with Ryley's suggestion that the oocyst wall appeared to contain waxy esters (1). However, we found that almost all of the fatty alcohols in both the wall and cytoplasm of the oocyst was free and not esterified into waxes.

It is interesting to compare the results from GLC of the nonsaponifiable lipids from *E. tenella* oocysts (Fig. 1) with those from GLC of the nonsaponifiable lipids from oocysts of the related species, *E. acervulina* (5). The retention times of several of the *E. acervulina* components relative to cholesterol suggest that these components might be fatty alcohols of 22-26 carbons.

GLC-MS of these fatty alcohols as TMSi ethers (or as deutero TMSi ethers) was found to have several advantages over GLC-MS of free alcohols. Unlike free alcohols, TMSi ethers produced molecular ions (Figs. 2 and 4), though these were of low intensity for saturated alcohols (Fig. 2). The two largest fragments produced by the free alcohols corresponded to loss of H_2O and loss of H_2O and H_2 from the molecular ion. That these two fragments differed by two atomic mass units tended to obscure the fact that each fatty alcohol contained some monounsaturated component which was not resolved chromatographically from the saturated homologue.

The TMSi ethers produced very intense ions which arise via loss of CH_3 from the molecular ion. Because of its intensity, the M-15 ion was useful for identifying two minor alcohols (C_{28} and C_{30}) for which material was extremely limited. This ion was readily identified because it is the only major ion differing by 6 mass units between the TMSi ether and TMSi-d_9 ether.

A final advantage of TMSi ethers is that the m/e 103 ion unique to primary alcohols is considerably more intense than the analogous m/e 31 ion of the free alcohol. For the deutero TMSi ethers, the corresponding fragment occurred at m/e 112. These data support the suggestion of Ryhage and Stenhagen that the m/e 31 fragment of the free alcohol is due to the $[CH_2OH]^+$ ion (7).

During sporulation, the wt of the nonsaponifiable lipids increased about 3-fold, attributed mainly to fatty alcohols (Table III). Fatty alcohols are generally synthesized by reduction of fatty acyl Coenzyme A esters to fatty aldehydes and subsequently to fatty alcohols (13-15). For unsporulated oocysts, no fatty acids longer than C_{18} were found even when GLC was done at temperatures as high as 280 C. These considerations imply that fatty acids may be elongated to the lengths of fatty alcohols and reduced during sporulation, which could account for the decrease in fatty acid content. This possibility is now under investigation. Reduction of the very long chain fatty acids following their synthesis must be very efficient, inasmuch as no fatty acids longer than C_{18} which might have escaped reduction, were found in sporulated oocysts.

In unsporulated oocysts, more than half of the fatty alcohols were found in the oocyst wall (Table III). Oocysts are noted for their resistance to dehydration and to various aqueous agents like acid, base, hypochlorite, and dichromate (1). The aqueous insolubility and chemical inertness of the wall fatty alcohols doubtlessly contribute to these properties.

During sporulation, newly synthesized fatty alcohols were not deposited in the oocyst wall but apparently remained in the cytoplasm (Table III). Whether these fatty alcohols are

part of the sporocyst, the sporozoite, or remain in the oocyst fluid surrounding the sporocysts is under investigation.

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Effect of Dietary Di-2-Ethylhexyl Phthalate on Lipid Biosynthesis in Selected Tissues from the Rat, In Vitro

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ABSTRACT

Di-2-ethylhexyl phthalate (DEHP), a plasticizer commonly used in the production of polyvinyl chloride plastics, has become an environmental pollutant. At the present time, the biological significance of phthalates in the environment is unknown. In the present studies, we observed that addition of DEHP to a stock diet of rats resulted in marked effects on incorporation of ^{14}C-acetate into lipid by liver and kidney slices; other organs, such as heart, testes, and aorta, were unaffected. Incorporation of ^{14}C-acetate into total lipid of liver (dpm/mg wet wt) from rats fed 0.5% or 1.0% DEHP for 10 or 18 days, respectively, was decreased to ca. 50% of control values. The decreased incorporation into liver lipid is not attributable to any one lipid fraction, inasmuch as incorporation into the phospholipid, sterol + diglyceride, free fatty acid, triglyceride, and sterol ester + hydrocarbon fractions was decreased 30-70% with respect to controls. In addition, the percent distribution of ^{14}C-acetate among the individual phospholipids was ca. 25% lower in phosphatidyl choline of the DEHP-fed rats. In rats fed 0.5% DEHP, incorporation of ^{14}C-acetate into total lipid of kidney was similar to control values, but incorporation into the triglyceride and sterol ester + hydrocarbon fraction was decreased 30-40%, whereas incorporation into the sterol + diglyceride fraction was increased 38%. Livers from DEHP-fed rats were ca. 20% larger than livers from control rats and, at the 0.5% level of DEHP feeding, testes wts were elevated; no significant changes were noted in wts of spleen, heart, aorta, kidney, or body wt gains in rats fed DEHP. These studies emphasize a subtle toxicity of phthalate esters not previously reported and emphasize the need for further biochemical studies to evaluate the effect of phthalates on biological systems.

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INTRODUCTION

Esters of phthalic acid are widely used in the industrial production of plastics, particularly the polyvinyl chloride or PVC type, and may account for as much as 40% of the wt of plastic products (1-3). The phthalate esters are not chemically bonded in the plastic but are interspersed throughout the plastic polymer matrix where they serve as intramolecular lubricants to impart the desired characteristics of flexibility to the products (1,3). Because the phthalates are not chemically bonded in the plastic products, they can, under various conditions, migrate from the plastic (1,2,4) and, as a result, have been found as contaminants of tissues from animals (5,6) and man (7-9), blood (10,11), milk (12), air (1), and water (1). This contamination of the environment by the phthalates probably reflects the widespread domestic and industrial use of PVC plastics

In general, phthalate esters have been reported to have a low order of acute toxicity in experimental animals (13-16) whereas, in chronic toxicity studies in numerous species, the effects of phthalate esters on body wt gain, organ size, and histological changes have been rather undramatic (3,16). However, recent studies have revealed more subtle toxicity effects of the phthalate esters, such as fetal resorption and birth defects (17) in experimental animals, as well as a toxicity to cells in culture (18,19).

In view of these recent findings, we decided to examine the effects of di-2-ethylhexyl phthalate (DEHP), the most widely used phthalate ester, on lipid metabolism in selected tissues in the rat.

EXPERIMENTAL PROCEDURES

Animals and Diets

Male Sprague-Dawley rats with intial wts of 300-340 g were individually caged with free access to food and water. The experimental diets were Purina Chow containing DEHP at a level of either 0.5% or 1.0% on a wt basis. The DEHP was added to the diets dissolved in diethyl ether (20 ml/Kg); an equivalent amount of ether was added to control diets as well. The ether was permitted to evaporate from the diets before feeding.

TABLE I

Effect of Dietary DEHP on Body Wt and Organ Wt in Rats[a]

Diet	Body wt (g)		Organ wt (% of body wt)		
	Initial	Final	Liver	Kidney	Testes
Control	330 ± 5[b]	346 ± 13	4.2 ± 0.1	0.80 ± 0.01	0.81 ± 0.05
DEHP (0.5%)	338 ± 4	356 ± 18	5.4 ± 0.4[c]	0.89 ± 0.06	1.00 ± 0.07[d]
Control	313 ± 5	350 ± 10	4.1 ± 0.3	0.79 ± 0.04	0.90 ± 0.02
DEHP (1.0%)	304 ± 3	356 ± 7	5.5 ± 0.2[c]	0.83 ± 0.03	0.86 ± 0.03

[a]Male Sprague-Dawley rats, caged individually, were fed a diet of Purina Chow (control group) or Purina Chow + di-2-ethylhexyl phthalate (DEHP) at a level of 0.5% or 1.0% for 10 and 18 days, respectively. Food and water were available ad libitum.

[b]Values are means ± SEM of six animals.

[c]Significantly different from control values ($P<0.01$).

[d]Significantly different from control values ($P<0.05$).

DEHP was obtained commercially (Eastman Kodak, Rochester, NY, cat. no. 4099) and purified by passing it through a glass column (25 cm long x 1.8 cm inside diameter) of washed and activated silicic acid (AR Grade, 100 mesh, Mallinckrodt Chemical Works, Jersey City, NJ). Silicic acid (18 g) was added to the column as a slurry in n-hexane. The DEHP to be purified (300 mg) was added to the top of the column dissolved in 1 ml of n-hexane and washed into the column with an additional 2-3 ml of n-hexane. The column was then eluted with 300 ml of 1% diethyl ether in n-hexane, followed by elution with 500 ml of 4% diethyl ether in n-hexane at flow rates of 3-4 ml/min; DEHP was recovered in the 4% diethyl ether:n-hexane eluate.

The purity of the DEHP was checked by thin layer chromatography (TLC) on Silica Gel G using two different solvent systems: (1) benzene:1,2-dichloroethane (1:1, v/v); (2) hexane:diethyl ether (95:5, v/v). Only one spot was obtained with each solvent system. The purity of the DEHP was reconfirmed with two gas liquid chromatographic columns: (1) 6 ft x 4 mm 15% diethylene glycol succinate on 60-80 mesh Gas Chrom P (Applied Science Labs, Inc., State College, PA); (2) 6 ft x 4 mm 10% SE52 - 5% XE60 on 80-100 mesh Gas Chrom Q (Applied Science Labs, Inc.). Only one peak was obtained with each column.

Tissue Preparation

The rats were killed by a blow to the head and their tissues rapidly excised. All subsequent procedures were performed at 0-5 C. Tissues were either sliced to a thickness of 0.5 mm with a Harvard tissue slicer (liver, kidney, heart) or minced on a glass plate with a spatula (testes) to yield material for the incubations.

Incubations

Tissue slices or tissue mince (500 mg) were incubated with 4 μCi sodium acetate-1-^{14}C (New England Nuclear Corp., Boston, MA, 58.6 mCi/mmol) at 37 C in 5.0 ml Krebs-Ringer-Bicarbonate buffer, pH 7.4, in a stoppered 25 ml Erlenmeyer flask containing streptomycin and penicillin (50 μg and 50 units/ml, respectively). Aortas were opened longitudinally and incubated as the whole tissue. Incubations were terminated with the addition of 3 ml methanol and then stored at -15 C until processed.

Analyses

The incubation mixtures were homogenized in a total volume of 50 ml chloroform:methanol (1:1, v/v). The homogenates were centrifuged to sediment the tissue residue, which was subsequently extracted with 3 x 10 ml of chloroform:methanol (2:1, v/v). The combined chloroform:methanol extracts were washed according to the method of Folch et al. (20). Aliquots of the extracts were counted to determine total radioactivity (21); additional aliquots were fractionated into phospholipids, sterol + diglyceride, free fatty acids, triglycerides, and sterol esters + hydrocarbons by TLC on Silica Gel G in a solvent system consisting of n-hexane:diethyl ether:acetic acid (146:50:4, v/v/v) (21). The individual lipid bands were visualized under UV light after spraying with rhodamine 6G (0.05% in ethanol), scraped, and counted (21). The individual phospholipids in the lipid extract were fractionated by TLC using the method of Skipski et al. (22). The individual phospholipids were detected with iodine vapor, scraped from the plates, and counted. Statistical analyses of the data were performed using Student's t-test for comparing unpaired samples.

TABLE II

Effect of 0.5% Dietary DEHP on Incorporation of Acetate-1-^{14}C into Lipids by Rat Tissue Slices, In Vitro[a]

Lipid fraction	Liver		Kidney	
	Control	DEHP	Control	DEHP
	dpm/g wet wt			
Phospholipid	697 ± 78[b]	426 ± 38[c]	2470 ± 221	2060 ± 131
Sterol + diglyceride	904 ± 190	397 ± 40[d]	1556 ± 163	2157 ± 186[d]
Free fatty acid	947 ± 140	501 ± 59[c]	1775 ± 144	2183 ± 119
Triglyceride	627 ± 107	372 ± 64[d]	1947 ± 218	1313 ± 102[d]
Sterol ester + hydrocarbon	348 ± 69	166 ± 15[d]	652 ± 63	384 ± 36[e]
Total	3523 ± 456	1862 ± 164[c]	8400 ± 433	8097 ± 348

[a]Male Sprague-Dawley rats were fed Purina Chow (control) or Purina Chow containing 0.5% di-2-ethylhexyl phthalate (DEHP) for 10 days. Tissue slices were prepared as described under Methods and incubated 3 hr at 37 C with 4 μCi sodium and 5 μmol sodium acetate carrier in a total volume of 5.0 ml of Krebs-Ringer-Bicarbonate buffer, pH 7.4. The incubation mixtures were extracted with chloroform:methanol (2:1, v/v) and the lipids fractionated by thin layer chromatography as described under Methods.

[b]Values are means ± SEM with six animals per group.

[c]Significantly different from control values ($P<0.02$).

[d]Significantly different from control values ($P<0.05$).

[e]Significantly different from control values ($P<0.01$).

RESULTS AND DISCUSSION

In our studies, addition of DEHP to the stock diet of rats was associated with changes in organ wts and tissue lipid metabolism, in vitro. In rats fed 0.5% DEHP for 10 days, wts of liver and testes were significantly elevated ($P<0.01$, $P<0.05$, respectively, Table I). Increased liver wt ($P<0.01$) was also observed in rats fed 1.0% DEHP for 18 days. In this group, testes wts were within normal limits, suggesting that testicular changes observed in animals fed 0.5% DEHP are transient in nature, disappearing with longer term DEHP feeding. This suggestion, however, is difficult to reconcile in view of the fact that feeding 0.375% DEHP to rats for 13 wk has been reported to result in testicular degeneration (14). The increased liver wts observed in our studies are in agreement with toxicological studies of DEHP feeding in rats (15,16). Although kidney wts tended to be marginally elevated in rats fed 0.5% and 1.0% DEHP (Table I), the significant increases in kidney size observed by others feeding DEHP at levels of 0.4% (15) and 0.5% (16) for 52 wk or 24 wk, respectively, were not observed in our study. It is possible that our feeding trials of 10 and 18 days did not provide sufficient time for gross kidney changes to develop. No changes in body wt gains or wts of spleen, heart, or aorta were observed at either level of DEHP feeding.

The changes in organ size observed in our studies prompted us to examine lipid biosynthesis from radioactive acetate in tissues from rats fed DEHP.

Table II shows the incorporation of ^{14}C-acetate (dpm/g wet wt) into total lipids and individual lipid fractions of liver and kidney in animals fed 0.5% DEHP. Incorporation of ^{14}C-acetate into total lipids was significantly reduced ($P<0.02$) in livers of rats fed DEHP whereas incorporation into total lipid of kidneys was unaffected. Decreased ^{14}C-incorporation into livers of DEHP-fed rats was reflected throughout the various lipid fractions, i.e., the phospholipids, sterol + diglyceride, free fatty acids, triglycerides, and sterol ester + hydrocarbon fractions; incorporation into all fractions was significantly decreased by DEHP feeding to ca. 50% of control values. These results suggest that the effect of DEHP on hepatic lipid biosynthesis is at a common point in the biosynthetic pathway of all the lipids, such as at the level of acetate activation.

Although 0.5% DEHP feeding did not alter significantly the incorporation of ^{14}C-acetate into total lipids of kidney (Table II), incorporation into the triglyceride and sterol ester + hydrocarbon fractions was significantly reduced ($P<0.05$ and $P<0.01$, respectively), as was the case with liver. In contrast to liver, however, incorporation of acetate into the sterol + diglyceride fraction of kidney lipids was significantly increased ($P<0.05$); incorporation into all other kidney lipid fractions did not differ significantly from control values.

Incorporation of ^{14}C-acetate into total lipids and the individual lipid fractions of heart and aortic tissue was also examined in these animals and found to be unaffected by 0.5%

DEHP feeding.

These data from Table II indicate that DEHP feeding is associated with alterations in lipid biosynthesis in some tissues (liver, kidney) but not in others (heart, aorta). In addition, specific alterations in lipid metabolism occurring with DEHP feeding can differ from tissue to tissue (liver vs kidney).

The feeding of DEHP at a level of 1.0% in the diet of rats produced results similar to experiments in which 0.5% DEHP was fed, with some exceptions, notably the kidney (Table III). As with 0.5% DEHP feeding, incorporation of ^{14}C-acetate into total lipids (dpm/g wet wt) of liver was significantly reduced ($P<0.01$) to ca. 50% of control values (Table III), suggesting that maximal inhibition of total hepatic lipid biosynthesis is achievable with the lower level (0.5%) of DEHP. In time-course studies (Fig. 1), ^{14}C-acetate incorporation into total hepatic lipid showed a steady increase over a 3 hr period in both control and DEHP-fed animals, indicating that the effects of DEHP observed in these in vitro studies is not attributabel to differences in tissue survival.

Incorporation of ^{14}C-acetate into individual lipid fractions of liver was also reduced in all fractions (Table III) with 1% DEHP feeding. Most marked decreases in incorporation were observed in the sterol + diglyceride, free fatty acid, and sterol ester + hydrocarbon fractions ($P<0.05$, $P<0.01$, and $P<0.001$, respectively). In kidney, the differences in incorporation of

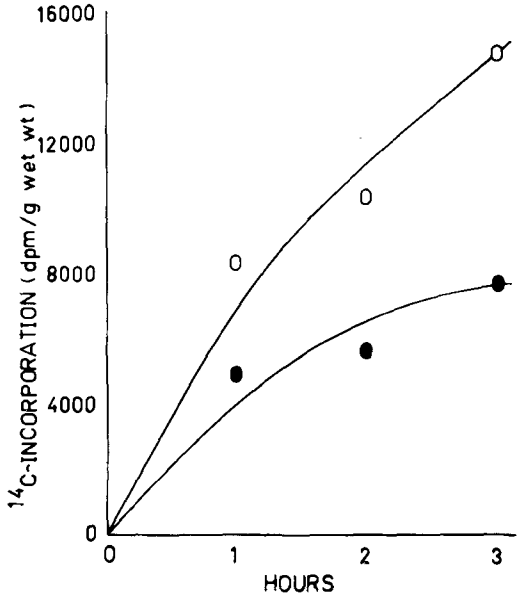

FIG. 1. Effect of dietary di-2-ethylhexyl phthalate (DEHP) on the rate of incorporation of acetate-1-^{14}C into total lipids of rat liver slices, in vitro. Slices of liver (500 mg) from rats fed Purina Chow (control, -○-○-) and rats fed Purina Chow containing 1.0% DEHP (-●-●-) for 18 days were incubated for up to 3 hr at 37 C with 4 μCi sodium acetate-1-^{14}C in a total volume of 5.0 ml of Krebs-Ringer-Bicarbonate buffer, pH 7.4. The incubation mixtures were extracted with chloroform:methanol (2:1, v/v) as described under Methods and the total lipid extract assayed for radioactivity. Values are the mean of two animals.

TABLE III

Effect of 1.0% Dietary DEHP on Incorporation of Acetate-1-^{14}C into Lipids by Rat Tissue Slices, In Vitro[a]

Lipid fraction	Liver (n=9)[b]		Kidney (n=6)	
	Control	DEHP	Control	DEHP
	dpm/g wet wt			
Phospholipid	2809 ± 443[c]	1849 ± 481	8630 ± 476	8464 ± 265
Sterol + diglyceride	693 ± 238	164 ± 67[d]	1171 ± 313	1363 ± 219
Free fatty acid	968 ± 50	601 ± 98[f]	7295 ± 475	6506 ± 554
Triglyceride	1740 ± 321	1270 ± 282	3760 ± 263	5000 ± 481
Sterol ester + hydrocarbon	699 ± 97	165 ± 37[e]	675 ± 101	784 ± 106
Total	6972 ± 327	4070 ± 708[f]	21530 ± 850	22117 ± 936

[a]Male Sprague-Dawley rats were fed Purina Chow (control) or Purina Chow containing 1% di-2-ethylhexyl phthalate (DEHP) for 18 days. Tissue slices were prepared as described under Methods and incubated 3 hr at 37 C with 4 μCi sodium acetate-1-^{14}C in a total volume of 5.0 ml of Krebs-Ringer-Bicarbonate buffer, pH 7.4. The incubation mixtures were extracted with chloroform:methanol (2:1, v/v) and the lipids fractionated by thin layer chromatography as described under Methods.

[b]n designates the number of animals per group.

[c]Values are means ± SEM.

[d]Significantly different from control values ($P<0.05$).

[e]Significantly different from control values ($P<0.001$).

[f]Significantly different from control values ($P<0.01$).

TABLE IV

Effect of Dietary DEHP on Percentage Distribution of Acetate-1-^{14}C Incorporated into Individual Phospholipids by Rat Liver Slices, In Vitro[a]

Diet	Origin	Lysolecithin	Sphingomyelin	Phosphatidyl choline	Phosphatidyl serine/inositol	Phosphatidyl ethanolamine
Control	12.42 ± 3.01[b]	9.53 ± 1.02	5.64 ± 0.78	36.40 ± 2.63	13.68 ± 1.07	23.11 ± 1.00
DEHP (0.5%)	21.86 ± 2.03[c]	8.75 ± 0.66	5.22 ± 0.71	27.20 ± 1.29[c]	11.73 ± 1.51	25.39 ± 1.94
Control	23.09 ± 2.73[d]	14.43 ± 2.15	5.03 ± 0.90	28.63 ± 2.02	12.03 ± 2.80	16.79 ± 1.28
DEHP (1.0%)	26.63 ± 3.27	11.64 ± 2.72	3.33 ± 0.53	22.20 ± 2.43	12.66 ± 2.67	23.53 ± 3.72

[a]Liver slices from male Sprague-Dawley rats fed 0.5% di-2-ethylhexyl phthalate (DEHP) for 10 days or 1.0% DEHP for 18 days were incubated 3 hr at 37 C with 4 μCi sodium acetate-1-^{14}C in a total volume of 5.0 ml of Krebs-Ringer-Bicarbonate buffer, pH 7.4. The incubation mixtures were extracted with chloroform:methanol (2:1, v/v) and the phospholipids fractionated by thin layer chromatography as described under methods.

[b]Values are the mean ± SEM with six animals per group.

[c]Significantly different from control values ($P<0.02$).

[d]Values are the mean ± SEM with eight animals per group.

acetate into the various lipid fractions observed in the animals fed 0.5% DEHP for 10 days were not apparent when DEHP was fed for the extended period of 18 days at the 1% level (Table III). Thus, it is possible that the kidney has the capacity to normalize or restore control over alterations in lipid metabolism observed in the shorter term feeding trial (Table II). The liver, on the other hand, may be unable to correct changes induced by DEHP feeding, particularly because incorporation of acetate into total hepatic lipids was significantly depressed to ca. 50% of control values at both levels of DEHP feeding (Tables II and III). Acetate incorporation into lipid by testes was examined only at the 1% level of DEHP feeding and did not differ from control values. It would be of interest, however, to reexamine lipid biosynthesis in testes inasmuch as, at the 0.5% level of DEHP feeding, testes wts were significantly elevated ($P<0.05$) (Table I). Besides decreased incorporation of ^{14}C-acetate into the phospholipid fraction of liver with DEHP feeding (Tables II and III), there were also certain changes in the percent distribution of label into the individual phospholipids (Table IV).

In rats fed 0.5% and 1.0% DEHP, respectively, 21.86 and 26.63% of the label in phospholipids remained with the origin and is unidentified; these values were greater than the corresponding control values (12.42 and 23.09, respectively), being significantly greater ($P<0.02$) in the livers from rats receiving 0.5% DEHP. Conversely, at both the 0.5% and 1.0% level of DEHP feeding, the percent distribution of label into phosphatidyl choline was reduced by ca. 25% compared to control values; this reduction was significant ($P<0.02$) in the 0.5% DEHP group. No significant differences in percent distribution were observed, at either level of DEHP, in lysolecithin, sphingomyelin, phosphatidyl serine + phosphatidyl inositol, or phosphatidyl ethanolamine, although the percent distribution of label into sphingomyelin tended to be reduced in livers of the 1.0% DEHP group.

With respect to the phospholipids, but aside from any effects of DEHP, we are unable to explain the observed difference in the percent of the total ^{14}C incorporation into the phospholipid fractions of the control animals in the two experiments shown in Tables II and III. In Table II, the fractional distribution of ^{14}C into phospholipids of controls was ca. 20% and in Table III, ca. 40%. Perhaps the differences are attributable to seasonal variations in animal response inasmuch as the 0.5% and 1.0% feeding trials (Tables II and III, respectively) were

separated by 6 months.

These studies demonstrate for the first time that DEHP feeding to rats is associated with a number of changes in tissue lipid biosynthesis from radioactive acetate. These changes are most pronounced in liver but also occur in kidney. Incorporation of acetate into total hepatic lipid is inhibited ca. 50% when DEHP is fed at a level of either 0.5% or 1.0% in a stock diet. The decreased incorporation of ^{14}C-acetate is reflected in a lower incorporation of label into the major lipid fractions of hepatic tissue. In addition, there is within the phospholipids a disproportionate reduction in ^{14}C-acetate incorporation into phosphatidyl choline.

The mechanism by which DEHP feeding alters tissue lipid metabolism and the importance of such alterations cannot be answered from these studies. However, it seems certain that the differences between tissues from control and DEHP-fed rats is not attributable to alterations in endogenous acetate pool sizes by DEHP because the effect of DEHP-feeding was observed with (Table II) and without (Table III) the addition of carrier acetate to the incubations. This addition of carrier acetate resulted, not unexpectedly, in a reduction of the total incorporation of ^{14}C-acetate into the tissues in Table II vs. Table III by virtue of its dilution effect on the ^{14}C-acetate. In addition to an inhibitory effect on hepatic lipid biosynthesis, it is feasible to speculate that DEHP (or a metabolite of DEHP) might also modify lipid catabolism. In this regard, it is interesting that addition of 0.1% DEHP to rat diets supplemented with 4% fat led to increased liver wts when compared to rats receiving either a fat free diet containing 0.1% DEHP or a 4% fat diet without DEHP (23); these results may reflect hepatic lipid accumulation resulting from a decrease in hepatic lipid catabolism.

It has been stated that the biological significance of phthalate esters in the environment is uncertain (1); the biochemical studies presented here emphasize a subtle toxicity of phthalate esters not previously reported and emphasize the need for further biochemical studies to evaluate the effect of phthalates on biological systems.

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Double Bond Location in Polyenoic Fatty Esters through Partial Oxymercuration[1]

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ABSTRACT

A rapid micro-procedure has been developed to locate double bonds in fatty acid methyl esters containing from one to four double bonds. Reaction of the ester with an equal molar amount of mercuric acetate in methanol and reduction with sodium borohydride, followed by hydrogenation, produce a mixture of monomethoxy alkanoates. The mass spectrum of this mixture is simpler and more definitive than that from the completely methoxylated polyenoate. Only one methoxyl group is present per molecule, and the mass spectrum of the mixture is indicative of all olefinic positions. Four intense ions are observed for all double bonds examined, except Δ3, where the double bond is represented by only two ions. Hydrogenation in a gas chromatograph reduces total analysis time to 1 hr.

INTRODUCTION

Unsaturated fatty esters do not produce spectra that are useful for determination of double bond positions; therefore, a suitable derivative must be made. To be useful, a derivative should be simple to make and reasonably volatile for good gas chromatography (GC). Its mass spectrum should contain intense ions that clearly reflect all double bond positions. Several procedures have been successfully used to locate double bonds in monoenoic fatty acid methyl esters (1). These procedures include epoxidation of the double bond (2), and epoxidation followed by either ring opening with dimethylamine to form N,N-dimethylamino alcohols (3) or reaction with NaI to form ketones (4). Because such derivatives give complex spectra and characteristic ions of low intensity, they are unsuited for double bond location in polyenes. Hydroxylation of monoenes with osmium tetroxide produces vicinal diols, which can be analyzed by gas chromatography-mass spectrometry (GC-MS) after conversion to more volatile trimethylsilyl (TMS) derivatives (5) or methyl ethers (6). Here, too, these derivatives of hydroxylated polyenes are difficult to analyze by GC-MS because their spectra become quite complex. Recently, the use of spectra of N-acylpyrrolidides of polyunsaturated fatty acids has been proposed as a method for double bond locations (7). Unfortunately, the important diagnostic ions have low abundances, making interpretation of unknowns difficult.

Oxymercuration-demercuration to form monomethoxy derivatives has been successfully used to locate double bonds in monoenoic fatty acids (8-10). This method has also been applied to polyenoic fatty acid methyl esters (10), but again the spectra are complex because many more ions must be defined. These ions result not only from primary cleavages at the methoxyl bearing carbon atoms, but also from secondary cleavages involving successive losses of methanol. The presence of large numbers of fragment ions and the low intensities observed for important higher mass fragments make these spectra difficult to interpret.

When we reduced partially oxymercurated polyenoic fatty acid methyl esters and hydro-

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TABLE I

Sources of Unusual Fatty Acids Used in Study

Fatty acid	Source	Reference[a]
trans-3,*cis*-9,*cis*-12-18:3	*Stenachaenium macrocephalum*	11
trans-5,*cis*-9,*cis*-12-18:3	*Thalictrium equilagiafolium*	12
All-*cis*-5,11,14,17-20:4	*Ephedra foliata*	13
All-*cis*-5,13-20:2	*Limnanthes douglassi*	14
All-*cis*-5,9,12-18:3	*Larix leptolepsis*	15
trans-3,*cis*-9,*cis*-12,*cis*-15-18:4	*Tecoma stans*	16

[a]Reference applies to genera and not necessarily species.

genated them in a GC, we produced a mixture of monomethoxy alkanoates. These compounds have simple spectra with four intense ions indicative of each double bond position in the polyene. The presence of only one methoxyl group per molecule minimizes competitive secondary fragmentations involving loss of methanol. The entire analysis including the GC-MS analysis takes < 1 hr. By this method, we located double bonds in nine different fatty acid methyl esters containing from two to four double bonds.

EXPERIMENTAL PROCEDURES

Methyl linoleate, methyl linolenate, and methyl arachidonate were purchased commercially. The remaining polyenoic fatty acid methyl esters were prepared and isolated from seed oils of known composition (Table I). The all-*cis*-5,9,12-18:3 and *trans*-3,*cis*-9,*cis*-12,*cis*-15-18:4 were isolated by high-pressure liquid chromatography of esters from their source seed oils on a 30 cm x 1/4-in. outside diameter C-18 μ-Bondapak column (Waters Associates, Milford, ME). The esters were eluted from the column with CH_3CN. The remaining esters were isolated by thin-layer chromatography on preparative (1 mm thick) 10 x 30 cm plates spread with Silica Gel G impregnated with 20% $AgNO_3$. Benzene was used as the developing solvent. Bands were visualized by spraying the plate with 2% dichlorofluorescein in ethanol and viewing under long wave UV light. Esters were recovered from the Silica Gel G by extracting with diethyl ether. Homogeneity in chain length and number of double bonds of recovered esters were verified by GC and GC-MS.

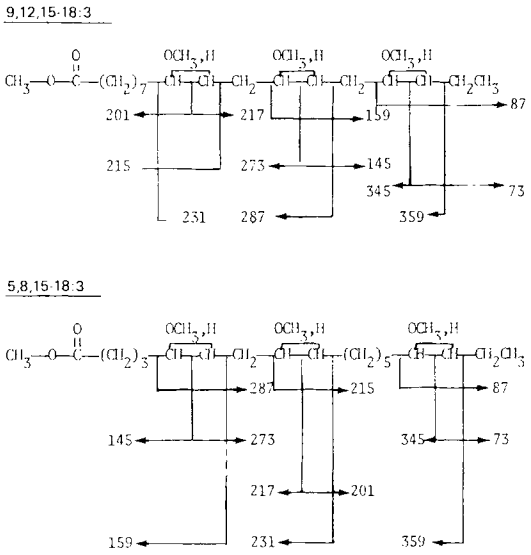
Samples were introduced into a Dupont 21-492-1 mass spectrometer through a Bendix 2625 gas chromatograph via a single-stage jet-type helium separator. All mass spectra were obtained by electron bombardment at 70 eV. The GC injector temperature was 200 C, and the transfer line and jet separator temperatures were 250 C. Two sets of columns and operating conditions were used. For hydrogenation GC-MS, hydrogen was the carrier gas and a 2-in. precolumn containing 1% palladium chloride on 42/60 mesh Chromosorb P was packed at the head of the column (17). The glass column (4 ft x 2 mm internal diameter) used was packed with either 3% Dexsil 300 or 3% Silar 5CP and programmed from 160-230 C at 2 C/min. Polyunsaturated fatty acid methyl esters were completely hydrogenated under these conditions. For GC-MS analyses without hydrogenation, helium was the carrier gas through 4 ft x 2 mm glass columns packed with 5% Apiezon L and programmed from 190-220 C at 2 C/min.

The monomethoxy alkanoate derivatives were formed as follows: Polyenoic fatty acid methyl esters (1-5 mg) and an equal molar amount of mercuric acetate were refluxed for 30 min in 1-2 ml of methanol. Solid sodium borohydride was added to the reaction mixture until no additional mercury precipitated. A portion was analyzed by hydrogenation GC-MS. This procedure hydrogenated all remaining double bonds. The resulting methyl alkanoate, monomethoxyalkanoates, and dimethoxyalkanoates were separated into three peaks. Reduction of the mercuric acetate adducts directly in the hydrogenation GC system instead of using sodium borohydride was also possible, but the catalyst became poisoned after five to seven injections, and hydrogenation was no longer complete.

RESULTS AND DISCUSSION

The primary mass spectral fragments in fully methoxylated derivatives of trienes contain from one to three methoxy groups, and when multiple methoxyl groups are present within a fragment, successive secondary fragments with losses of methanol are observed. Low intensities for some important primary fragments and the large number of secondary fragment ions make spectra complicated. Furthermore, in the low resolution spectrum of a trimethoxyalkanoate derivative of a triene, observance of the particular fragments is not enough to establish structure because there will be one other possible structure that will give completely identical fragments. This possibility exists because two fragment structures with an observed *m/e* value can be drawn from either end of the alkanoate depending upon placement of the methoxyl groups. For example, Scheme 1 shows the fragment ions expected for the trimethoxy stearates from 9,12,15-18:3 and 5,8,15-18:3. All fragments observed in one compound would be expected in the other.

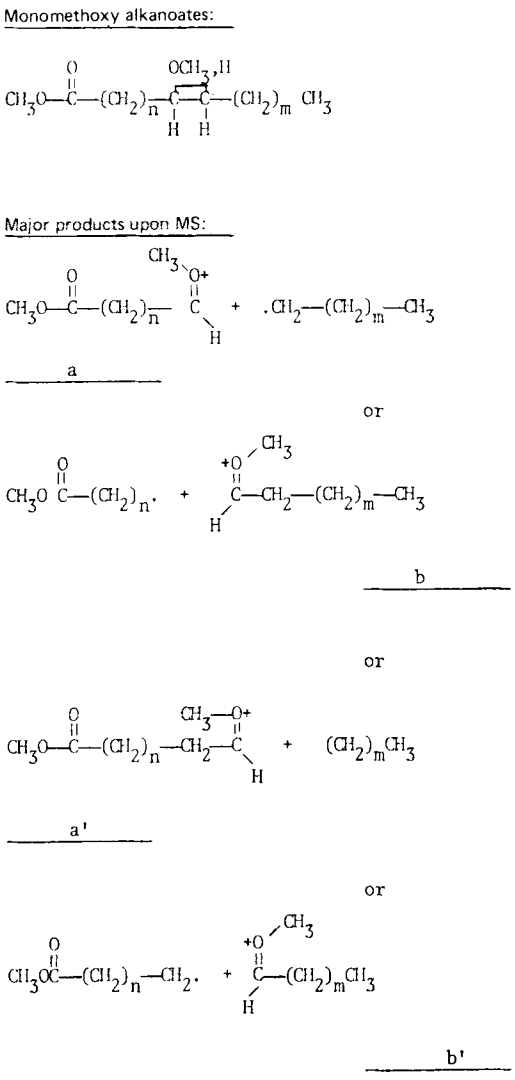
Although we did not have 5,8,15-18:3 to analyze, the mass spectrum for it might be distinguishable from the derivative of 9,12,15-18:3 by careful examination of both mass spectra, paying particular attention to the intensities of secondary fragments 32 amu less than *m/e* 145 and 159 or *m/e* 201 and 215. The *m/e* 145 and 159 fragments from the trimethoxy alkanoates from 9,12,15-18:3 contain two methoxyl groups and would be more likely to lose methanol than the *m/e* 145 and 159 fragments in the alkanoates from 5,8,15-18:3



SCHEME 1. Fragments expected for fully methoxylated 9,12,15-18:3 and 5,8,15-18:3.

that have only one methoxyl group. Similarly, the *m/e* 201 and 215 fragments from the alkanoates from 5,8,15-18:3 contain two methoxyl groups, although they contain only one in the 9,12,15-18:3 alkanoates. Of course, high resolution spectra of either of these derivatives would be unique because the number of carbon, oxygen, and hydrogen atoms differ in the proposed fragments, although nominal masses are the same. Detection of a second triene in the presence of another would be difficult based on the spectra of their trimethoxy alkanoates derivatives, particularly for naturally occurring mixtures of 5,9,12-18:3 and 9,12,15-18:3, which have been found together in the past (18), because important fragments for identification of Δ5 (145, 159, 287, 273) are identical to fragments obtained for Δ9 and Δ12.

Reaction of the polyenoic fatty acid methyl ester with an equal molar amount of mercuric acetate, rather than an excess as proposed by Minnikin and coworkers (9,10), produced mostly monomethoxy mercurated adducts with small amounts of dimethoxy mercurated adducts and some unreacted starting material. The mass spectra of these monomethoxy alkenoates are not easily interpreted; however, hydrogenation produces a mixture of monomethoxyalkanoates, which then have simple mass spectral fragmentation patterns clearly indicative of the position of the methoxyl group. These monomethoxyalkanoates are easily separated from the alkanoates and dimethoxyalkanoates for analysis by GC-MS.



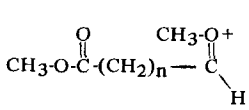
SCHEME 2. Major fragments observed for monomethoxyalkanoates.

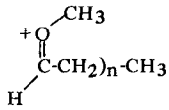
Major fragmentations observed in a monomethoxy alkanoate are presented in Scheme 2. Two intense fragment ions, a and b, locate the position of the methoxyl group along the chain. In methoxy derivatives of fatty acids, two sets of these ions (a and a' [a + 14 amu] and b and b' [b + 14 amu]) are observed because methanol adds to either of the carbon atoms of the original double bond.

Fragments expected for various double bond positions are listed in Table II. The expected ions in the monomethoxyalkanoate derivative from any double bond position of a polyenoic fatty acid methyl ester can be calculated using Table II.

TABLE II

Expected Fragments for Methoxy Derivatives from Various Double Bond Positions





a			b		
Double bond position (Δ)	a*	a'*	Double bond position (ω)	b*	b'*
3		131	3	87	73
4	131	145	4	101	87
5	145	159	5	115	101
6	159	173	6	129	115
7	173	187	7	143	129
8	187	201	8	157	143
9	201	215	9	171	157
10	215	229	10	185	171
11	229	243	11	199	185
12	243	257	12	213	199
13	257	271	13	227	213
14	271	285	14	241	227
15	285	299	15	255	241
16	299	313	16	269	255
17	313	327	17	283	269

*Mass spectral fragments.

The ester function does not direct the addition of methanol except for Δ2, Δ3, and Δ4 double bonds in which the methoxyl group is attached preferentially to the unsaturated carbon atom farthest from the carboxyl group (8).

Although the GC peak of the methoxyalkanoate mixture obtained for a diene, triene, or tetraene may look symmetrical, consecutive mass spectra show that when ω3 bonds are present, positional isomers are partially separated across the peak. To obtain a representative spectrum for the monomethoxyalkanoates present in a mixture, successive spectra can be taken across the peak and added together. The spectrum obtained for the monomethoxystearates derived from methyl linolenate (9,12,15-18:3) is shown in Figure 1. The three sets of a and a′ ions, indicative of the double bond positions, are observed at *m/e* 201 and 215 (Δ9), 243 and 257 (Δ12), and 285 and 299 (Δ15). The three sets of b and b′ ions are observed at *m/e* 171 and 157 (ω9), 129 and 115 (ω6), and 87 and 73 (ω3). The intensities of the six a and a′ ions are similar, ranging from 15 to 30% of the base peak. The b and b′ ions, however, show a marked decrease in intensity at increasing *m/e* value. Less abundant ions are observed at 267, 253, 225, 211, 183, and 169 due to loss of methanol from the a and a′ ions. Loss of methanol is not observed from the b and b′ ions.

Ions and their relative intensity values were

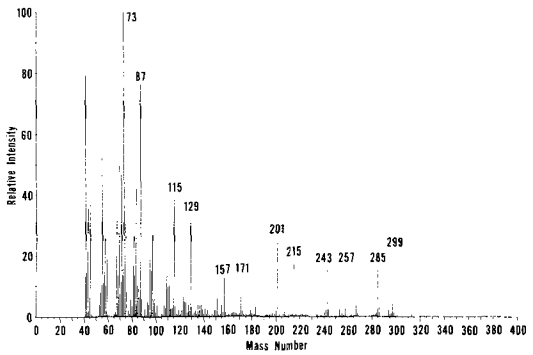


FIG. 1. Monomethoxystearates from 9,12,15-18:3.

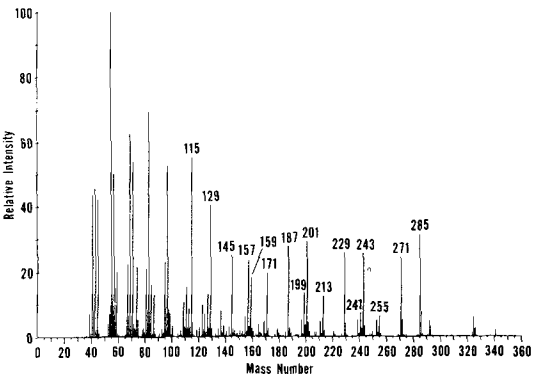


FIG. 2. Monomethoxyeicosanoates from 5,8,11,14-20:4.

TABLE III

Characteristic Ions and Intensities Used to Identify Double Bond Positions in Dienes, Trienes, and Tetraenes Analyzed

Fatty acid	a_1	a_1'	b_1'	b_1	a_2	a_2'	b_2'	b_2	a_3	a_3'	b_3'	b_3	a_4'	a_4	b_4'	b_4
All-*cis*-9,12-18:2	201(44)	215(39)	157(31)	171(37)	243(34)	257(44)	115(99)	129(50)								
All-*cis*-9,12,15-18:3	201(30)	215(21)	157(14)	171(15)	243(17)	257(17)	115(40)	129(34)	285(16)	299(21)	73(100)	87(79)				
trans-5,*cis*-9,*cis*-12-18:5	145(17)	159(6)	213(3)	227(4)	201(18)	215(35)	157(29)	171(12)	243(18)	257(31)	115(72)	129(33)				
trans-3,*cis*-9,*cis*-12-18:3	.....	131(28)	241(7)	.....	201(49)	215(33)	157(26)	171(30)	243(33)	257(44)	115(87)	129(45)				
trans-3,*cis*-9,*cis*-12,*cis*-15-18:4	.....	131(2)	241(3)	.....	201(41)	215(34)	157(26)	171(29)	243(34)	257(31)	115(49)	129(46)	285(22)	299(25)	73(100)	87(86)
All-*cis*-5,11,14,17-20:4	145(12)	159(11)	241(5)	255(3)	229(22)	243(15)	157(14)	171(16)	271(22)	285(19)	115(45)	129(36)	313(13)	327(16)	73(70)	87(67)
All-*cis*-5,8,11,14-20:4	145(25)	159(18)	241(7)	255(6)	187(28)	201(29)	199(14)	213(13)	229(26)	243(25)	157(23)	171(20)	271(24)	285(31)	115(55)	129(41)
All-*cis*-5,13-22:2	145(59)	159(42)	283(14)	269(16)	257(65)	271(66)	157(61)	171(52)								

used to identify the double bonds in the dienes, trienes, and tetraenes examined (Table III). The monomethoxystearates derived from methyl linoleate (9,12-18:2) had intense fragments at *m/e* 201 and 215 (Δ9 a and a′), 243 and 257 (Δ12 a and a′), 157 and 171 (ω9 b and b′), and 115 and 129 (ω6 b and b′). Ions at 225, 211, 183, and 169 result from loss of methanol from a and a′ ions. All characteristic fragments had intensities of > 30% of the base peak. The monomethoxybehenates from 5,13-22:2 had fragments at *m/e* 145 and 159 (Δ5 a and a′), 257 and 271 (Δ13 a and a′), and 157 and 171 (ω9 b and b′), with intensities of 40-66% of the base peak. The fragments at *m/e* 283 and 269 (ω17 b and b′) had much smaller intensities but were still ca. 15% of the base peak. Again, loss of methanol is observed for the a and a′ fragments.

The monomethoxystearates derived from *trans*-5,*cis*-9,*cis*-12-18:3 had intense ions at *m/e* 145 and 159 (Δ5 a and a′), 201 and 215 (Δ9 a and a′), 243 and 257 (Δ12 a and a′), 213 and 227 (ω13 b and b′), 157 and 171 (ω9 b and b′), and 115 and 129 (ω6 b and b′). Ions containing the 5 and 6 methoxy from the Δ5 double bond are less intense than the ions arising from the Δ9 or Δ12 bond. Presumably, this intensity difference results from either a slower reaction rate in the adduct formation near the carboxyl group or a reaction that forms a hydroxyl at C-5 as reported by Gunstone and Inglis (8). The all-*cis*-5,9,12-18:3 gave similar results to those for the *trans*-5,*cis*-9,*cis*-12-18:3 The *trans*-3,*cis*-9,*cis*-12-18:3 had only one set of ions (*m/e* 131 and 241) arise from the Δ3 double bond. They are also lower in intensity than ions from the Δ9 and Δ12 bonds.

Three tetraenes were analyzed. In the spectrum of the derivative from 5,11,14,17-20:4, the a series ions (145 and 159 [Δ5], 229 and 243 [Δ11], 271 and 285 [Δ14], and 313 and 327 [Δ17]) had intensities of 12-22% of the base peak whereas the b series ions (73 and 87 [ω3], 115 and 129 [ω6], 157 and 171 [ω9], and 241 and 255 [ω15]) showed much greater stability for lower *m/e* value.

In the spectrum of the derivative from methyl arachidonate (Fig. 2) (5,8,11,14-20:4), the a series ions (145 and 159 [Δ5], 187 and 201 [Δ8], 229 and 243 [Δ11], and 271 and 285 [Δ14]) had intensities of 18-31% of the base peak whereas the b series ions (115 and 129 [ω6], 157 and 171 [ω9], 199 and 213 [ω12], and 241 and 255 [ω15]) showed the same intensity ratios previously observed. The spectrum of the derivative of *trans*-3,*cis*-9,*cis*-12,*cis*-15-18:4 showed similar a and b series

intensities to 9,12,15-18:3 with the addition of the ions from a Δ3 double bond (131[12] and 241 [3]).

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^{13}C Nuclear Magnetic Resonance Spectroscopy of Saturated, Unsaturated, and Oxygenated Fatty Acid Methyl Esters[1,2]

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ABSTRACT

Chemical shifts have been assigned to all the separate signals in the ^{13}C nuclear magnetic resonance spectra of methyl stearate, oleate, and petroselinate by means of the second and third atom isotope effects in spectra of specifically deuterated esters. Spectra of almost all the isomeric hydroxy, acetoxy, and oxo stearates were also measured. From the assignments, the effects of the introduction of a double bond or an oxo, hydroxy, or acetoxy group at different positions on the fatty acid chain on the ^{13}C nuclear magnetic resonance spectra were determined. All the isomeric oxo stearates and most of the hydroxy and acetoxy stearates can be distinguished and identified by their ^{13}C spectra.

INTRODUCTION

Elucidation of structures of many natural products has been simplified in the last few years by the use of ^{13}C nuclear magnetic resonance (NMR) spectroscopy. The chemical shift range is ca. 200 ppm for ^{13}C spectra compared to only ca. 10 ppm for ^{1}H spectra. When this investigation was undertaken, there had been no extensive studies of ^{13}C spectra of fatty acids, but it appeared that the method could be useful in structure determination of lipids, particularly of mixtures of compounds with such similar structures that other spectroscopic or chromatographic methods would be unsuccessful. In addition, because the carbons in long chain compounds are in similar environments, useful analytical estimations could probably be made from the intensities of the spectral lines.

Because differences between chemical shifts of corresponding carbons in isomeric fatty esters were expected to be very small, the procedure followed in this study was to assign unambiguously all the separate signals in the spectrum of methyl stearate and then to determine the effect of introducing a double bond or an oxygenated functional group.

Comparison of the ^{13}C NMR spectrum of a compound with that of the corresponding specifically deuterated compound is a convenient method of signal assignment. In general, it is known that monodeuteration of a CH_2 group converts the signal of that carbon to a triplet with $J_{^{13}C\text{-}D}$ of ca. 20 Hz. and also causes an upfield (negative) displacement of ca. 0.4 ppm (first atom isotope effect) (1). Conversion of CH_2 to CD_2 usually causes the signal of that carbon to disappear (2), probably because of increased relaxation time or decreased nuclear Overhauser effect (3). Small upfield displacements were also sometimes observed in the signals of adjacent carbons (second atom isotope effect) (1,4).

In a preliminary investigation of ^{13}C spectra of some deuteriooctadecanoates, small negative third atom isotope effects were observed in addition to the expected second atom effects, when two or more deuterons were attached to a carbon (5). Also, signals due to the second and third carbons, i.e., carbons α and β to the deuterated carbon in the usual lipid nomenclature, were broadened 3-5 fold by long range ^{13}C-D coupling (5). Thus, because signals of five carbons are affected by deuteration of one carbon, this method was used to assign the signals of the saturated and unsaturated esters described in this investigation. The effects of vicinal ^{13}C-D coupling have also been observed in spectra of alicyclic compounds (6).

EXPERIMENTAL PROCEDURES

Natural abundance ^{13}C NMR spectra were obtained with a Varian XL-100-15 spectrometer in the Fourier transform mode at 25.2 M Hz with proton noise decoupling (2 K Hz band width). For most spectra, data length was 8192 points, and spectra measured at 1000 Hz sweep width and acquisition time 4 S (0.25 Hz/point) were accurate to ± 0.02 ppm. In most cases the 29-32 ppm region was also examined with a 20 S acquisition time (0.1 Hz/point), giving shifts accurate to ± 0.01 ppm. The spectral window was selected so that signal foldback did not interfere with signals being observed.

Esters were examined as 15% (w/v) solutions in $CDCl_3$. Signals of nonoxygenated esters were assigned by means of spectra of the fol-

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[1]Presented in the Symposium on NMR analysis of lipids at the AOCS Fall Meeting, Philadelphia, October 1974.

[2]National Research Council of Canada No. 150 87.

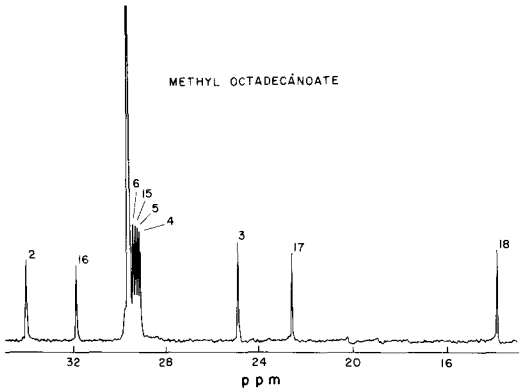

FIG. 1. ^{13}C Nuclear magnetic resonance (NMR) spectrum of methyl stearate; 12-36 ppm region (sweep width 1000 Hz).

lowing deuterated compounds: methyl 2-D_2, 4-D_2, 7-D, 7-D_2, 12-D_2, 17-D, 17-D_2, and 16,18-D_5 stearates; methyl 2-D_2 and 12-D_2 oleates; and methyl 2-D_2 petroselinate. Spectra measured on mixtures of methyl 6-, 7-, 12-, and 13-hydroxy, 12-acetoxy, and 12-oxo stearates (0.150 g each) with methyl stearate (0.300 g) in 2 ml $CDCl_3$ were used to determine differences between the signals of the oxygenated compounds and the corresponding signals in the stearate spectrum.

Preparation of some deuterated esters was described previously (7), and synthesis of the rest will be reported later. The oxygenated esters were prepared in a previous investigation (8).

RESULTS AND DISCUSSION

Methyl Stearate

The ^{13}C NMR spectrum (Fig. 1) contains nine one carbon signals and a large composite signal (neglecting those due to C-1 and the ester methoxyl carbon). Signals due to C-16, C-17, and C-18 at the end of the chain can be assigned from the work of Grant on hydrocarbons (9), and those due to C-2 and C-3 from the work of Lippma (10) and Roberts (11). The four signals due to C-4, C-5, C-15, and C-6 can be assigned from the spectra of the deuteriostearates. Central portions of the spectra are shown in Figure 2; the highest field signal can be assigned to C-4 because, in the spectrum of 2-D_2 stearate, it has moved slightly upfield and broadened considerably. This assignment is confirmed by the spectrum of 4-D_2 stearate, which does not show a C-4 signal; the signal assigned to C-5 has broadened and moved upfield appreciably, and that assigned to C-6 has also broadened but has moved upfield only slightly.

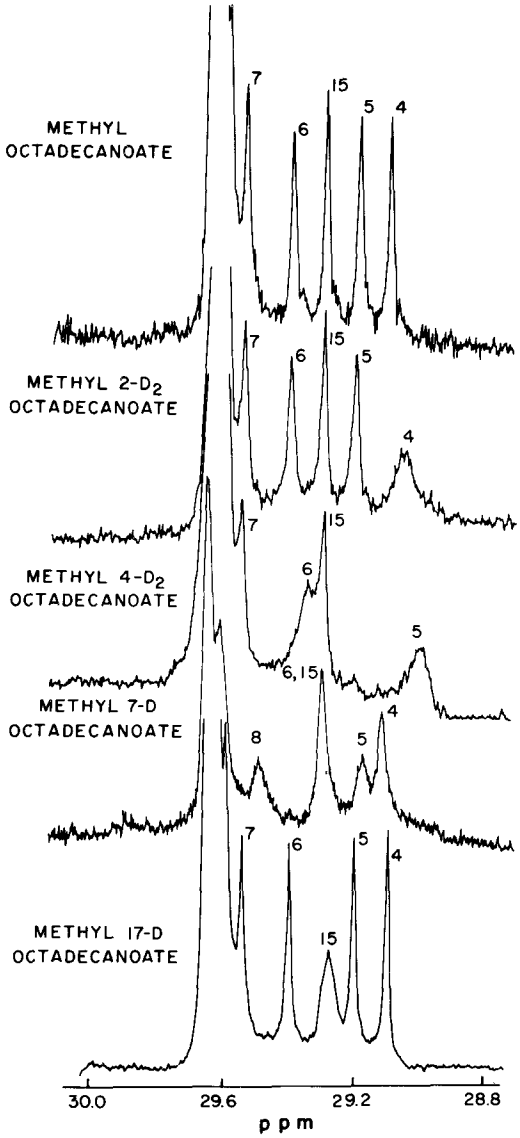

FIG. 2. ^{13}C Nuclear magnetic resonance (NMR) spectra of methyl stearate, methyl 2-D_2, 4-D_2, 7-D, and 17-D stearates; 28.8-30.0 ppm region (100 Hz sweep width).

The spectrum of 7-D stearate confirms these assignments, although the upfield displacements of the C-5 and C-6 signals are smaller inasmuch as they are affected by one deuteron only (5). The signal partially resolved from the upfield side of the large composite peak is assigned to C-7 because it does not appear in the spectrum of 7-D stearate. It is presumably a triplet obscured by the broad signal assigned to C-8, which has moved upfield from the composite peak.

TABLE I

^{13}C Chemical Shifts of Methyl Stearate[a]

Carbon	Shift	Carbon	Shift
1	174.01	10	29.65
2	34.05	11	29.65
3	24.92	12	29.65
4	29.13	13	29.65
5	29.21	14	29.65
6	29.40	15	29.29
7	29.52	16	31.91
8	29.65	17	22.70
9	29.65	18	14.05
OCH_3	51.31		

[a]In ppm relative to TMS (tetramethylsilane).

The last signal is assigned to C-15 because, in the spectrum of 17-D stearate, it has moved upfield and broadened. It was also modified in a similar way in spectra of 17-D_2 and 16,18-D_5 stearates (5). Thus, the signals in the spectrum of stearate can be assigned as in Table I. Signals of C-8 to C-14 has all been given the value 29.64 ppm, the center of the peak from 29.58 to 29.72, but some of them presumably differ from this value. While this work was in progress, Batchelor et al. (12) reported similar chemical shifts for the methyl stearate carbons; the assignments were made by comparing spectra of a number of esters of different chain length and by ^{13}C labeling.

Methyl Oleate and Petroselinate

Figure 3 shows spectra of methyl oleate and petroselinate. Signals of carbons α to the double bond, 8 and 11 in oleate, and 8 and 5 in petroselinate, are displaced upfield by ca. 2.4

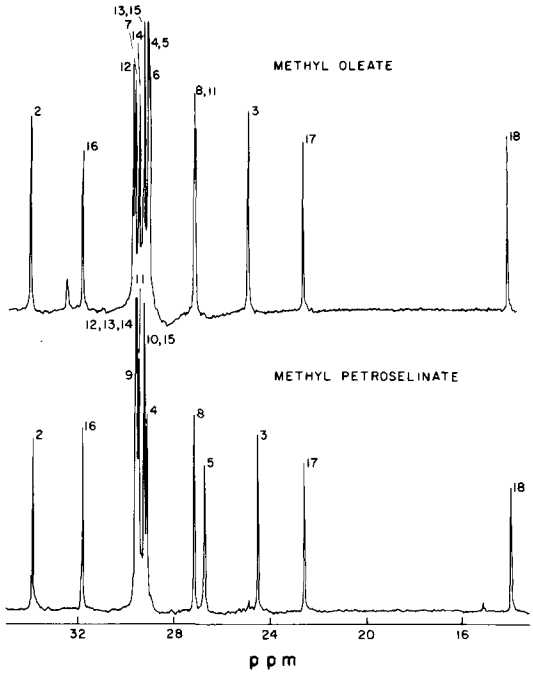

FIG. 3. ^{13}C Nuclear magnetic resonance (NMR) spectra of methyl oleate and petroselinate; 12-36 ppm region (1000 Hz sweep width). The small peak downfield of C-16 signal of methyl oleate is due to C-8 and C-11 of elaidate (13) present as an impurity in the oleate.

TABLE II

^{13}C Chemical Shifts of Methyl Oleate and Petroselinate[a]

	Oleate		Petroselinate	
Carbon	Shift	Change from stearate	Shift	Change from stearate
1	173.91		173.80	
2	34.09		33.97	-0.08 (δ)
3	24.96		24.60	-0.32 (γ)
4	29.13		29.21	+0.08 (β)
5	29.13	-0.08 (δ)	26.83	-2.38 (α)
6	29.09	-0.31 (γ)	128.98	+99.49
7	29.68	+0.16 (β)	130.28	+100.76
8	27.22	-2.42 (α)	27.22	-2.42 (α)
9	129.56	+99.92	29.72	+0.08 (β)
10	129.80	+100.16	29.33	-0.31 (γ)
11	27.18	-2.46 (α)	29.56	-0.08 (δ)
12	29.76	+0.12 (β)	29.66	
13	29.33	-0.31 (γ)	29.66	
14	29.52	-0.12 (δ)	29.66	
15	29.33		29.33	
16	31.90		31.92	
17	22.70		22.69	
18	14.09		14.09	
OCH_3	51.31		51.31	

[a]In ppm relative to TMS (trimethylsilane).

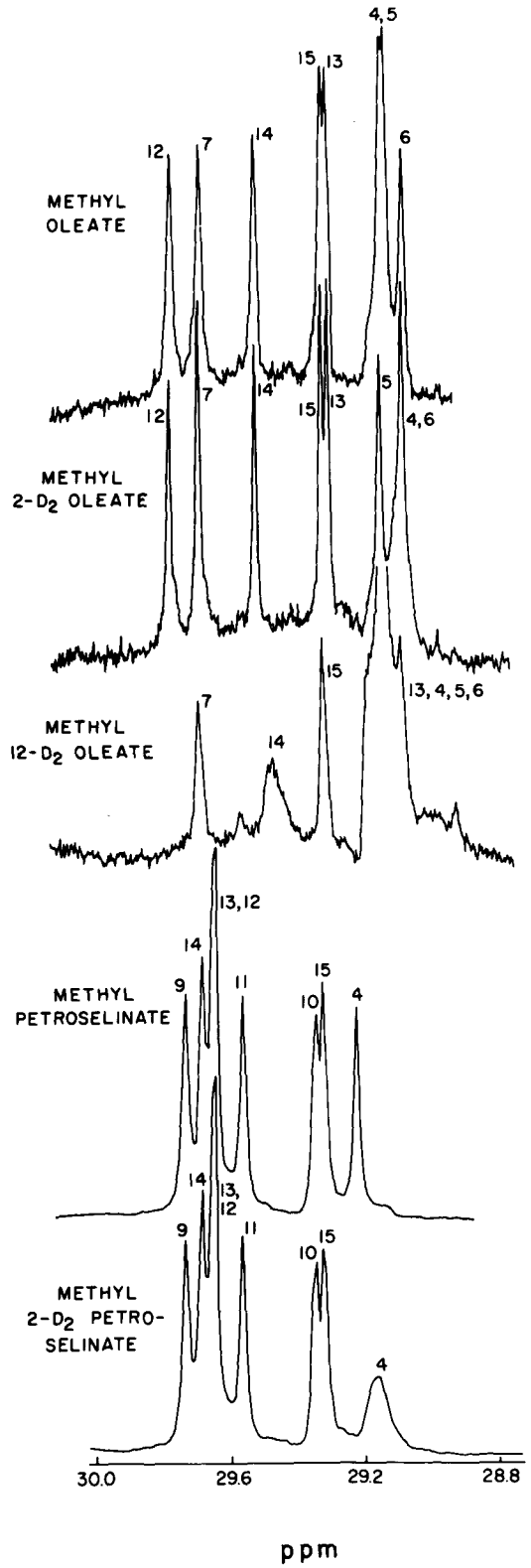

FIG. 4. ^{13}C Nuclear magnetic resonance (NMR) spectra of methyl oleate, methyl 2-D_2 and 12-D_2 oleate, methyl petroselinate, and 2-D_2 petroselinate; 28.8-30.0 ppm region (100 Hz sweep width).

ppm. Earlier work (13) suggested an upfield shift of ca. 1-3 ppm for α carbons of *cis* olefins. The central portion of the methyl oleate spectrum is shown expanded in Figure 4; the signal due to C-4 was assigned from the spectrum of 2-D_2 oleate and the other signals from that of 12-D_2 oleate.

Thus, the C-12 signal has disappeared and signals due to C-7 and C-15 (which is scarecely changed from its position in stearate) are not affected by deuteration. The signal due to C-14 has broadened and moved slightly upfield and that due to C-13 has moved upfield appreciably. The highest field signal is assigned to C-6 (the carbon γ to the double bond) because of the relatively high field displacement of C-3 in the spectrum of petroselinate (Fig. 3 and Table II).

In the central part of the ^{13}C spectrum of petroselinate (Fig. 4, lower part), the signal due to C-4 is assigned from the spectrum of 2-D_2 petroselinate. Signals due to C-9, C-10, and C-11 are assigned from the displacements observed for corresponding carbons 4, 3, and 2 on the carboxyl side of the double bond and also from those in the oleate spectrum.

Chemical shifts of the signals in the spectra of oleate and petroselinate are shown in Table II along with the changes relative to the signals in the stearate spectrum. Thus, for carbons near the double bond, the average α, β, γ, and δ changes are -2.42, +0.11, -0.31, and -0.09, respectively. The positive β displacement is particularly to be noticed.

These smaller double bond effects were not observed earlier (13), but Batchelor et al. (12) reported similar displacements using ^{13}C labeling and lanthanide shift reagents. The signals at 128.80 ppm in the oleate spectrum was assigned to C-10 because it was markedly broadened in the spectrum of 12-D_2 oleate; the signals of the double bond carbons of petroselinate were then assigned by analogy. This particular result agrees with the assignments made by Batchelor et al. (14) by comparison of spectra of unsaturated esters with double bonds at various distances from the ester group.

The spectrum of methyl petroselinate suggests that signals of carbons at the same distance from the double bonds are not significantly changed relative to each other from their positions in the stearate spectrum. Thus, the signals of C-5 and C-8, which were 0.43 ppm apart in the stearate spectrum, are

TABLE III

Change in ^{13}C Chemical Shifts on Conversion of Stearate to Hydroxy, Acetoxy, and Oxo Stearates (Average of Isomers 6- to 13-)[a]

Carbons	Hydroxy	Acetoxy	Oxo
Oxygenated carbon	+42.2	+44.70	+181.10
α	+7.80	+4.40	+13.10
β	-4.00	-4.40	-5.75
γ	+0.06	-0.20	-0.40
δ	-0.06	-0.20	-0.25
ε	-0.09	-0.10	-0.20
ζ	-0.05	-0.05	-0.08

[a]Downfield displacements are positive.

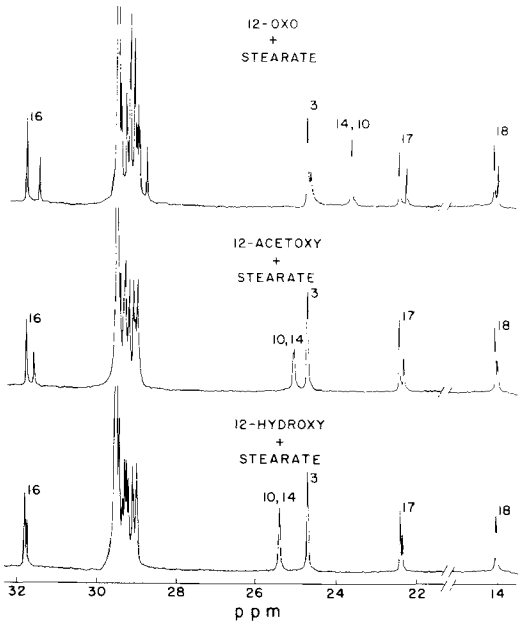

FIG. 5. ^{13}C Nuclear magnetic resonance (NMR) spectra of mixtures of methyl 12-hydroxy, 12-acetoxy, and 12-oxo stearates with methyl stearate (1:2); 13.5-32.5 ppm region (500 Hz sweep width).

0.39 ppm apart in petroselinate, so that the double bond effects appear to be simply superimposed on the stearate shifts. There are also other useful differences between spectra of oleate and petroselinate, in shifts of C-5 and C-3 particularly, which are quite sufficient to distinguish the two and probably also to estimate composition of mixtures.

Oxygenated Esters

Plant lipids not infrequently contain oxygenated fatty acids and these sometimes occur as mixtures of positional isomers. Identification of oxygenated C_{18} esters by gas liquid chromatography (GLC) (8), ^1H NMR (15), and thin layer chromatography (TLC) (16) had been investigated previously when it was found that mixtures of isomers with the functional group attached near the center of the chain were particularly difficult to analyze. It was previously found that several hydroxy myristates derived from a plant wax component could be distinguished by ^{13}C NMR (17). It was useful, therefore, to investigate spectra of a number of isomeric C_{18} esters with an oxygenated substituent near the center of the chain to determine the extent to which ^{13}C spectra could identify, distinguish, and estimate these components in mixtures. The oxo, hydroxy, and acetoxy isomers are considered together because they are closely related chemically.

Roberts et al. (18) showed that, in spectra of secondary alcohols, carbons α to CHOH were displaced downfield ca. 8 ppm and β carbons were displaced upfield ca. 4 ppm. Spectra of some acyclic ketones also indicated appreciable displacement of α and β carbon signals (19). For both hydroxy and oxo compounds, however, the effects on γ, δ, etc., carbons were uncertain; also, it appeared that spectra of acetylated hydroxy compounds had not been studied

Comparison of ^{13}C spectra of methyl 6- to 13- hydroxy, acetoxy, and oxo esters with that of stearate gave the results in Table III. Although some of the differences from the stearate shifts are very small, they appear to be real, as can be seen in Figure 5, which shows spectra of mixtures of 12-hydroxy, 12-acetoxy, and 12-oxo stearate with stearate (all in ratio 1:2). The small γ to ζ carbon effects are all upfield (negative) except for the small downfield effect on the γ carbon of hydroxy esters. This effect was clearly seen in spectra of the mixture of 13-hydroxy and stearate (difference in C-16 signals), of 12-hydroxystearate (overlap of C-6 and C-15 signals), and of the mixture of 7-hydroxy and stearate (difference in C-4 signals). In the spectrum of the mixture of 6-hydroxy and stearate, however, the C-3 signal of 6-hydroxy was upfield of that of stearate by 0.8 Hz; this difference may be due to interaction with the ester group.

As expected, there are large downfield displacements of α carbon signals and smaller upfield displacements of β carbon signals. For acetoxy esters, α carbon signals are very close to that of C-2 and β carbon signals very close that that of C-3, making unambiguous assignment difficult. The effects on the γ to ζ carbons are largest in the case of oxo esters (as also appears from Fig. 5).

TABLE IV

Differences Between ^{13}C Chemical Shifts of Pairs of Carbons α and β to CHOH, CHOAc, and CO Groups

Position of oxygenated group	α Carbons (Hz)				β Carbons (Hz)			
	Observed				Observed			
	CHOH	CHOAc	CO	Calculated[a]	CHOH	CHOAc	CO	Calculated[a]
13	-	1	1	-	9	9	9	9
12	-	-	-	-	-	0.5	-	-
11	-	2-3	1	-	-	5	1	-
10	1	1-2	1	?	1	1	2	?
9	2	2	3	?	2	2	3	3
8	4	-	5	3	5	0.4	7	5
7	8	5	11	6	10	9	13	11
6	12	9	16	8	11	11	16	13

[a]*Calculated values obtained from the differences between the chemical shifts of these carbons in the stearate spectrum.*

Applications to Identification and Analysis of Oxygenated Esters

If displacements of chemical shifts caused by introduction of an oxygen containing substituent are simply superimposed on the stearate shifts, then those shifts which are different in the stearate spectrum should differ by the same amount in the spectrum of the oxygenated derivative, which should make it possible to identify many isomers. Table IV lists the differences between chemical shifts of pairs of α carbons and of pairs of β carbons in spectra of the three types of ester and compares them to the differences between shifts of these carbons in the stearate spectrum.

In 13-substituted esters, there is very little difference between the α carbon signals (C-14 and C-12) and none is expected, but there is an observed and expected difference of 9 Hz between β carbon signals (C-15 and C-11). There is virtually no difference between α or β carbon signals in 12-substituted esters and none expected; the coincident β signals appear in Figure 5. The 11- and 10-substituted esters were also not expected to show differences, and none were observed except in the spectrum of 11-acetoxy stearate where α carbons differed by 2-3 Hz and β carbons by 5 Hz.

The other isomers, 9-, 8-, 7-, and 6-substituted, showed differences in α and β carbon signals which were noticeably greater when the substituent was closer to the ester group. In fact, some observed values for 6-substituted esters were considerably larger than those expected, presumably because of interaction between the functional groups, so that chemical shifts are not completely additive. Unexpected results were also found for 8-acetoxy ester, no differences being observed when differences of 3 Hz and 5 Hz for α and β carbons, respectively, were expected.

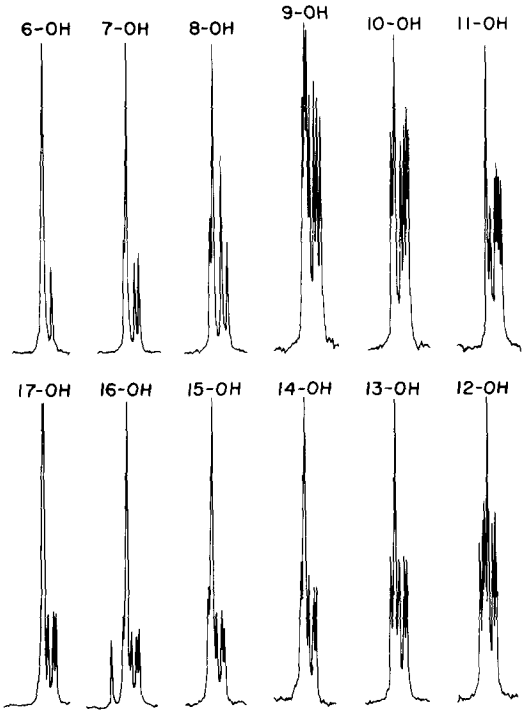

FIG. 6. Central portions (29-30 ppm region) of ^{13}C nuclear magnetic resonance (NMR) spectra of methyl 6- to 17-hydroxystearates.

The results showed that of the eight hydroxy isomers with substituents from C-6 to C-13, only 10-, 11-, and 12-hydroxy stearates could not be clearly identified from ^{13}C NMR spectra. As acetates, however, 11-acetoxy ester can be distinguished. Also 10-, 11-, and 12-oxo esters can be distinguished by means of the difference in the C-16 and C-17 signals caused by the long range effect of the oxo group. Thus, the C-16 signal of 11-oxo is 5 Hz upfield of

C-16 in 10-oxo, and the C-17 signal in 11-oxo is 2 Hz upfield of C-17 in 10-oxo, and, in 12-oxo, this signal is a further 2 Hz upfield.

When signals are separated by 2 or more Hz, mixtures of isomers can also be analyzed, and it appears that amounts of isomers can be estimated fairly accurately from intensities of signals inasmuch as, when signals due to the same carbon in two isomers are being compared, relaxation times usually do not differ appreciably. Figure 5 shows that signal intensities are ca. proportional to amounts of components in the mixtures.

Both isomers containing 18 carbons and those with fewer carbons (17) can be distinguished by ^{13}C NMR but it would be difficult to identify all oxygenated esters with more than 18 carbons if the substituent were near the center of the chain. Useful indications of substitutent position can, however, be obtained by examination of the 29-30 ppm region of the spectrum containing signals of carbons near the center of the chain which have been little affected by substituents. These signals are illustrated in Figure 6, which shows this region of the spectrum for the 6- to 17-hydroxy stearates. When OH is near the ester end, then the only upfield signal is that of C-15, and in 7-OH the signal due to C-4 also appears. In 8-OH, the C-5 signal is present as well, although it coincides with that of C-15 (positive γ effect). In 9-, 10-, and 11-OH, the four signals due to C-4, C-5, C-15, and C-6 are all present. In 12-OH, the C-15 signal now coincides with that of C-6. The region is less useful for 13- to 17-OH isomers, but all show signals due to C-4, C-5, and C-6 with the C-15 signal missing because it has been displaced to much lower or higher field. Thus, this part of the spectrum would indicate the position of an OH group on one of the first 7 CH_2 groups or indicate that it was on one of the last 5 CH_2 groups.

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β-Oxidative Cleavage of Octanoyl- and Dodecanoyl-CoA in Rat Liver Cytoplasm

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ABSTRACT

[12-^{14}C] Dodecanoyl-CoA and [8-^{14}C] octanoyl-CoA were tested as substrates for shortening the chain by two carbon atoms using both the 105,000 x g soluble fraction and the sonicated mitochondrial fraction of rat liver homogenate as the enzyme source. Both substrates were metabolized by the cytoplasmic enzymes giving rise to the accumulation of intermediates of the β-oxidation process without formation of two carbon units from the methyl carbon of the acyl residue. A new method is described which allows quantitative estimation of volatile fatty acids formed by β-oxidation of dodecanoyl- and octanoyl- Coenzyme A.

INTRODUCTION

In a previous communication, we reported that long chain acyl-CoA esters are catabolized by the 105,000 x g soluble fraction of rat liver homogenate (1). It was shown that chain shortening occurs with the formation of intermediates of the β-oxidation process (1). However, degradation catalyzed by the soluble enzymes differs definitely from the mitochondrial β-oxidation. Both oleoyl- and stearoyl-CoA are catabolized by the soluble enzymes to their homologous acids of 16 and 14 carbon atoms with only trace amounts of shorter acids being formed. In contrast, the solubilized mitochondrial enzymes convert the substrates to only water soluble acids under the same conditions (1). To evaluate the chain shortening of medium chain fatty acids, [8-^{14}C] octanoyl-CoA and [12-^{14}C] dodecanoyl-CoA were incubated with soluble rat liver enzymes. Quantitative estimation of the radioactivity associated with the metabolites of these substrates by radio-gas chromatographic analysis of their methyl esters was rendered impossible owing to their high volatility. Therefore, a method was developed which is independent of the total recovery of the acids. This method is based on the addition of known amounts of unlabeled acids at the end of the incubation, transformation of the acids into the much less volatile p-bromophenacyl esters, and evaluation of the molar radioactivity of each ester purified by thin layer chromatography (TLC), preparative gas chromatography, and crystallization.

Degradation patterns similar to those obtained by incubation of long chain acyl-CoA (1) were observed. Insignificant amounts of radioactivity were found associated with acetic acid. Considering that substrates labeled at the methyl carbon were used, this result indicates that no complete degradation of the molecules to acetate units occurs.

MATERIALS AND METHODS

Materials

[U-^{14}C] Stearic acid was obtained from (Amersham, Buckinghamshire, UK); sodium [8-^{14}C] octanoate and [12-^{14}C] dodecanoic acid from SORIN (Saluggia, Italy). Nonradioactive fatty acids, 1-undecene, 1-heptene, and the bisulphite derivative of hexadecanoic aldehyde were RS (Reagent Speciale) reagents purchased from Carlo Erba S.p.A. (Milan, Italy); p-bromophenacyl bromide was obtained from Schuchardt GmbH (Hohenbrunn, West Germany); BSTFA (N,O-bis-[trimethylsilyl] trifluoroacetamide) was purchased from Pierce Chemicals (Rockford, IL). and oxidized nicotinamide adenine dinucleotide (NAD^+) and CoA were obtained from Boehringer (Mannheim, West Germany).

Preparative Gas-Liquid Chromatography

A Carlo Erba Gaschromatograph model GV (flame ionization detector), equipped with 1m long glass sylanized column packed with SE 30 1% on GasChrom Q (80-100 mesh), internal diameter 0.8 cm, was used. Operation conditions for the analysis of p-bromophenacyl esters were as follows: programmed column temperature from 110 C to 140 C at 3 C/min to purify p-bromophenacyl acetate and p-bromophenacyl butyrate and from 150 C to 240 C to purify the esters of longer chain acids; injector temperature was 250 C. Helium flow was 200 ml/min. At the column end, a splitting device was employed which diverted 90% of the injected mixture (2-3 mg of esters in 20-50 μl of ethyl acetate) to a container cooled with dry ice in acetone where the compounds corresponding to each chromatographic peak were recovered.

Thin Layer Chromatography

DC Fertigplatten Kieselgel 60 F_{254}, 20 x 20 cm, obtained from Merck (Darmstadt, West Germany) were used both for analytical and preparative purposes. Benzene:ethyl acetate (95:5, v/v) was used as the solvent mixture to separate p-bromophenacyl esters of fatty acids and benzene:ethyl acetate (50:50, v/v) to purify the p-bromophenacyl esters of 3-hydroxyacids.

Radio-Gas Chromatography and Counting

Instruments and conditions used were as reported previously (1).

Gas-Liquid Chromatography-Mass Spectrometry

An LKB 9000 instrument was used; the temperatures of the molecular separator and ion source were 270 C and 290 C, respectively. The energy of the electrons was 70 eV and the ion current 60 μA. Gas chromatographic conditions were as follows: SE 30 1% column, 2 m long, internal diameter 0.3 cm, programmed temperature from 110 C to 250 C at 3 C/min.

Synthesis of 3-Hydroxyacids

Ethyl 3-hydroxyoctanoate, 3-hydroxydodecanoate, and 3-hydroxyoctadecanoate were prepared by the Reformatsky procedure from hexanoic, decanoic, and hexadecanoic aldehydes, respectively (2). Hexanoic and decanoic aldehydes were obtained by ozonolysis of 1-heptene and 1-undecene followed by reductive decomposition of the ozonides with triphenylphosphine.

Hexadecanoic aldehyde was obtained by hydrolysis of the bisulphite derivative. The ethyl esters were saponified with KOH in 60% methanol, and the 3-hydroxyacids extracted with ether were transformed into their methyl esters with diazomethane. The ester were purified by column chromatography on Kieselgel (70-230 mesh ASTM, Merck, Darmstadt) and saponified to give pure 3-hydroxyacids. 3-Hydroxyoctanoic and 3-hydroxydodecanoic acids were crystallized from hexane and 3-hydroxyoctadecanoic acid from acetone. The chemical purity and the identity of the 3-hydroxyacids were checked by preparing the trimethylsilyloxy derivatives of their methyl esters and by analysis with GLC-MS. Derivatives were prepared by treating each acid (2 mg) with diazomethane in ether and by adding BSTFA (20 μl) and pyridine (3μl) to the residue after the ether was evaporated.

The purity was always $>$ 99%. The identity of the compounds was confirmed by the peaks corresponding to the molecular ion (M^+), to the molecular ion minus 15 (M^+-15), and the peak at m/e 175 $[(CH_3)_3SiO\text{-}CH\text{-}CH_2COOCH_3]^+$ present in the mass spectra (1).

p-Bromophenacyl esters

The acids were added to a solution of p-bromophenacylbromide in dimethylformamide (0.08 g/ml) containing triethylamine (0.15 ml/ml). An excess of acid with respect to p-bromophenacyl bromide (1.2 moles/mole) was used to minimize the formation of p-bromophenacyl alcohol by hydrolysis of bromide under the reaction conditions. After heating 2 hr at 60 C, addition of water, extraction with diethyl ether, and evaporation of the solvent, the esters were obtained. These were purified from small amounts of the p-bromophenacyl alcohol by preparative TLC. The esters recovered (80-85% yield) were crystallized from benzene:hexane mixtures. Melting points were in agreement with those reported in the literature (3). The identity of the esters was confirmed by mass spectrometry; the derivatives of 3-hydroxyacids were analyzed via direct inlet in the ion source because decomposition was observed if analyzed via gas chromatography inlet. Due to the isotope distribution of bromide present in the molecule (4), two equally intense peaks were present in the spectra at m/e corresponding to the calculated mol wt $\pm$ 1, respectively. Characteristic ions (m/e 183 and 185) corresponding to the fragment $[BrC_6H_4CO]^+$ were present in the spectra of all the examined p-bromophenacyl esters; in addition, the spectra of 3-hydroxyesters showed two peaks (m/e 285 and 287) originating from the fragment $[BrC_6H_4COCH_2OCOCH_2CHOH]^+$.

Synthesis of Radioactive Substrates

The radioactive acids were diluted with the corresponding nonradioactive acids; the molar activities were as follows: [8-^{14}C] octanoic acid, 1890 dis/min/nmol; [12-^{14}C] dodecanoic acid, 1860 dis/min/nmol; and [U-^{14}C] stearic acid, 10,000 dis/min/nmol. Acyl-CoA was prepared by the method of Goldman and Vagelos (5) in 30-50% yield.

Enzyme Preparation and Incubation Conditions

Livers from Sprague-Dawley rats killed after 12 hr fasting were homogenized and fractioned as described previously (1). Incubations were carried out by shaking 90 min in a Dubnoff incubator at 37 C and were stopped by addition of 2N KOH in 60% methanol. Incubation conditions were as follows: substrate, 0.01 mM;

inorganic phosphate, 75 mM; sucrose, 50 mM; adenosine triphosphate, 2.5 mM; CoA, 0.1 mM; NAD^+, 0.5 mM; ethylenediaminetetraacetic acid, 2.5 mM; Mg^{++}, 5 mM; and arsenite, 10 mM; pH 7.4. When [U-^{14}C] stearoyl-CoA was the substrate, the final volume was 1 ml and the incubation was carried out in 10 ml test tubes whereas, when either [8-^{14}C] octanoyl-CoA or [12-^{14}C] dodecanoyl-CoA was the substrate, the final volume was 12 ml and the incubation was carried out in 150 ml Erlenmeyer flasks.

Extraction, Purification and Analysis Conditions of Incubations Conducted with [U-^{14}C] Stearoyl-CoA as the Substrate

Two samples of each experiment were worked up and analyzed by radio-gas chromatography as reported previously (1). To the other samples, 56.4 mg (0.2 mmol) stearic acid, 60 mg (0.2 mmol) 3-hydroxyoctadecanoic acid, and 25.4 mg (0.1 mmol) hexadecanoic acid were added, and the obtained mixture was saponified 2 hr at 60 C. The acids were then extracted with diethyl ether after acidification of the mixture with hydrochloric acid and saturation with sodium chloride; the ether phase was evaporated under vacuum and a solution of p-bromophenacyl bromide (0.4 mmol) in 2 ml dimethylformamide containing 0.3 ml triethylamine was added to the residue. p-Bromophenacyl esters prepared as described above were applied to four Kieselgel plates (20 x 20 cm). After elution with benzene:ethyl acetate (95:5, v/v), two bands were scraped off the plates; the more polar one corresponded to p-bromophenacyl 3-hydroxyoctadecanoate (80-85 mg) whereas the less polar band contained both p-bromophenacyl stearate and hexadecanoate (155-165 mg). The products were extracted from the Kieselgel with diethyl ether. p-Bromophenacyl 3-hydroxyoctadecanoate was further purified by TLC using benzene:ethyl acetate (50:50, v/v); the ester recovered from the plates was crystallized three times from benzene. From 50 to 55 mg of pure ester were obtained. The mixture of p-bromphenacyl stearate and hexadecanoate obtained from the less polar zone was subjected to preparative GLC in the conditions reported above. The compounds corresponding to each chromatographic peak were recovered and crystallized three times from hexane. The crystallized compounds (40-45 mg of p-bromphenacyl stearate and 20-25 mg of p-bromophenacyl hexadecanoate) were analyzed by GLC-MS to confirm their chemical purity which was always $> 99\%$. The molar radioactivity (dis/min per mmol) of purified p-bromophenacyl esters was determined by counting two weighed amounts (5-10 mg) of each sample. Radioactivity associated with each acid was calculated as described in Table I.

Extraction, Purification, and Analysis Conditions of Incubations Conducted with [12-^{14}C] Dodecanoyl-CoA as the Substrate

To the incubation mixture treated with KOH, 20 mg (0.1 mmol) of dodecanoic acid, 21.6 mg (0.1 mmol) 3-hydroxydodecanoic acid, 17.2 mg (0.1 mmol) decanoic acid, 14.4 mg (0.1 mmol) octanoic acid, 23.2 mg (0.2 mmol) hexanoic acid, 17.6 mg (0.2 mmol) butyric acid, and 24 mg (0.4 mmol) acetic acid were added. After saponification at 60 C for 2 hr, acidification with hydrochloric acid, and saturation with sodium chloride, the acids were extracted with diethyl ether. Triethylamine (0.5 ml) was added to the solution to minimize the loss of short chain acids during evaporation of the solvent. The evaporation was carried out under vacuum, avoiding any heating of the solution which caused partial loss of short chain acids despite the presence of triethylamine. The residue was treated with p-bromophenacyl bromide as described for the incubation with stearoyl-CoA as the substrate. The mixture containing p-bromophenacyl esters was applied to ten Kieselgel plates (20 x 20 cm) and, after development with benzene:ethyl acetate, four bands were recovered from the plates which corresponded to the p-bromophenacyl esters of 3-hydroxydodecanoic acid, acetic acid, butyric acid, and medium chain acids. The residue obtained by extraction with diethyl ether of the band containing the p-bromophenacyl ester of 3-hydroxydodecanoic acid was further purified by preparative TLC using benzene:ethyl acetate (50:50, v/v) as the solvent. The ester recovered from the plates was crystallized several times from benzene. The residue obtained by extraction with diethyl ether of each of the remaining three bands was subjected to preparative GLC under the conditions reported above. Yields of p-bromophenacyl esters of acids in different incubation experiments were as follows: 3-hydroxydodecanoic, 18-22 mg; acetic, 10-15 mg; butyric, 14-18 mg; hexanoic, 20-24 mg; octanoic, 16-21 mg; decanoic, 22-25 mg; and dodecanoic, 20-25 mg. The percent radioactivity associated with each acid after incubation was calculated as reported in the preceding paragraph.

Extraction, Purification, and Analysis Conditions of Incubations Conducted with [8-^{14}C] Octanoyl-CoA as the Substrate

After addition to each sample of 14.4 mg (0.1 mmol) octanoic acid, 16 mg (0.1 mmol) 3-hydroxyoctanoic acid, 23.2 mg (0.2 mmol)

TABLE I

Percent Radioactivity[a] Found in Acids after Incubation[b] of [U-^{14}C]Stearoyl-CoA, [12-^{14}C]Dodecanoyl-CoA, and [8-^{14}C] Octanoyl-CoA with Subcellular Fractions of Rat Liver Homogenate[c]

	Substrate											
	[U-^{14}C] Stearoyl-CoA[c]				[12-^{14}C] Dodecanoyl-CoA				[8-^{14}C] Octanoyl-CoA			
Acid	A	A boiled	B	B boiled	A	A boiled	B	B boiled	A	A boiled	B	B boiled
Stearic	44.5	98.2	9.6	99.1	-	-	-	-	-	-	-	-
3-Hydroxyoctadecanoic	6.2	0.1	1.0	0.2	-	-	-	-	-	-	-	-
Hexadecanoic	10	0	0.7	0.1	-	-	-	-	-	-	-	-
Dodecanoic	-	-	-	-	27.4	101.0	1.4	99.0	-	-	-	-
3-Hydroxydodecanoic	-	-	-	-	10.1	0.2	0.2	0.0	-	-	-	-
Decanoic	-	-	-	-	28.2	0.1	0.5	0.1	-	-	-	-
Octanoic	-	-	-	-	9.9	0.0	0.6	0.1	52.3	99.3	5.0	101.0
3-Hydroxyoctanoic	-	-	-	-	-	-	-	-	10.7	0.1	0.0	0.0
Hexanoic	-	-	-	-	1.0	0.0	4.5	0.0	10.5	0.1	5.5	0.0
Butyric	-	-	-	-	0.1	0.1	28.2	0.2	0.5	0.0	33.6	0.1
Acetic	-	-	-	-	1.4	0.0	8.1	0.2	0.7	0.0	4.1	0.0

[a]% Radioactivity associated with each acid was calculated as follows: $\frac{m.r_s \times n}{S} \times 100$ where m.r = dis/min per mmol of the purified p-bromphenacyl ester; n=mmol of nonlabeled acid added after incubation; S = total radioactivity of the incubated substrate.

[b]Incubation conditions: either 105,000 x g soluble proteins (A) 5 mg/ml or 105,000 x g soluble proteins from sonicated mitochondria (B) 0.5 mg/ml; substrate 0.01 mM, sucrose 50 mM, adenosine triphosphate 2.5 mM, oxidized nicotinamide adenine dinucleotide 0.5 mM, CoA 0.1 mM, ethylenediaminetetraacetic acid 2.5 mM, phosphate 75 mM, Mg^{++} 5 mM; arsenite 10 mM; pH 7.4.

[c]Values reported represent the average of three different enzyme preparations on which each assay was made in duplicate: there was always < 5% difference between duplicates and <10% between enzyme preparations.

[d]Percent radioactivity associated with acids after incubation of this substrate was also calculated by triangulation of the radioactivity peaks associated in the radio-gas chromatogram to methyl stearate (43.7%), 3-hydroxyoctadecanoate (8.2%), and hexadecanoate (7.9%), considering 100% the incubated radioactivity.

hexanoic acid, 17.6 mg (0.2 mmol) butyric acid, and 24 mg (0.4 mmol) acetic acid, the samples were treated as described above for incubations with dodecanoyl-CoA. Yields of p-bromophenacyl esters of acids in different incubation experiments were as follows: 3-hydroxyoctanoic, 15-18 mg; acetic, 11-16 mg; butyric 14-17 mg; hexanoic, 12-22 mg; and octanoic, 16-18 mg.

RESULTS AND DISCUSSION

We reported previously (1) that long chain fatty acyl-CoA are degraded in vitro via a β-oxidation process by the 105,000 x g soluble fraction of rat liver homogenate. After incubation of uniformly labeled oleoyl- and stearoyl-CoA, a considerable fraction of the radioactivity was associated with intermediates of 18,16, and 14 carbon atoms, whereas only trace amounts of radioactivity were associated with shorter acids (1). This prompted us to check if shorter acyl CoA are also degraded by soluble enzymes. Octanoyl- and dodecanoyl-CoA labeled at the methyl carbon were used as the substrates in this study to follow the complete degradation of the molecule through the formation of acetate units from the last two carbon atoms. The absence of radioactivity associated with these compounds would definitely exclude the formation of acetate units deriving from the last two carbon atoms of the acyl chain. Due to their high volatility, the quantitative recovery of short chain fatty acids from the incubation mixtures is quite impossible because the procedure requires the concentration of a dilute solution of the acids; moreover, the amounts recovered are different for the various acids. To solve this problem, radioactivity associated with the acids after incubation was determined by a new method which gives reproducible values with respect to their amounts independently from quantitative recovery. For this purpose, known amounts of nonlabeled acids were added at the end of the incubation time. After saponification and acidification, the acids were extracted with diethyl ether. The radioactivity associated with the acids was then calculated from the molar radioactivity of their p-bromophenacyl esters prepared and purified as described in Materials and Methods. Since nonlabeled acids were added before any other treatment of the incubation mixture, the final results are independent of the total recovery of the acids. However, to obtain enough of the pure p-bromophenacyl esters for reliable weighing and counting, at least 0.1-0.2 mmol of each nonlabeled acid had to be added. The radiochemical purity of p-bromophenacyl esters was checked by crystallizing the purified acid several times; no change of the molar radioactivity was observed by repeated crystallization. The identity and the chemical purity were determined by GLC-MS; purity was always > 99% and retention times and mass spectra corresponded to those of authentic p-bromphenacyl esters. To check the validity of the analytical method, experiments using [U-^{14}C] stearoyl-CoA were performed. The transformation of the substrate was evaluated by determining the radioactivity associated with unchanged substrate and with two metabolites, both by radio-gas chromatography of the methyl esters of the acids extracted after incubation, as reported previously (1), and from the molar radioactivity of p-bromophenacyl esters as described in Materials and Methods. For this purpose, stearic, 3-hydroxyoctadecanoic, and hexadecanoic acids were added to the mixture at the end of the incubation. As shown in Table I, results obtained with the new analytical method (44.5% of radioactivity associated with stearic acid after incubation with soluble enzymes) are in good agreement with those obtained by radio-gas chromatography (43.7%, Table I, *c*) and those reported previously (45.6%) (1). Values for 3-hydroxyoctadecanoic and hexadecanoic acids determined using the two analytical methods were also in agreement. The sum of the analyzed compounds in incubation samples of [U-^{14}C] stearoyl-CoA with soluble enzymes accounts for 60.7% of the incubated radioactivity. The remaining 39.3% is considered associated with other catabolites as demonstrated previously (1). Moreover, 98.2% of the incubated radioactivity was found associated with stearic acid when labeled stearoyl-CoA was incubated with boiled soluble enzymes (Table I). From this data, it appears that the new analytical method gives reliable results in comparison with the previously described method.

Using the described procedure, the degradation products obtained by incubation of [8-^{14}C] octanoyl-CoA and [12-^{14}C] dodecanoyl- CoA both by soluble enzymes and sonicated mitochondrial enzymes were analyzed. The percent transformation of octanoyl-CoA by soluble enzymes was of the same order as that of stearoyl-CoA. Considerable amounts of radioactivity were found associated with 3-hydroxyoctanoic acid and hexanoic acids (Table I) and no radioactivity with butyric and acetic acids. The transformation of dodecanoyl-CoA was also of the same order as that of stearoyl-CoA and, in this case, radioactivity was found in 3-hydroxydodecanoic, decanoic, and octanoic acids. Again, trace amounts

of radioactivity were present in butyric and acetic acids. In incubations with octanoyl-CoA and dodecanoyl-1 CoA, the radioactivity was 74.7% and 78.1%, respectively. Radioactivity up to 100% can be considered associated with other possible intermediates of the oxidative catabolism, such as the 2,3- and 3,4- unsaturated acids and the 3-hydroxyacids with carbon chain shorter than that of the substrate. Therefore, it can be concluded that soluble enzymes catalyze only a partial degradation of medium chain acyl-CoA as found before for long chain derivatives (1).

The degradation of octanoyl and dodecanoyl-CoA by solubilized mitochondrial enzymes was also determined in order to compare the oxidative patterns. The transformation of both substrates was much higher than with soluble enzymes. In fact, only 1.4% and 5% of the incubated radioactivity was found in dodecanoic and octanoic acids in incubations with dodecanoyl-CoA and octanoyl-CoA, respectively (Table I). Radioactivity associated with the seven acids analyzed in incubations with dodecanoyl-CoA and with the five analyzed acids in those with octanoyl-CoA as substrate represented 43.5% and 48.2%, respectively. Radioactivity up to 100% should be associated with nonanalyzed intermediates of the oxidative degradation of the substrate, with acetic acid, or with other compounds deriving from the catabolism of acetic acid. Most of the radioactivity of the analyzed acids was found in butyric acid and acetic acid (28.2% and 33.6%, respectively), indicating that the oxidative pattern differs greatly from that observed with cytoplasmic enzymes. Stewart et al. (6) reported recently that free intermediates of the β-oxidation are formed when octanoyl-CoA is oxidized by enzymes solubilized from acetone dried mitochondria. In particular, they presented evidence for the formation of 3-hydroxyoctanoyl- and hexanoyl-derivatives during the incubation. Under our experimental conditions, using mitochondrial enzymes solubilized by sonication and longer incubation times, the formation of free intermediates was demonstrated by the radioactivity found in hexanoic and butyric acids both from octanoyl-CoA and dodecanoyl-CoA, whereas only small amounts of radioactivity were found associated with longer chain acids and 3-hydroxyacids.

From the reported data, it derives that cytoplasmic enzymes catalyze the β-oxidative cleavage of medium chain fatty acyl-CoA esters. The degradation pattern of medium chain acyl-CoA esters is similar to that of long chain acyl-CoA derivatives (1). The finding that trace amounts of radioactive acetic acid are formed from substrates labeled at the methyl carbon atom confirms that the acyl chain is only partially degraded by the cytoplasmic enzymes.

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SHORT COMMUNICATIONS

Relationship Between Desaturation and Chain Elongation of Palmityl-CoA in Rat Liver Microsomes

ABSTRACT

The relationship between desaturation and chain elongation of palmityl-CoA was studied in rat liver microsomes. Enzyme activities for desaturation and chain elongation were stimulated by re-feeding starved rats. The activities of desaturation and chain elongation in re-fed rats were four- and twofold greater than those in normal rats, respectively. When a sonicated dispersion of lecithin was added to the incubation medium containing both reactions, chain elongation activity, especially formation of stearic acid, was stimulated and desaturation activity was apparently depressed.

INTRODUCTION

The enzymes catalyzing desaturation and chain elongation of fatty acids are located in the microsomal fraction of rat liver and show identical cofactor requirements (1).

It is well known that the desaturation reaction is substantially affected by various physiological conditions of animals, such as dietary changes, age, and diabetes (2-4), and also partially regulated by the amounts of some endogenous materials, such as α-glycerophosphate, adenosine triphosphate (ATP), fatty acids, and phospholipids (2,5-8).

On the other hand, the chain elongation reaction is also affected by the chain length and degree of unsaturated fatty acyl-CoA used as substrate (9), the amounts of ATP and reduced pyridine nucleotides as cofactors (10) and fatty acids (11). Furthermore, the effect of the nutritional state of the animal on chain elongation has recently been reported by Sprecher and by our laboratory (12,13).

However, little is known about the relationship between desaturation and chain elongation reactions in rat liver microsomes.

In this paper, we deal with the effect of dietary alteration of rats and sonic dispersion of lecithin on both desaturation and chain elongation of palmityl-CoA under aerobic conditions.

MATERIALS AND METHODS

Palmityl-1-^{14}C-CoA (58.2 mCi/mM) was obtained from New England Nuclear Corp. (Boston, MA). Malonyl-CoA, palmityl-CoA, reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Lecithin was prepared from egg yolk by the method of Faure (14) and purified by the same procedure as described previously (15).

Female albino rats of the Wistar strain, weighing 80-120 g, were used. Some animals were starved for 48 hr and then fed ad libitum for 24 hr on a commercial diet obtained from Oriental Yeast Co. (Tokyo, Japan). These rats are referred to as "re-fed rats."

The rats were killed by cervical fracture and livers were excised and homogenized in 3 vol of cold 0.25 M sucrose solution. The homogenates were centrifuged 15 min at 12,000 g. The resultant supernatant was then centrifuged 60 min at 100,000 g. The microsomal pellet was washed with 0.25 M sucrose solution using the ultracentrifugal procedure. The washed microsomes were suspended in an aliquot of 0.1 M phosphate buffer (pH 7.4) containing 0.25 M sucrose. The suspension was used as the enzyme source. The incubation mixture contained 45 μM palmityl-CoA, 3.4 μM palmityl-1-^{14}C-CoA, 100 μM malonyl-CoA, 6 mM β-mercaptoethanol, 3 mM NADH, 3 mM NADPH, 25 mM sucrose, and microsomes (protein 0.8 mg). The final volume was adjusted to 1 ml with 0.1 M phosphate buffer (final concentration 100 mM, pH 7.4). Incubation was performed at 30 C under air with constant shaking. At the end of the incubation time, the reaction was stopped by addition of 1 ml of 10% methanolic-KOH and then heated 30 min at 70 C. After acidification with 6N-HCl, extraction of lipids and isolation of fatty acids were performed by the procedure described previously (13). Rates of desaturation and chain elongation of palmityl-CoA were calculated from the peak area of radioactivity of each fatty acid on a radiochromatogram obtained by the radio-gas liquid

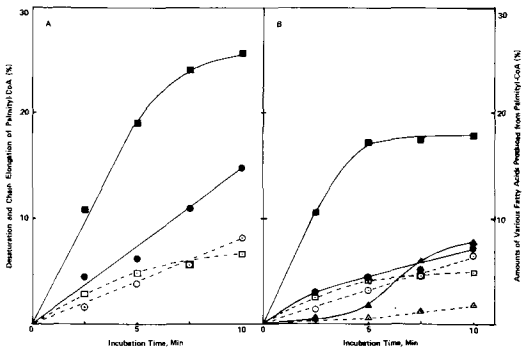

FIG. 1. Relationship between desaturation and chain elongation of palmityl-CoA in liver microsomes of normal and re-fed rats. Incubation conditions were described in the text. **1A**: ■——■ chain elongation in re-fed rats; ●——● desaturation in re-fed rats; □——□ chain elongation in normal rats; ○——○ desaturation in normal rats. **1B**: ■——■ palmitoleic acid in re-fed rats; ●——● stearic acid in re-fed rats; ▲——▲ octadecenoic acid in re-fed rats; □——□ palmitoleic acid in normal rats; ○——○ stearic acid in normal rats; △——△ octadecenoic acid in normal rats.

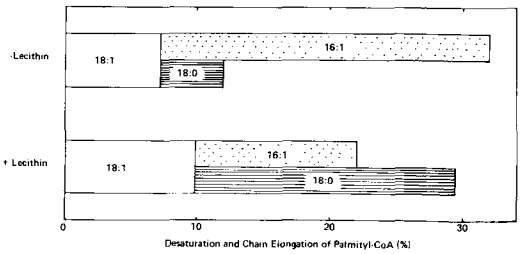

FIG. 2. Effect of a sonicated dispersion of lecithin on desaturation and chain elongation of palmityl-CoA in liver microsomes of re-fed rats. Incubation conditions were described in the text, except for the use of 360 μg of lecithin dispersion. Incubation was carried out at 30 C for 7 min. Prior to incubation, 0.8 mg of microsomal protein and the dispersion were preincubated at 0 C for 60 min. The values (%) for the desaturation and chain elongation reactions were indicated as $C_{16:1}$ + $C_{18:1}$ acids (▨) and as $C_{18:0}$ + $C_{18:1}$ acids (▤), respectively.

chromatographic method as described previously (13).

RESULTS AND DISCUSSION

We have previously reported that, by using radio-gas liquid chromatography after the incubation of palmityl-CoA in a medium containing malonyl-CoA, NADH, NADPH, and KCN under anaerobic conditions, chain elongation activity in liver microsomes of re-fed rats is ca. twofold greater than that of normal rats (13).

In this paper, we first examined the alteration of the activities of both desaturation and chain elongation of palmityl-CoA in liver microsomes of rats fasted for 48 hr and then re-fed a balanced diet for 24 hr. As shown in Figure 1A, both the enzyme activities in liver microsomes of normal and re-fed rats were linear at least to 5 min of incubation under the present experimental conditions. Desaturation and chain elongation activities were not very different in normal rats during the incubation. However, both enzyme activities were markedly stimulated by re-feeding starved rats, i.e., the desaturation and chain elongation activities in re-fed rats were four- and twofold greater than those in normal rats, respectively. Accordingly, as shown in Figure 1B, the major fatty acids derived from palmityl-CoA were palmitoleic and stearic acids in normal rats and palmitoleic acid in re-fed rats. In addition, formation of octadecenoic acid in re-fed rats was markedly increased by prolonging incubation. Although octadecenoic acid derived from palmityl-CoA is expected to contain oleic and vaccenic acids, we have not determined the ratio of these monoenoic acids.

These results suggest that the enzyme for desaturation may be induced more rapidly than the enzymes catalyzing chain elongation by re-feeding starved rats.

We have also reported that chain elongation activity in normal and re-fed rats under anaerobic conditions is stimulated by addition of a sonicated dispersion of lecithin and decreased by acetone extraction or phospholipase C treatment of liver microsomes, and that the activity decreased by acetone or phospholipase C treatments was partially or completely restored by addition of the dispersion (13). Prior to investigating the effect of a sonicated dispersion of lecithin on both reactions, the effect of the dispersion of palmityl-CoA desaturation in liver microsomes of re-fed rats was studied in the absence of malonyl-CoA. Even though 560 μg of lecithin was added to the incubation medium, palmityl-CoA desaturation was not affected. However, we have found that chain elongation of palmityl-CoA is stimulated ca. 70% by addition of 560 μg of lecithin (13).

On the basis of these results, the effect of a sonicated dispersion of lecithin on desaturation and chain elongation of palmityl-CoA in liver microsomes of re-fed rats was studied. As shown in Figure 2, desaturation activity (as $C_{16:1}$ + $C_{18:1}$) in re-fed rats was greater than chain elongation activity (as $C_{18:0}$ + $C_{18:1}$), as in the results in Figure 1A. However, when the dispersion was added to the incubation medium containing both reactions, chain elongation activity, especially formation of stearic acid, was substantially stimulated and desaturation

activity was apparently depressed. Apparently, palmityl-CoA as substrate may be preferentially introduced into the chain elongation pathway rather than into the desaturation pathway by addition of the dispersion.

The relationship between enzyme activity and phospholipid content in rat liver microsomes has been most recently reported by Belina et al. (16). They have indicated that the sex difference in some specific phospholipids is well related to the corresponding sex difference in drug metabolizing activity. We have also observed that chain elongation activity is not stimulated by addition of α-glycerophosphate and lysolecithin instead of lecithin (13). Accordingly, some specific phospholipids, especially lecithin, may paly an important role in enzymatic reactions as one of the regulators of both reactions in rat liver microsomes.

Although no definite information is now available on the mechanisms of stimulation of the chain elongation reaction and inhibition of the desaturation reaction in rat liver microsomes by the dispersion, the stimulatory effect of the dispersion on chain elongation in the incubation medium containing both reactions may be due to increased formation of an active form of enzyme or substrate and/or to elevated formation of enzyme-substrate complex for the chain elongation reaction by the dispersion. However, whether lecithin added to the incubation system in vitro as the dispersion acts in a similar physical state as compared to one in rat liver microsomes in vivo remains to be clarified.

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Hypolipidemic Principles of *Cicer Arietinum:* Biochanin-A and Formononetin

ABSTRACT

Two isoflavones, biochanin-A and formononetin isolated from gram *Cicer arietinum*, have been shown to possess hypolipidemic properties for Triton WR-1339 induced hyperlipidemia in male albino rats, when administered as a crude extract or as individual compounds.

INTRODUCTION

As a result of extensive feeding trials on rats, rabbits and humans, it is apparent that gram (*Cicer arietinum*) has been found to reduce the elevated serum total cholesterol and free esterified cholesterol content of α- and β-lipoproteins of rats and rabbits fed hypercholesterolemia inducing diet (1-5). The protective role of gram appears similar to that of estrogens. Biochanin-

activity was apparently depressed. Apparently, palmityl-CoA as substrate may be preferentially introduced into the chain elongation pathway rather than into the desaturation pathway by addition of the dispersion.

The relationship between enzyme activity and phospholipid content in rat liver microsomes has been most recently reported by Belina et al. (16). They have indicated that the sex difference in some specific phospholipids is well related to the corresponding sex difference in drug metabolizing activity. We have also observed that chain elongation activity is not stimulated by addition of α-glycerophosphate and lysolecithin instead of lecithin (13). Accordingly, some specific phospholipids, especially lecithin, may paly an important role in enzymatic reactions as one of the regulators of both reactions in rat liver microsomes.

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ABSTRACT

Two isoflavones, biochanin-A and formononetin isolated from gram *Cicer arietinum*, have been shown to possess hypolipidemic properties for Triton WR-1339 induced hyperlipidemia in male albino rats, when administered as a crude extract or as individual compounds.

INTRODUCTION

As a result of extensive feeding trials on rats, rabbits and humans, it is apparent that gram (*Cicer arietinum*) has been found to reduce the elevated serum total cholesterol and free esterified cholesterol content of α- and β-lipoproteins of rats and rabbits fed hypercholesterolemia inducing diet (1-5). The protective role of gram appears similar to that of estrogens. Biochanin-

A (5,7-dihydroxy-4′-methoxy isoflavone) and formononetin (7-hydroxy-4′-methoxy isoflavone), known to have estrogenic properties (6,7), have been isolated from gram. We describe here the hypolipidemic effect of biochanin-A and formononetin present in a crude ethanol extract of gram.

METHODS

Biochanin-A and formononetin were isolated from germinated gram as described earlier (8) with the modification that, instead of dialysis, the germs were directly extracted with alcohol. The alcoholic extract of germs was evaporated under vacuum to a thick slurry and filtered. The yield of petroleum-ether washed powder (crude isoflavone fraction) with respect to germ wt was 0.18%. For the evaluation of hypocholesterolemic property of crude isoflavone fraction, three groups each of 10 young male albino rats, weighing 90-100 g, were fed a basal diet (9).

The basal diet consisted of (%) casein, 15.0; sucrose, 68.3; hydrogenated vegetable oil, 10.0; salt mixture, 4.0; cellulose, 2.0; vitamin mixture, 0.5; and choline chloride, 0.2. The salt mixture contained (% in the mixture) NaCl, 4.6; $Na_2HPO_4 \cdot H_2O$, 9.3; K_2HPO_4, 25.6; $CaH_4(PO_4)_2 \cdot H_2O$, 14.5; $Fe(C_6H_5O_7) \cdot .5H_2O$, 3.2; $Ca(C_3H_5O_3)_2 \cdot 5H_2O$, 34.9; $MgSO_4$, 7.0; and KI, 0.9. Each 100 g of vitamin mixture contained (in mg) riboflavin, 150; thiamin, 100; nicotinic acid, 1,000; pyridoxin, 100; cyanocobalamin, 1; panthothenic acid, 500; folic acid, 50; ascorbic acid, 3,750; vitamin E, 100; vitamin A, 250,000 IU; Vitamin D_2, 20,000 IU; and sucrose to 100 g. In addition, the diet of group II and of group III contained 1% cholesterol and 0.2% cholic acid. The diet of group III was supplemented with 0.15% (w/w) crude isoflavone fraction. Each morning each group of animals was given a restricted diet (10 g/day/rat) to ensure the same food intake. After 3 weeks of dietary regimen, the animals were anesthetized with ether, blood was withdrawn by cardiac puncture to obtain serum, and the aorta was removed immediately. Total and ester cholesterol in serum and aorta were determined by the Bloor method (10), phospholipids were determined by the Marinetti method (11), and the triglycerides were estimated by the procedure of Van Handel and Zilversmit (12). At the end of treatment, there was no significant difference in the average body wt of rats in group II (128±14 g) and group III (135±7 g), and the average liver wts of both groups were the same.

For further purification, crude isoflavone fraction was dissolved in acetone, applied on a Silica Gel G column (14.0 x 4 cm), and eluted with 2%, 3%, and 5% ethylacetate-benzene. Biochanin-A followed by formononetin appeared in 5% ethylacetate-benzene fraction. After evaporation of the pooled effluent, the residue was crystallized in ethanol at room temperature. The purity of each compound was checked by thin layer chromatography (TLC) using ethylacetate-benzene (10%), color reagents (13), and ultraviolet spectrum. The recrystallized Biochanin-A (uncorrected mp, 214-215 C) and formononetin (uncorrected mp, 255-256 C) were used for evaluation of their hypolipidemic properties.

Three groups each of five young male albino rats, weighing 150 g (range 140-160 g), were fasted for 24 hr and intraperitoneally injected with 300 mg Triton WR-1339/kg/body wt dissolved in 0.15M NaCl at a concentration of 45.0 mg/ml (14,15). Biochanin-A or formononetin (7.5 mg) was suspended in 2 ml of 0.25% aqueous methyl cellulose, which provided the total screening dose of 15.0 mg/kg for 150 g rat in 4 ml. Each rat first received two 2 ml doses by gastric intubation immediately after the Triton injection and the second dose 20 hr later. The control group received 4 ml of 0.25% aqueous methyl cellulose in two doses. Fasting was continued during the post-Triton period. Blood was withdrawn by cardiac puncture 43 hr after Triton administration under ether anesthesia. Serum and liver lipids were estimated as described earlier, except that cholesterol was measured by the method of Zlatkis et al. (16). Liver lipids were extracted by the method of Folch et al. (17). Statistical significance was calculated by Student's *t*-test.

RESULTS AND DISCUSSION

As is shown in Table I, the serum total cholesterol level in rats fed 1% cholesterol-added diet was elevated to ca. 700 mg/100 ml, and the supplementation of crude isoflavone fraction to the cholesterol diet depressed this elevation 34.9%. In contrast, phospholipid levels revealed no significant change in experimental groups. The cholesterol:phospholipid ratio, believed to be an index of atherogenecity, was markedly decreased by supplements of the crude fraction. Ester and free cholesterol were reduced to the extent of 30.2% and 48.1%, respectively. However, triglycerides remained uneffected. The supplement also depressed the total aorta cholesterol.

It is evident from Table II that biochanin-A significantly ($P<0.01$) reduced all lipid parameters of the Triton-treated rats. Formononetin did signficantly ($P<0.01$) reduce all serum lipid

TABLE I

Effect of Crude Isoflavone Fraction on Serum and Aorta Total Cholesterol (Mean ± Standard Error in mg/100 ml Serum)

Lipids	Group I Basal diet	Group II Basal + cholesterol + cholic acid	Group III Basal + cholesterol + cholic acid + isoflavone fraction	Percent[a] reduction
Serum				
Total cholesterol	76 ± 8	700 ± 60	456 ± 57[b]	34.9
Ester cholesterol	57 ± 6	516 ± 50	360 ± 49[c]	30.2
Free cholesterol	19 ± 2	185 ± 15	96 ± 12[d]	48.1
Phospholipids	208 ± 14	395 ± 38	386 ± 63	2.2
Triglycerides	74 ± 11	407 ± 17	405 ± 11	--
Cholesterol:phospholipid ratio	0.37	1.77	1.18	33.3
Aorta				
Total cholesterol (mg/100 g wet wt)	133 ± 13	357 ± 38	221 ± 17[e]	38.0

[a]Compared to group II.

[b]Significantly different from group II ($P<0.01$).

[c]Significantly different from group II ($P<0.05$).

[d]Significantly different from group II ($P<0.001$).

[e]Significantly different from group II ($P<0.01$).

TABLE II

Effect of Biochanin-A and Formononetin on Serum Lipids of Triton Induced Hyperlipidemia (Mean ± Standard Error in mg/100 ml Serum)

Lipids	Triton treated	Triton + biochanin-A treated	Reduction (%)	Triton-formononetin treated	Reduction (%)
Total cholesterol	421 ± 33	272 ± 9[a]	35.4	305 ± 11[b]	27.6
Phospholipids	880 ± 59	567 ± 10[c]	35.6	673 ± 80[d]	23.5
Triglycerides	912 ± 67	603 ± 45[c]	33.9	710 ± 19[a]	22.1

[a]Significantly different from Triton treated ($P<0.01$).
[b]Significantly different from Triton treated ($P=0.01$).
[c]Significantly different from Triton treated ($P<0.001$).
[d]Significantly different from Triton treated ($P<0.1$).

parameters except phospholipids. Relatively, biochanin-A appears more active than formononetin. These isoflavones did not show significant effect on liver lipids except formononetin, which caused significant reduction only in liver phospholipids (Triton treated: 6518±228; biochanin-A treated: 5844±309; formononetin treated: 5382±278 mg/100 g wet wt) to 17.4% ($P<0.02$). The significant hypocholesteremic action of biochanin-A and formononetin suggests it as a potential drug, provided it is well tolerated in man. The hypocholesterolemic properties of gram may be attributed to the isoflavones of gram. It is likely that these isoflavones act as proesterogens. However, until more is known about the metabolism of isoflavones, little can be said about their possible role as precursors of estrogen. It is likely that these isoflavones show their hypocholesterolemic activity because of their structural similarity to estrogen, stilbestrol (18).

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trans-6-Hexadecenoic Acid in the Spadefish *Chaetodipterus faber*

ABSTRACT

Fatty acid methyl esters were prepared from spadefish *(Chaetodipterus faber)* liver oil and were analyzed by open tublar gas chromatography. The unusual fatty acid *trans*-6-hexadecenoic acid was identified as a major monoenoic component by reductive ozonolysis and by mass spectrometry of the di-trimethylsilyl ether. Very minor amounts of 7-methyl-7-hexadecenoic acid were found.

INTRODUCTION

Spadefish are representative of many of the reef fishes and are found on rocky bottoms and in wrecks of the Atlantic from Cape Cod to Rio de Janeiro and the West Indies (1). An excellent food fish prized by many anglers, the spadefish is not caught in sufficient numbers to be of substantial commercial value.

The presence of *trans*-6-hexadecenoic acid ($t16{:}1\omega10$) has been reported in several marine organisms (2-8). During routine investigations, we found spadefish liver oil to contain a relatively high concentration of an unidentified monounsaturated fatty acid component which was tentatively identified as $t16{:}1\omega10$ according to its gas chromatographic (GC) behavior. This study was undertaken to confirm the structure of this fatty acid.

EXPERIMENTAL PROCEDURES

Spadefish were obtained at the jetties near Charleston, SC, in July 1975. The liver was extracted by the method of Bligh and Dyer (9). The chloroform layer was evaporated to dryness in a rotary evaporator, and fatty acid methyl esters were prepared in sealed tubes in 3% methanolic H_2SO_4 heated to 60 C for 2 hr. The fatty acid methyl esters were purified by thin layer chromatography (TLC) on silica gel (Supelcosil 12A, Supelco Inc., Bellefonte, PA) by developing in chloroform. The plate was sprayed with 0.1% 2',7'-dichlorofluorescein (Fisher Scientific Co., Atlanta, GA), and the fatty acid methyl ester band was extracted from the adsorbent with chloroform:methanol: water (50:50:1). The solvent was evaporated to dryness, and the fatty acid methyl esters were taken up in hexane.

Methyl esters were chromatographed on a Hewlett-Packard 5711-A gas chromatograph equipped with a 45.7 m x 0.25 mm inside diameter open-tubular stainless steel column coated with Silar-5CP (Perkin-Elmer Corp., Norwalk, CT). GC temperature conditions were: injector 300 C, column 170 C, detector 300 C. Helium carrier gas pressure was 50 psig. All equivalent chain length (ECL) values were also obtained under identical conditions on a butanediol succinate (BDS) coated open tubular column of the same dimensions and manufacturer. Integration values were obtained on a Vidar 6300 digital integrator. Fatty acid methyl esters were separated according to degree of unsaturation on TLC plates coated with silica gel (Supelcosil 12A) impregnated with 5% $AgNO_3$. Plates were developed in chloroform at 5 C and were sprayed with 2',7'-dichlorofluorescein. Each monounsaturated band was gas chromatographed under the conditions described above. A portion of each band was hydrogenated and another was subjected to ambient temperature reductive ozonolysis (10-12). Ozonolysis products were chromatographed on a Beckman GC-45 gas chromatograph fitted with a 1.8 m x 4 mm glass column packed with 8% OV-101 on 80/100 mesh Gas-Chrome Q (Applied Science Laboratories, State College, PA). After a 3 min isothermal period at 60 C, the column oven was programmed from 60 C to 260 C at 25 C/min. A portion of the uppermost band was also derivatized to the di-trimethylsilyl ether after oxidation with OsO_4 (13) and was chromatographed on a Finnigan gas chromatograph-mass spectrometer (GC-MS) fitted

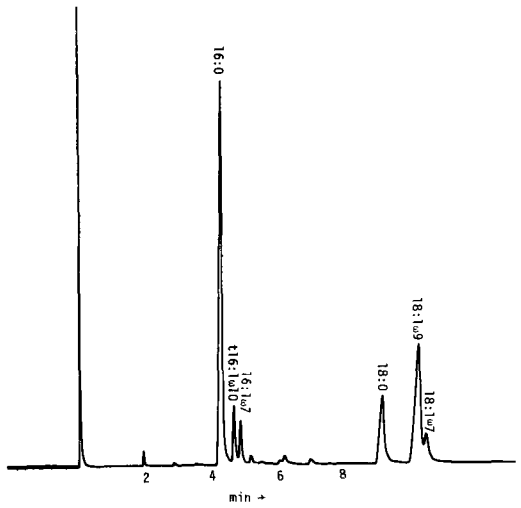

FIG. 1. A partial gas chromatogram of spadefish liver oil fatty acid methyl esters which were gas chromatographed on an open tublar column coated with Silar-5CP.

TABLE I

Principal Fatty Acids of Total Lipids of the Spadefish *Chaetodipterus faber*

Fatty acid	Total lipids (% by wt)
14:0	0.9
16:0	33.9
trans 16:1ω10	4.6
16:1ω7	3.3
ECL[a] 16.48	1.1
ai[b] 17:0	0.5
17:0	2.0
17:1ω8	1.1
18:0	10.2
18:1ω9	20.0
18:1ω7	3.9
20:1ω9	0.9
20:1ω7	0.5
20:4ω6	2.9
20:5ω3	2.2
22:4ω6	2.2
22:5ω3	2.5
22:6ω3	3.6
Others[c]	7.3

[a]Equivalent chain length (ECL) values given in this table were determined on Silar-5CP under previously stated conditions.

[b]The abbreviations i and ai designate iso and anteiso branched chain fatty acids.

[c]Only those fatty acids accounting for > 0.5% of the total identified fatty acids are included in the table. Minor fatty acids accounting for less than this percentage included 12:0, i14:0, 15:0, i16:0, i17:0, 16:3ω3, 7-methyl-7-hexadecenoic acid, i18:0, i19:0, ai19:0, 19:0, ECL 19.19, 19:1ω8, 20:0, 20:1ω11, 20:2ω6, 20:4ω3, 21:0, ECL 21.06, 22:1ω11, 22:1ω9, 22:5ω6.

with a 1.2 m x 2 mm glass column packed with 2% OV-17 on 100/120 mesh Supelcon AW-DMCS (Supelco Inc. Bellefonte, PA).

RESULTS AND DISCUSSION

As shown in Figure 1, GC of the total methyl esters revealed two 16:1 peaks with ECL values of 16.22 and 16.33 on Silar-5CP (16.23 and 16.29 on BDS). After chromatographing the methyl esters by TLC on $AgNO_3$ impregnated silica gel, the monounsaturates were resolved into four bands. The uppermost band (Rf 0.37) contained the component of interest (ECL 16.22 on Silar-5CP) in almost pure form. After hydrogenation, this ECL value shifted to 16.00, eliminating a branched structure. Based on the separation of *cis* and *trans* isomers by $AgNO_3$-TLC (14), it was concluded that the double bond configuration of this component was *trans* (RF 0.21 for *cis* 16:1ω7). Reductive ozonolysis yielded a 10 carbon aldehyde and a 6 carbon aldehydic ester. GC-MS of the methyl di-trimethylsilyl ether yielded diagnostic ions at m/e 217 and 229. These data confirmed the structure of this component as *trans*-6-hexadecenoic acid (*t*16:1ω10). Although probably present in minor amounts, other ω10 chain lengths were not detected. Minor amounts of 7-methyl-7-hexadecenoic acid representing <0.5% of the total lipid fatty acids were noted.

The fatty acid profile of spadefish liver lipid is generally representative of marine fish oils, with the exception of palmitic acid, the predominant fatty acid in *Chaetodipterus*. It accounted for almost 34% of the fatty acids, which is two- to threefold the relative % by wt of palmitate found in cod liver oil (15), commercial redfish and flounder oils (16), and pilchard oil (17).

The fatty acid *t*16:1ω10 has been reported in the lipids of various marine organisms, including several marine turtles (5,6,8), coelenterates (2-4), and fish (7). In Table I, it is shown that in *Chaetodipterus* *t*16:1ω10 comprised a considerable portion of the total lipid fatty acids and its relative % by wt exceeded that of 16:1ω7. Because coelenterates appear to be a common food source of this fatty acid in certain fish (7) and turtles (5,6,8), the substantial presence of *t*16:1ω10 in *Chaetodipterus* could be a reflection of a high dietary intake of such organisms.

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[Received November 19, 1975]

Biosynthesis of Prostaglandin E_1 by Human Seminal Vesicles

ABSTRACT

[1-^{14}C] 8,11,14-Eicosatrienoic acid was converted into prostaglandin E_1 when incubated with a homogenate of human seminal vesicles. No conversion could be detected with homogenates of human prostate or testis.

INTRODUCTION

Occurrence of prostaglandins in seminal fluid of man (1-3) and sheep (1,4) is well documented. In addition, prostaglandin-like material has been isolated from monkey (5) and goat (6) seminal fluid. Prostaglandins have also been found in accessory genital organs, such as the vesicular gland and ampulla ductus deferens of sheep (1). More recently, prostaglandins (PGE_1, PGE_2, $PGF_{1\alpha}$, and $PGF_{2\alpha}$) were isolated from testicular tissue of rat (7) and swine (8).

Biosynthesis of prostaglandins from certain polyunsaturated fatty acids by homogenates of the vesicular gland of sheep was described in 1964 (9,10). An acetone powder prepared from bull seminal vesicles (11) and homogenates of guinea pig lung (12) also catalyzed the formation of prostaglandins from added unsaturated fatty acids. Since then, it has become apparent that a vast number of tissues from many species are capable of prostaglandin biosynthesis (see ref. 13 and references cited therein).

We are presently exploring prostaglandin biosynthesis in organs of the genital tract of the human male. Results obtained with homogenates of seminal vesicles, prostate, and testis will be given in this communication.

EXPERIMENTAL PROCEDURES

[1-^{14}C] 8,11,14-Eicosatrienoic acid was prepared from 1-chloro-7,10,13-nonadecatriene and Na^{14}CN, followed by hydrolysis of the nitrile (14). The specific radioactivity was 1.5 Ci/mol and the chemical and radiochemical purity > 97%.

The body of a 40 year old man was obtained ca. 1.5 hr after sudden violent death. The seminal vesicles, prostate, and testes were rapidly excised and placed in ice cold modified Bucher medium (20 mM KH_2PO_4, 72 mM K_2HPO_4, 27.6 mM nicotinamide, and 3.6 mM $MgCl_2$, pH 7.4). Part of the organs were minced and homogenized in medium (1:4, w/v) with a Potter-Elvehjem type homogenizer (Table I). [1-^{14}C] 8,11,14-Eicosatrienoic acid, 50 μg in 50 μl of ethanol, was added to the whole homogenates and the mixtures incubated with shaking at 37 C for 45 min. Five volumes of ethanol were added, and precipitated protein was filtered off. The filtrate was diluted with water, acidified to pH 3, and extracted twice with diethyl ether. Material obtained after evaporation of the ether was treated with diazomethane and subjected to thin layer radiochromatography with water saturated ethyl

TABLE I

Incubation of [1-^{14}C] 8,11,14-Eicosatrienoic Acid

Organ	Tissue wt (g)	Yield of labeled prostaglandin E_1 (%)
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Prostate	3	0
Testis	4	0

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Testis	4	0

acetate as solvent. The methyl esters of PGE_1, $PGF_{1\alpha}$, and 19-hydroxy-PGB_1 were used as references.

RESULTS AND DISCUSSION

Thin layer radiochromatographic analysis of the esterified product obtained after incubation of [1-^{14}C] 8,11,14-eicosatrienoic acid with seminal vesicle homogenate showed a major peak close to the solvent front corresponding to unconverted 8,11,14-eicosatrienoic acid and a smaller peak (20% of the radioactivity applied to the plate) that cochromatographed with the methyl ester of PGE_1 (R_F=0.69). No other peak of radioactivity was detected. The labeled prostaglandin was eluted from the silica gel, converted into the O-methyloxime–trimethylsilyl ether derivative, and subjected to gas liquid radiochromatography (column, 1% OV-1; column temperature, 210 C). The mass tracing showed two pairs of partially separated peaks corresponding to the *syn/anti* isomers of the O-methyloxime–trimethylsilyl ether derivatives of PGE_2 (C-24.0 and C-24.5) and PGE_1 (C-24.3 and C-24.8) (15). These prostaglandins were present in ca. equal amounts. The radioactivity tracing showed two peaks which coincided with the mass peaks of the derivative of PGE_1. It could be calculated from the size of the peaks of the radioactivity tracing and from the specific radioactivity of the incubated [1-^{14}C] 8,11,14-eicosatrienoic acid that only a small part of the PGE_1 isolated was derived from the added 8,11,14-eicosatrienoic acid. The identities of the two prostaglandins were conclusively established by mass spectrometric analysis using authentic materials as references.

Analysis by thin layer radiochromatography of the esterified products isolated after incubation of [1-^{14}C] 8,11,14-eicosatrienoic acid with homogenates of human prostate and testis showed only one peak at the solvent front corresponding to unreacted fatty acid. These data showed that human seminal vesicles are capable of formation of PGE_1 from added 8,11,14-eicosatrienoic acid and that this organ contains PGE_1 and PGE_2 and/or is capable of formation of these prostaglandins from endogenous precursor acids. The finding that prostaglandin biosynthesis could not be demonstrated with homogenates of human prostate and testis indicated that prostaglandins occurring in human seminal fluid are formed in the seminal vesicles. This is in agreement with the work of Eliasson (6), who found a correlation between the content of fructose and prostaglandin in different portions of "split-ejaculates," indicating that prostaglandins are formed in the seminal vesicles.

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ERRATUM

In "Identification of 3,6,9,12,15-Octadecapentaenoic Acid in Laboratory-Cultured Dinoflagellates" (*Lipids* 10:395 [1975]), two errors appear in Table II. On the EGSS-Y column, the equivalent chain length of 18:3ω3 should be 20.00 and that of 20:5ω3 should be 22.78.

acetate as solvent. The methyl esters of PGE_1, $PGF_{1\alpha}$, and 19-hydroxy-PGB_1 were used as references.

RESULTS AND DISCUSSION

Thin layer radiochromatographic analysis of the esterified product obtained after incubation of [1-^{14}C] 8,11,14-eicosatrienoic acid with seminal vesicle homogenate showed a major peak close to the solvent front corresponding to unconverted 8,11,14-eicosatrienoic acid and a smaller peak (20% of the radioactivity applied to the plate) that cochromatographed with the methyl ester of PGE_1 (R_F=0.69). No other peak of radioactivity was detected. The labeled prostaglandin was eluted from the silica gel, converted into the O-methyloxime–trimethylsilyl ether derivative, and subjected to gas liquid radiochromatography (column, 1% OV-1; column temperature, 210 C). The mass tracing showed two pairs of partially separated peaks corresponding to the *syn/anti* isomers of the O-methyloxime–trimethylsilyl ether derivatives of PGE_2 (C-24.0 and C-24.5) and PGE_1 (C-24.3 and C-24.8) (15). These prostaglandins were present in ca. equal amounts. The radioactivity tracing showed two peaks which coincided with the mass peaks of the derivative of PGE_1. It could be calculated from the size of the peaks of the radioactivity tracing and from the specific radioactivity of the incubated [1-^{14}C] 8,11,14-eicosatrienoic acid that only a small part of the PGE_1 isolated was derived from the added 8,11,14-eicosatrienoic acid. The identities of the two prostaglandins were conclusively established by mass spectrometric analysis using authentic materials as references.

Analysis by thin layer radiochromatography of the esterified products isolated after incubation of [1-^{14}C] 8,11,14-eicosatrienoic acid with homogenates of human prostate and testis showed only one peak at the solvent front corresponding to unreacted fatty acid. These data showed that human seminal vesicles are capable of formation of PGE_1 from added 8,11,14-eicosatrienoic acid and that this organ contains PGE_1 and PGE_2 and/or is capable of formation of these prostaglandins from endogenous precursor acids. The finding that prostaglandin biosynthesis could not be demonstrated with homogenates of human prostate and testis indicated that prostaglandins occurring in human seminal fluid are formed in the seminal vesicles. This is in agreement with the work of Eliasson (6), who found a correlation between the content of fructose and prostaglandin in different portions of "split-ejaculates," indicating that prostaglandins are formed in the seminal vesicles.

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Biosynthesis of Molecular Species of CDP-Diglyceride from Endogenously-Labeled Phosphatidate in Rat Liver Microsomes[1]

B.J. HOLUB and **J. PIEKARSKI**, Department of Nutrition, College of Biological Science, University of Guelph, Guelph, Ontario, Canada

ABSTRACT

The biosynthesis of [^{14}C] CDP-diglyceride was studied using rat liver microsomes which were endogenously labeled with [^{14}C] phosphatidic acid by preincubation of unlabeled microsomes with *sn*-[^{14}C] glycerol-3-phosphate and appropriate cofactors. The formation of CDP-diglyceride from radioactive phosphatidate showed an absolute requirement for CTP and $MgCl_2$. The newly formed [^{14}C] CDP-diglyceride was characterized by thin layer chromatography (TLC), isotopic labeling from radioactive CTP, and its ability to serve as substrate for the microsomal enzyme, CDP-diglyceride:inositol phosphatidyltransferase. The distributions of radioactive glycerol-3-phosphate among the various chemical classes of microsomal [^{14}C] phosphatidate and [^{14}C] CDP-diglyceride were determined following argentation TLC of their 1,2-diglyceride acetate derivatives. Most of the radioactivity among the phosphatidic acids was present in the monoenoic (36%) and dienoic (33%) molecular species, whereas 10, 8, 4, and 8% were associated with the saturates, trienes, tetraenes, and polyenes, respectively. Similar distributions of radioactivity were found among the corresponding classes of newly formed CDP-diglyceride. Only a slight enrichment of radioactivity in the tetraenoic CDP-diglyceride was found relative to the corresponding phosphatidates. Therefore, under the conditions of study, the microsomal CTP:phosphatidate cytidylyltransferase produces mainly monoenoic and dienoic species of CDP-diglyceride and shows little specificity towards different molecular species of phosphatidic acids. The present results suggest also that the arachidonoyl phosphatidate derived from the microsomal acylation of *sn*-glycerol-3-phosphate is not likely the major source of arachidonic acid in liver phosphatidylinositol.

[1]Presented in part at the Annual Meeting of the Canadian Federation of Biological Societies, Winnipeg, June 1975.

INTRODUCTION

It is now well recognized that intact phosphatidic acid enters directly into phosphatidylinositol during the de novo biosynthesis of this phospholipid, with CDP-diglyceride serving as the metabolic intermediate in this transformation (1-4). The formation of CDP-diglyceride and phosphatidylinositol involves the enzymes CTP:phosphatidate cytidylyltransferase (EC 2.7.7.41) and CDP-diglyceride:inositol phosphatidyltransferase (EC 2.7.8.11), respectively, which are located primarily in the endoplasmic reticulum fraction of cells (3,5-7). Because of this metabolic sequence, the fatty acid composition of the product, phosphatidylinositol, might be expected to bear a close resemblance to that of phosphatidic acid. However, liver phosphatidylinositol is greatly enriched in tetraenoic (arachidonoyl) molecular species but contains only low levels of monoenoic and dienoic molecules (8), whereas the exact converse is true for phosphatidic acid isolated from this same tissue (9,10). It might be logically speculated, therefore, that the microsomal CTP:phosphatidate cytidylyltransferase could generate a much higher proportion of tetraenoic relative to monoenoic and dienoic species of CDP-diglycerides by specifically reacting with tetraenoic phosphatidate, which would account for the high level of arachidonic acid in phosphatidylinositol. An alternative pathway for the incorporation of arachidonate into phosphatidylinositol involves the acylation of lysophosphatidylinositol (11-14). The purpose of the work described here was to determine the molecular species of CDP-diglyceride produced in liver microsomes by the cytidylyltransferase so as to assess the significance of this reaction for the entry of arachidonate into phosphatidylinositol.

MATERIALS AND METHODS

Materials

The *sn*-[^{14}C] glycerol-3-phosphate (131 mCi/mmol), [2-^{14}C] cytidine 5'-triphosphate (56 mCi/mmol), and [2-^{3}H] inositol (2800 mCi/mmol) were purchased from the New England Nuclear Corp. (Boston, MA). They were diluted with *rac*-glycerol-3-phosphate

(Sigma Chemical Co., St. Louis, MO) and CTP (Calbiochem, La Jolla, CA) to obtain the desired sp act. Adenosine triphosphate (ATP), Coenzyme A (CoA), and *myo*-inositol were obtained from Calbiochem. Standard and carrier lipids for thin layer chromatography (TLC) were purchased from Serdary Research Laboratories (London, Ontario) and Applied Science Laboratories (State College, PA) or isolated from rat liver lipid extracts. [^{14}C]CDP-diglyceride and [^{3}H]phosphatidylinositol were enzymatically prepared from [^{14}C]CTP and [^{3}H]-inositol and employed as standard lipids for TLC using methods similar to those described elsewhere (3,15). All chemicals and solvents were of analytical grade.

Animals and Microsomal Preparations

The animals were male Wistar rats weighing 150-170 g (Woodlyn Farms, Guelph, Ontario). They were housed in individual cages and fed Purina lab chow and water ad libitum prior to experimentation. Animals were sacrificed by cervical fracture and the livers were rapidly excised. Rat liver homogenates were prepared by homogenizing the livers in 4 vol of 0.25 M sucrose containing 20 mM Tris-HCl buffer (pH 7.4) and 0.6 mM ethylenediaminetetraacetic acid (EDTA) using a Potter-Elvehjem homogenizer with a Teflon pestle. Rat liver microsomes (unlabeled) were then isolated as previously described (15). Microsomal protein was measured by the method of Lowry et al. (16) using bovine serum albumin as the standard.

Radioactive microsomes containing [^{14}C]phosphatidic acid were prepared as follows. Unlabeled microsomal preparations (5 mg protein) were incubated with 1.2 mM ATP, 0.03 mM CoA, 2.0 mM $MgCl_2$, and 1.2 mM *sn*-[^{14}C]-glycerol-3-phosphate in 1.0 ml of medium containing 50 mM Tris-HCl buffer (pH 7.4). After a 30 min reaction period at 37 C, the incubation media containing the microsomes from at least 15 such incubations were pooled and mixed with 50 ml of the chilled homogenizing medium. The microsomal pellet was again isolated by ultracentrifugation and resuspended in fresh medium. In one such experiment using 3.0×10^6 cpm of labeled glycerol-3-phosphate per ml of incubation medium, the resulting microsomal preparation contained 8.2 nmol of newly synthesized [^{14}C]phosphatidate per mg protein with a sp act of 2,500 cpm per nmol. The sp act of the various microsomal preparations depended upon that of the glycerol-3-phosphate which was employed. The labeled microsomes used in the present investigations contained 90 ± 3% (mean ± SE) of the total lipid radioactivity as phosphatidic acid and the remaining 10% in other lipid fractions (diglyceride, monoglyceride, lysophosphatidic acid, etc.). Only 0.6 ± 0.1% of the lipid radioactivity was present in the CDP-diglyceride fraction.

Enzyme Assay Procedures

Unless stated otherwise in the text, the assay for studying the CTP:phosphatidate cytidylyltransferase using microsomes containing [^{14}C]phosphatidic acid was as follows. The basic system contained 50 mM Tris-HCl buffer (pH 7.4), 1.6 mM CTP, 0.54 mg of labeled microsomes, and 20 mM $MgCl_2$ (added 15 sec after all other components) in a total volume of 0.5 ml. Incubations were conducted at 37 C for various times as indicated in the text. The reactions were stopped by the addition of methanolic-HCl and the lipids extracted (3). Aliquots of the purified lipid extract were taken for scintillation counting in Aquasol (New England Nuclear Corp., Boston, MA) after removal of the solvent (15). The remaining portion was taken for TLC, as described below, after the addition of suitable amounts of carrier lipids in order to monitor the biosynthesis of [^{14}C]CDP-diglyceride.

The labeled CDP-diglyceride formed from [^{14}C]phosphatidic acid was isolated from total lipid extracts by TLC and used as a substrate for the microsomal CDP-diglyceride:inositol phosphatidyltransferase. The radioactive CDP-diglyceride was diluted with unlabeled carrier to a sp act of 10,000 cpm/μmol. The reaction mixture contained 50 mM Tris-HCl buffer (pH 8.2), 200 μM [^{14}C]CDP-diglyceride, 0.05 mg Tween-20, 5.0 mM $MnCl_2$, 500 μM inositol, and 0.4 mg of unlabeled microsomal protein in a final vol of 0.5 ml. Control incubations were conducted in the absence of added inositol. All incubations were conducted for 45 min at 37 C. Reactions were quickly terminated as described above, and 0.5 mg of pig liver phosphatidylinositol was added as carrier to total lipid extracts prior to monitoring the formation of radioactive phosphatidylinositol by TLC (see below).

Lipid Analyses

The biosynthesis of CDP-diglyceride from microsomes containing [^{14}C]phosphatidate was routinely measured by applying purified lipid extracts to Silica Gel H Plates (0.5 mm thickness) and developing in a mixture of chloroform:methanol:acetic acid:water (50:24:4:8, v/v/v/v) as described elsewhere (3). [^{14}C]CDP-diglyceride was eluted from the gel scrapings by duplicate elutions with chloroform:methanol:acetic acid:water (50:39:1:10,

v/v/v/v) according to a modification of Arvidson's procedure (17) after spraying the chromatograms with a dichlorofluorescein solution and visualizing under ultraviolet light. Control experiments revealed that at least 71% of the radioactivity associated with purified samples of [^{14}C] CDP-diglyceride spotted on thin layer plates could be recovered after development and elution from the gel scrapings. For identification purposes, radioactive lipids were also separated on Silica Gel H in chloroform:methanol:acetic acid:water (25:15:4:2, v/v/v/v) and chloroform:methanol:28% ammonia (65:25:4, v/v/v) and on Silica Gel G containing 0.5 M oxalate using light petroleum:acetone:formic acid (76:24:0.2, v/v/v) as described (18,19). Lipids were identified by co-chromatography with both unlabeled and radioactive standards. The biosynthesis of labeled phosphatidylinositol from [^{14}C] CDP-diglyceride was followed by TLC in chloroform:methanol:28% ammonia (65:25:4, v/v/v). The radioactivity associated with the various lipid fractions was determined as previously described (20).

The distribution of radioactivity among the various molecular species of [^{14}C] phosphatidic acid was determined after conversion of the radioactive lipid to 1,2-diglycerides using a phosphatidate phosphohydrolase preparation from rat liver (21, 22). The 1,2-diglycerides were isolated by TLC and converted to their diglyceride acetate derivatives as described (8) prior to argentation TLC. Alternatively, the phosphatidic acid and CDP-diglyceride were subjected to acetolysis (8,13) under conditions where $< 4\%$ of the total fatty acids in these lipids underwent intermolecular exchange as verified by control experiments with mixtures of standard phosphatidic acids and CDP-diglycerides. The 1,2-derivatives represented over 91% of the resultant diglyceride acetates that were formed. The purified 1,2-diglyceride acetates were then separated into their respective molecular species by argentation TLC in chloroform: methanol (100:2.5, v/v), after adding appropriate amounts of carrier 1,2-diglyceride acetates prepared from rat liver lecithin, and radioactivity was measured following elution from the gel scrapings (8). The radioactive diglyceride acetates applied to the silver nitrate plates could be recovered to the extent of 88.6 ± 6.7% (mean ± SE) following development and elution from the gel. The distributions of radioactivity among the various molecular species of liver phosphatidic acids using the two different methods for forming the 1,2-diglyceride acetate derivatives were essentially identical. The endogenously synthesized [^{14}C] phosphatidate which was isolated from the microsomal preparations was also subjected to hydrolysis with phospholipase A_2 (*Crotalus adamanteus*, Sigma Chemical Co., St. Louis, MO) as described (23), and the resulting [^{14}C] lysophosphatidic acid was isolated following TLC (9). Following acetolysis, the purified 1-monoglyceride diacetates were mixed with the corresponding acetates of standard monoglycerides (1-palmitoyl, 1-oleoyl, 1-linoleoyl, etc.) obtained from Nu-Chek-Prep (Elysian, MN). The lipid mixture was then subjected to argentation TLC and the radioactivity in the various molecular classes was determined as described above.

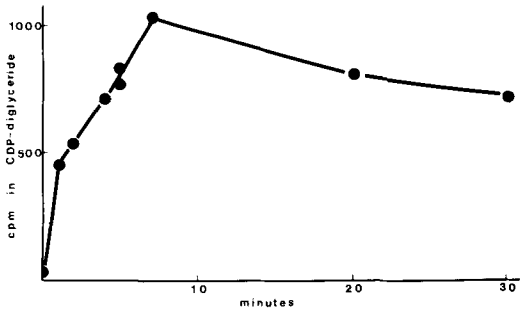

FIG. 1. Effect of incubation time on conversion of [^{14}C] phosphatidate into CDP-diglyceride. Incubation mixtures contained CTP (1.6 mM), $MgCl_2$ (20 mM), and 0.54 mg of labeled microsomes (containing 5,200 cpm of [^{14}C] phosphatidate) in a total volume of 0.5 ml of 50 mM Tris-HCl buffer (pH 7.4).

RESULTS

Figure 1 gives the time course for the formation of CDP-diglycerides using the labeled microsomal preparations containing [^{14}C] phosphatidic acid. The amount of newly synthesized CDP-diglyceride increased steadily with incubation times of increasing duration and reached a maximum at 7 min. At this latter point, 19.5% of the original [^{14}C] phosphatidate had been converted to [^{14}C] CDP-diglyceride. With extended incubation times, there was a decrease in the amount of [^{14}C] CDP-diglyceride present, which suggests some degradation of this lipid was occurring. For most experiments on CDP-diglyceride biosynthesis, incubation times of 1-5 min were routinely employed since CDP-diglyceride formation was rising steadily during this interval.

The effect of CTP concentration on the synthesis of CDP-diglyceride is given in Figure 2. The amount of CDP-diglyceride formed increased with increasing concentrations of the added nucleotide and approached a plateau between levels of 0.81-2.7 mM. For standard reaction conditions, the concentration of CTP was maintained at 1.6 mM.

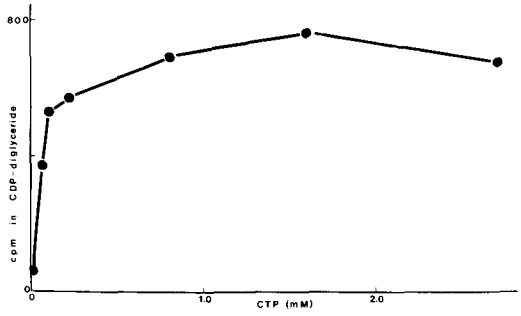

FIG. 2. Effect of varying the concentration of CTP on conversion of [^{14}C] phosphatidate into CDP-diglyceride. Incubation mixtures also contained $MgCl_2$ (20 mM) and 0.54 mg of labeled microsomes (containing 5,200 cpm of [^{14}C] phosphatidate) in a total volume of 0.5 ml of 50 mM Tris-HCl buffer (pH 7.4). Incubation time was 5 min.

TABLE I

Requirements for Conversion of Microsomal [^{14}C] Phosphatidate to CDP-diglyceride[a]

Omission	Radioactivity in CDP-diglyceride[c] (cpm)
None[b]	832
CTP	66
$MgCl_2$	84
CTP, $MgCl_2$	69

[a]Incubations were conducted for 5 min using same microsomal preparations as in Figures 1 and 2.

[b]Refers to complete system which contained CTP (1.6 mM), $MgCl_2$ (20 mM), and 0.54 mg of microsomal protein.

[c]Unincubated microsomes contained 87 cpm in the CDP-diglyceride fraction. The net appearance of 745 cpm (832-87) in the CDP-diglyceride fraction was accompanied by a loss of 711 cpm from the phosphatidic acid fraction relative to the unincubated (control) microsomal preparations.

Table I gives the cofactor requirements for the biosynthesis of [^{14}C] CDP-diglyceride from microsomal [^{14}C] phosphatidate. The requirement for CTP and $MgCl_2$ was essentially absolute since the radioactivity appearing in the CDP-diglyceride fraction when either was omitted from the assay medium was no greater than for unincubated microsomes. It has been previously demonstrated (3) that the microsomal CTP:phosphatidate cytidylyltransferase has a requirement for CTP and $MgCl_2$ when exogenous phosphatidic acid is used as the substrate. When the microsomal preparations containing [^{14}C] phosphatidate were incubated with [^{14}C] CTP of high sp act rather than unlabeled CTP, 97% of the radioactivity incorporated into lipid from the nucleotide was found to be present in the labeled CDP-diglyceride formed from [^{14}C] phosphatidate. These latter experiments employed unincubated samples and [^{14}C] microsomes incubated with unlabeled CTP as the appropriate controls. The radioactivity appearing in CDP-diglyceride from [^{14}C] CTP when microsomes containing [^{14}C] phosphatidate were employed was determined by substracting the radioactivity appearing in the CDP-diglyceride fraction when the same microsomal preparations were incubated with unlabeled CTP.

The labeled CDP-diglyceride which was synthesized from microsomal [^{14}C] phosphatidate was routinely isolated from thin layer chromatograms and tested as a substrate for the CDP-diglyceride:inositol phosphatidyltransferase present in rat liver microsomes. As seen in Table II, 51% of the radioactivity originally associated with CDP-diglyceride was converted to phosphatidylinositol when free inositol was added to the incubation medium, while the conversion was no more than 3% when inositol was excluded. Reisolation of the unreacted

TABLE II

Conversion of Radioactive CDP-diglyceride formed from Microsomal [^{14}C] Phosphatidate into Phosphatidylinositol[a]

Lipid fraction	cpm in fraction[b] minus inositol	plus inositol
CDP-diglyceride	948 (97.0)	369 (41.6)
Phosphatidylinositol	29 (3.0)	455 (51.3)
Other	0 (0.0)	63 (7.1)

[a]Radioactive CDP-diglyceride (200 μM) containing 1,000 cpm was incubated 45 min in the presence or absence of added inositol (500 μM) under standard assay conditions as described in Materials and Methods. Incubation medium also contained $MnCl_2$ (5.0 mM), 0.05 mg Tween-20, unlabeled microsomes (0.4 mg protein), and 50 mM Tris-HCl buffer (pH 8.2) in a final volume of 0.5 ml.

[b]The data in parentheses represent the percent distributions of the recovered radioactivity among the lipid fractions.

CDP-diglyceride (41.6% of total radioactivity) and reincubation under identical conditions gave a further inositol-dependent transformation of radioactive CDP-diglyceride into phosphatidylinositol.

The results in Table III give the percent distribution of radioactive glycerol-3-phosphate among molecular species of the microsomal substrate, [^{14}C]phosphatidic acid, and the reaction product of the CTP:phosphatidate cytidylyltransferase, [^{14}C]CDP-diglyceride. Most of the total radioactivity in the phosphatidate was present in the monenoic (36%) and dienoic (33%) species, while the saturates, trienes, tetraenes, and polyenes (>tetraenes) contained 10, 8, 4, and 8% of the radioactivity, respectively. These distributions were similar to those obtained among the corresponding molecular species of phosphatidate isolated from rat liver following the administration of labeled glycerol in vivo (10) or after its addition to liver slices in vitro (13,24). Characterization of the nature of the fatty acids in the 1-position of the [^{14}C]phosphatidic acid (Materials and Methods) revealed that 76, 15, 4, and 4% of the total acids were saturated, monoenoic, dienoic, and polyenoic (>dienoic), respectively. These results indicate an asymmetric distribution of saturated and unsaturated fatty acids among the 1- and 2-positions of the [^{14}C]phosphatidate, used as substrate for the CTP:phosphatidate cytidylyltransferase, in agreement with the distributions found for phosphatidate isolated from pooled rat livers (9,10). Thus, Possmayer et al. (9) found that saturated acids represented 84 and 11% of the total fatty acids at the 1- and 2-positions, respectively, of natural phosphatidate.

Table III also gives the distributions of radioactivity among the various molecular species of [^{14}C]CDP-diglycerides which were formed from the labeled phosphatidic acids. Under the conditions of the experiments reported in Table III, 7-15% of the microsomal [^{14}C]phosphatidic acid was converted into CDP-diglyceride at intervals from 1-5 min, respectively. In almost all cases, the percent distribution of total radioactivity among the various classes was not significantly different from the corresponding phosphatidic acids. The amount of radioactivity (5.5% of total) in the tetraenoic species of CDP-diglycerides was slightly greater (by 34%) than that in tetraenoic phosphatidate. The distributions of radioactivity among the various chemical classes of CDP-diglyceride were not significantly altered from those given in Table III when the concentration of CTP was lowered from 1.6 to 0.06 mM.

TABLE III

Distribution of Radioactivity among Molecular Species of Microsomal [^{14}C]Phosphatidic Acid and Newly Formed [^{14}C]CDP-diglyceride[a]

Chemical classes	Distribution of radioactivity (%)	
	Phosphatidic acid	CDP-diglyceride
Saturates	10.5 ± 4.1	10.2 ± 3.5
Monoenes	36.0 ± 4.2	33.3 ± 3.5
Dienes	33.4 ± 3.3	32.6 ± 3.4
Trienes	7.8 ± 1.1	9.6 ± 0.8
Tetraenes	4.1 ± 0.4	5.5 ± 1.0
Polyenes	8.2 ± 1.1	8.9 ± 1.8

[a]All values are given as means ± SE for [^{14}C]phosphatidate in the various microsomal preparations employed and for the [^{14}C]CDP-diglyceride which was formed during incubations of 1-5 min under standard assay conditions as given in Materials and Methods. The data in the table are derived from experiments with six different preparations of microsomes containing [^{14}C]phosphatidate. The results for [^{14}C]CDP-diglyceride are from six sets of analyses (each set consisting of pooled samples of labeled CDP-diglyceride from 5-15 separate incubations).

DISCUSSION

It has been well established in various mammalian tissues that phosphatidic acid can react with CTP to generate the liponucleotide CDP-diglyceride in the presence of the enzyme CTP:phosphatidate cytidylyltransferase (1-4). The CDP-diglyceride, in turn, can react with free inositol to produce phosphatidylinositol with the liberation of CMP due to the enzyme CDP-diglyceride:inositol phosphatidyltransferase (1,2,25). This sequence of metabolic reactions is known to provide for the de novo biosynthesis of phosphatidylinositol, and both enzymes are localized predominantly in the microsomal fraction of liver cells (3,5-7). Based on this sequence, one might well anticipate a close similarity in the fatty acid composition of phosphatidic acid and phosphatidylinositol isolated from rat liver, since intact phosphatidate itself enters directly into phosphatidylinositol during the synthesis of this latter phospholipid. Contrary to expectation, however, the fatty acid composition of phosphatidylinositol is markedly different from that of phosphatidic acid (8-11). Thus, arachidonate, oleate, and linoleate represent ca. 40, 3, and 3% of the total fatty acids in liver phosphatidylinositol (11), whereas these same acids contribute 7, 23, and 22%, respectively, to the total in phosphatidic acid (9,10). The striking preponderance of tetraenoic (arachidonoyl) molecular species in phosphatidylinositol relative to phosphatidic acid might be readily explained if the CTP:phosphatidate cytidylyltransferase were highly

specific for tetraenoic phosphatidic acids so as to produce largely tetraenoic CDP-diglycerides for the synthesis of phosphatidylinositol. The aim of the present investigations was to test this hypothesis and to characterize the complement of molecular species of CDP-diglycerides produced by the CTP:phosphatidate cytidylyltransferase in rat liver microsomes.

The experiments reported herein on the microsomal CTP:phosphatidate cytidylyltransferase were specifically designed to use endogenously labeled [^{14}C]phosphatidate as the substrate so to try to depict to some extent the conditions of this reaction in vivo. The problem of solubility with exogenously added lipid substrates in an aqueous medium often renders the results from such studies difficult to interpret (26). The microsomal [^{14}C]phosphatidate was prepared by incubating unlabeled microsomes in the presence of radioactive glycerol-3-phosphate and appropriate cofactors (ATP, CoA, $MgCl_2$) without the addition of exogenous fatty acids. It was deemed desirable to utilize this latter approach so that the molecular species composition of the [^{14}C]phosphatidate, which was newly formed by the acylation of glycerol-3-phosphate with endogenous fatty acids, would better represent that of the naturally occurring lipid. It has been widely reported that microsomal preparations from liver (27) and other tissues (28,29) do have a substantial pool of endogenous fatty acids for the acylation of glycerol-3-phosphate, since the addition of fatty acids does not greatly increase the acylation rate using generating systems for fatty acid activation (27,28) such as that employed herein. The distributions of radioactivity among the various molecular classes of phosphatidic acid formed from the microsomal acylation of [^{14}C]glycerol-3-phosphate were similar to in vivo findings with labeled glycerol (10). This is not too surprising in view of the fact that liver acyl-CoA:*sn*-glycero-3-phosphate acyltransferase and acyl-CoA:1-acyl-*sn*-glycero-3-phosphate acyltransferase show high activity in the microsomal fraction (27,30) although considerable activity resides in the mitochondrial fraction as well (31). The [^{14}C]phosphatidate contained largely saturated fatty acids at the 1-position of the glycerol moiety, even though 90% of the molecular species of phosphatidic acid were unsaturated (predominatly monoenes and dienes). These latter patterns were consistent with those reported for phosphatidate isolated from rat liver (9,10).

The possible preference of the CTP:phosphatidate cytidylyltransferase towards certain molecular species of microsomal phosphatidate was tested by comparing the distributions of radioactivity among the various chemical classes of newly synthesized CDP-diglyceride with those of phosphatidic acid. Any specificity would be expected to manifest itself by differences in the distributions within the two types of lipid. The percent distributions of radioactivity in all chemical classes of CDP-diglyceride did not differ significantly from the corresponding phosphatidic acids. Although the difference was not statistically significant when tested at the 5% level, there was a slight enrichment in the proportion of total radioactivity in the tetraenoic species of CDP-diglycerides as compared to the tetraenoic phosphatidic acids. These results indicate that little or no preference of the CTP:phosphatidate cytidylyltransferase is exhibited towards the various molecular species of microsomal phosphatidic acid which differ in their degree of unsaturation. An almost exclusive specificity of the transferase towards tetraenoic phosphatidic acids relative to monoenoic plus dienoic species might have been anticipated in order to attribute the high level of arachidonate in hepatic phosphatidylinositol, but not in phosphatidic acid (8-10), to this reaction alone. The microsomal cytidylyltransferase, as studied herein, produces mainly monoenoic plus dienoic species of CDP-diglyceride (66% of total) and only minor amounts of tetraenoic molecules (6% of total). These latter findings are in fairly good agreement with both in vivo (11) and in vitro (13) findings using labeled glycerol, which suggested that the phosphatidic acid pathway involving CDP-diglyceride produces a complement of phosphatidylinositols with a relatively high proportion of monoenoic plus dienoic species.

The question arising from this work that needs to be answered concerns the metabolic pathway(s) responsible for the high levels of arachidonic acid in liver phosphatidylinositol. There is considerable evidence from both in vivo (11,12,32) and in vitro (13,14) experiments to indicate that the acylation of lysophosphatidylinositol with arachidonate is an important route for the entry of this acid into phosphatidylinositol. However, the possibility that arachidonoyl species of liver CDP-diglyceride can be synthesized from other sources, such as from mitochondrial biosynthesis (33).or by the specific utilization of arachidonoyl phosphatidate derived from diglyceride kinase reactions in microsomes or other cellular fractions (34,35), cannot be excluded at the present time. It is also important to note that Thompson and MacDonald (36) have recently reported upon the isolation of CDP-diglyceride from beef liver and found its fatty acid composition to resemble phosphatidylinositol from

this same tissue. Besides some of the potential sources mentioned above, they also proposed (36) that CDP-diglyceride could be derived by the back reaction of the CDP-diglyceride:inositol phosphatidyltransferase (37,38).

To summarize the findings reported herein, the microsomal CTP: phosphatidate cytidylyltransferase gives rise to mainly monoenoic and dienoic species of CDP-diglycerides when studied under the present conditions. In conclusion, the present results suggest that arachidonoyl phosphatidic acid derived from the microsomal acylation of *sn*-glycerol-3-phosphate does not provide the major source of arachidonate in liver phosphatidylinositol.

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Unsaponifiable Matter of Green and Blue-Green Algal Lipids as a Factor of Biochemical Differentiation of Their Biomasses: I. Total Unsaponifiable and Hydrocarbon Fraction

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ABSTRACT

As part of a program to study the chemical composition of algal biomasses, the composition of the unsaponifiable matter of the lipids of ten algal species (five *Myxophyceae* and five *Chlorophyceae*) was investigated. The total unsaponifiable content, its general composition, and the components of the hydrocarbon fraction are discussed in the present paper. The unsaponifiable content of green algae is constantly higher than that of the blue-green ones, with the exception of *Chlorella*. In both algal classes, the major components are hydrocarbons and sterols. Blue-green algae are richer in hydrocarbons, whereas the green ones contain higher amounts of sterols. In most of the species examined, at least 48 components are present in the hydrocarbon fraction. Each algal species shows a characteristic gas liquid chromatography pattern, but n-C_{17} is always one of the most abundant components. Generally, the prokaryotic blue-green algae show a simpler hydrocarbon composition than the eucaryotic green algae, which contain higher amounts of high mol wt components. Unsaturated hydrocarbons are generally present in very limited quantities, with the exception of *Spirulina* sp. and *Chlorella* sp., which contain a C_{17} alkene. Green algae also contain appreciable amounts of a C_{27} monoene and of squalene.

INTRODUCTION

As a logical development of the research on the chemical composition of algal biomasses performed at the Centro di Studio dei Microrganismi Autotrofi of Florence and on the fundamental constituents of algae (proteins, fatty acids, pigments, nucleic acids, etc.) (1-13), we have studied the unsaponifiable fraction of algal lipids. Only scattered and contradictory data, very often obtained with inadequate analytical methods, are available in the literature regarding this lipid fraction. Systematic studies on a few constituents of algal lipid unsaponifiables, however, have been reported (14,15). On the other hand, the study of the unsaponifiable fraction is of fundamental interest because of the various implications that its components have with chemotaxonomic, evolutionary, and cytologic problems. Furthermore, algal biomasses are more and more considered a source of animal feed and even human food, and detailed knowledge of their constituents is therefore of primary importance.

In the present communication, we wish to report an investigation performed on some representative species of both green and blue-green microalgae. Some of the species studied, such as those of the genus *Spirulina*, previously had been studied extensively from different points of view (1-3,9,12,13,16,17). The total unsaponifiable content of algal lipids, its general composition, and the component of the hydrocarbon fraction are discussed in detail. The accompanying paper describes the composition of the terpenic alcohol and sterol fractions (18).

MATERIALS AND METHODS

Organisms

The organisms studied were *Spirulina platensis* strain Mao I and Mao II isolated from Lake Mombolo (Republic of Chad), *Spirulina* sp. from Lake Texcoco (Mexico), *Calothrix* sp. strain 59 P1 from hot water springs of Vinadio (Italy), *Nostoc commune* strain 40 from rice fields of Poggio a Caiano (Italy), *Scenedesmus quadricauda* 156V from the river Quebrada Marco Parra (Venezuela), *Chlorella* sp. strain 101 S from an alpine soil (Switzerland), *Uronema gigas* and *Uronema terrestre* from the culture collection of Göttingen University (W. Germany), and *Selenastrum gracile* strain 30A from an Australian lake.

Culture Conditions

All the organisms reported in this paper were grown outdoors in circular vessels equipped with a stirring device and bubbling tubes for air

TABLE I

Total Lipids and Unsaponifiable Matter Content of the Algal Biomasses Examined

Microalgae	Total lipids	Unsaponifiables	
		On lipids	On biomass
Myxophyceae			
Spirulina platensis Mao I	12.05	9.68	1.17
Spirulina platensis Mao II	12.20	10.67	1.30
Spirulina sp. (Mexico)	11.00	11.28	1.24
Calothrix sp.	8.30	14.37	1.19
Nostoc commune	4.80	10.19	0.49
Chlorophyceae			
Scenedesmus quadricauda	17.80	14.50	2.59
Chlorella sp.	12.70	10.56	1.34
Uronema gigas	20.70	16.57	3.44
Uronema terrestre	18.90	23.76	4.49
Selenastrum gracile	25.50	17.00	4.34

and carbon dioxide (95:5) (19). (The outdoor cultures were unialgal but, with the exception of the three *Spirulina* strains, they were started with a heavy inoculum from a pure culture and, moreover, were performed to minimize contamination with other microorganisms. In fact, at the harvest of the biomasses, the level of contamination, mostly bacterial, was very low [20]. Because it has been estimated that the amount of contaminants was in the range of 0.01% of the algal biomass, the interference of bacterial metabolites with the analytical results must be considered quite negligible.) The algal cells were harvested when the culture was in the linear phase of growth.

The three *Spirulina* strains were grown on an inorganic medium developed in Florence and the *Nostoc* and *Calothrix* species on a modified Chu's 10 medium (21). The green algae were grown on a modified Kolkwitz medium (22), with the exception of *Selenastrum*, for which the Kessler medium (23) was used. All cells were harvested by centrifugation, washed twice with physiological solution, then freeze dried.

Lipid Extraction and Preparation of Unsaponifiables

The freeze dried algal mass (30-40 g) was ground in a mortar with sand and extracted for 24 hr with methanol:chloroform (1:2, v/v) in the Paquot apparatus (24). The solvent was totally removed in a rotary evaporator, and the lipid residue was determined after drying to constant wt in a desiccator. The total lipids were then saponified with methanolic KOH 1N by refluxing for 3 hr. After addition of 2 vols of distilled water, the unsaponifiables were extracted with ethyl ether; the ether solution was passed on a column of alumina (25), taken to dryness, and the residue weighed after drying to constant wt.

Thin Layer Chromatography (TLC) Fractionation of the Unsaponifiables

The unsaponifiables were fractionated by TLC using 20 x 20 cm plates spread with a 0.3 mm layer of Silica Gel G (25). On the preparative plates, 30 mg of sample were applied in a continuous band. Development was obtained with hexane:ethyl ether (1.5:1 v/v).

Spots were visualized with a 0.2% solution of 2,7-dichlorofluorescein (sodium salt) in 95% ethanol and observed under UV light. For densitometric determination, the plates were sprayed with a saturated solution of $K_2Cr_2O_7$ in 80% H_2SO_4 and heated in an oven 25 min at 180 C. A Joyce Chromoscan densitometer was used. The hydrocarbon bands visualized under UV light were scraped from the preparative plates and recovered by extracting with hexane.

Examination of the Hydrocarbon Fraction

The hydrocarbon fraction was rechromatographed on plates spread with a 0.3 mm layer of Silica Gel G containing 30% $AgNO_3$ (26). The developing solvent was a 8:2 (v/v) mixture of benzene:hexane. After development, the plates were sprayed with the alcoholic solution of 2,7-dichlorofluorescein and the bands, visualized under UV light, were recovered from the absorbent with ethyl ether. The total hydrocarbon fraction and the single bands recovered from argentation TLC were analyzed by gas liquid chromatography (GLC). A C. Erba model C gas chromatograph equipped with temperature programmer and flame ionization detector was used. The column was a 2mm x 4mm inside diameter (ID) glass packed with 3% JXR on 80-100 mesh Gas Chrom Z. Temperature was programmed from 130 to 280 C at 5 C/min. The injector temperature was 270 C; detector temperature, 280 C; carrier gas, N_2, 30-35 ml/min.

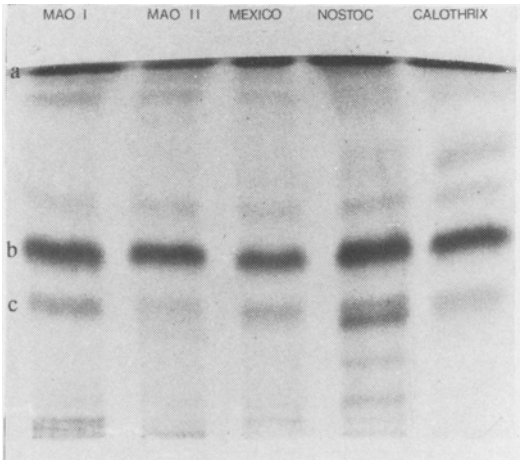

FIG. 1. Thin layer chromatography separation of the unsaponifiable matter of blue-green algae (visualized by charring with H_2SO_4 - $K_2Cr_2O_7$ at 180 C). a = hydrocarbons, b = terpenic and aliphatic alcohols, c = sterols.

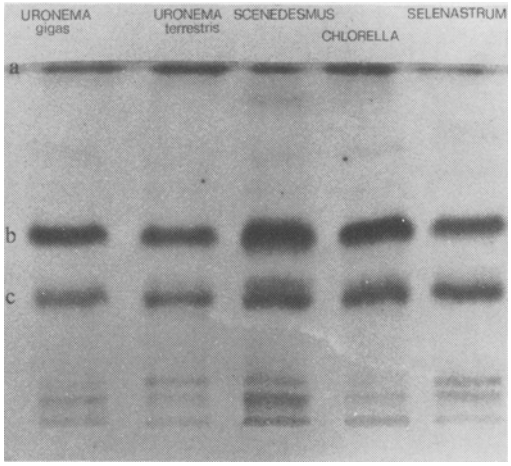

FIG. 2. Thin layer chromatography separation of the unsaponifiable matter of green algae (visualized by charring with H_2SO_4-$K_2Cr_2O_7$ at 180 C). a = hydrocarbons, b = terpenic and aliphatic alcohols, c = sterols.

Mass spectrometric (MS) analyses were done at the Centro di Gascromatografia-Spettrometria di Massa of the University of Bologna. An LKB 9000 combination gas chromatograph-mass spectrometer equipped with a 2m x 4mm ID glass column packed with 1% SE 30 on acid washed and silanized 100-200 mesh Gas Chrom P was used. The gas chromatographic (GC) conditions were the same as those used for the conventional GLC; the He separator was maintained at 30 C and the ion source at 290 C. Ionizing potential was 70 eV, and the ionizing current 60 μA. Spectra were recorded on the apex of the GC peak.

RESULTS AND DISCUSSION

Both the total lipid and unsaponifiable content of the algal biomasses are reported in Table I. As can be noted, the total lipid content of green algae is constantly higher than that of the blue-green ones, the mean being 19.1 for the former and 9.7 for the latter. Among the green algae, only the *Chlorella* strain shows a lipid content of the magnitude of the *Myxophyceae*. Also, the unsaponifiable percentage, calculated both on total lipids and on the biomass, is always higher in the green algae than in the blue-green ones, again with the exception of *Chlorella*.

The fractionation of the unsaponifiables by TLC on Silica Gel G shows further differences between the two algal classes. Figure 1 illustrates the thin layer separation of the five blue-green algae as visualized by spraying with sulfuric acid saturated with potassium bicromate.

The same components appear to be present in the unsaponifiables of the five green algae (Fig. 2). However, the hydrocarbon spot is generally fainter, while the sterol spot is more intense. The presence of other minor components can be detected, but the most evident difference between green and blue-green algae appears to be the different relative content of hydrocarbon and sterol. Although the densitometric determination cannot be considered an exact quantitative measurement, it allows a rapid evaluation of these differences when used for comparison only. The relative amounts of hydrocarbons, terpenic alcohols, and sterols present in blue-green and green algae, as determined by photodensitometry of the thin layer plate are reported in Table II.

The three major bands – hydrocarbons, terpenic alcohols, and sterols – of the unsaponifiable matter of each algal species were recovered and studied separately by means of preparative plates. The hydrocarbon band, which has a yellow orange color, also contains carotenoid hydrocarbons which were determined separately.

The total carotenoid and carotene contents of the algae studied are reported in Table III. Both the total carotenoid and carotene contents of the two algal classes appear to vary within a wide range but do not seem to afford a differentiation criterion. It should be noted, however, that the carotenoid content, as determined in this work, does not necessarily correspond with the actual carotenoid content of the algal species examined, but rather to the

TABLE II

Relative Composition of Algae Unsaponifiables from Densitometric Measurements

Microalgae	Hydrocarbons	Alcohols	Sterols
Myxophyceae			
Spirulina platensis Mao I	37.3	39.3	8.2
Spirulina platensis Mao II	32.7	52.2	5.3
Spirulina sp. (Mexico)	40.6	40.0	8.0
Calothrix sp.	48.0	39.6	7.8
Nostoc commune	41.8	40.4	14.5
Mean value	40.1	42.3	8.8
Chlorophyceae			
Scenedesmus quadricauda	16.8	43.3	23.0
Chlorella sp.	27.3	44.4	21.8
Uronema gigas	31.7	43.6	17.3
Uronema terrestre	36.0	37.1	21.7
Selenastrum gracile	23.1	35.5	28.1
Mean value	27.0	40.8	22.4

TABLE III

Carotenoid Content of Algae Unsaponifiables

Microalgae	Carotenoid (%)		β-carotene (%)	
	On unsaponifiables	On biomass	On unsaponifiables	On biomass
Myxophyceae				
Spirulina platensis Mao I	8.79	0.103	4.46	0.052
Spirulina platensis Mao II	5.00	0.065	3.21	0.042
Spirulina sp. (Mexico)	5.05	0.062	2.92	0.036
Calothrix sp.	6.85	0.081	1.96	0.023
Nostoc commune	2.24	0.011	0.93	0.004
Chlorophyceae				
Scenedesmus quadricauda	5.99	0.155	0.51	0.013
Chlorella sp.	2.91	0.039	1.65	0.022
Uronema gigas	2.78	0.096	0.84	0.029
Uronema terrestre	1.08	0.048	0.11	0.005
Selenastrum gracile	2.37	0.103	0.59	0.026

fraction of these compounds which remains in the unsaponifiables obtained by the method described above.

The hydrocarbon fraction of the unsaponifiables of the ten algal species was further studied by GC on 2m packed JXR columns. Some typical GC traces are reproduced in Figure 3.

In most of the species examined, at least 48 components–some of which are not completely resolved in the experimental conditions adopted–are present, although their relative amounts vary within a wide range, and some of them may be absent in some species. Each algal species shows a characteristic GC pattern, and different strains of the same species, as in *Spirulina*, may show remarkable quantitative differences. Identification of most of the components was accomplished both by comparison of their retention times with those of suitable model compounds and by the combination GC-MS. The presence of unsaturated hydrocarbons was further checked by separating the total hydrocarbon fraction by TLC on Silica Gel G-$AgNO_3$ plates. However, the small amount of some of the components did not allow a complete and unequivocal identification of all the compounds present.

Table IV summarizes the data for the hydrocarbon composition of the ten species of algae analyzed.

The two strains of *Spirulina platensis*, Mao I and Mao II, and the strain of *Spirulina* sp. (Mexico) contain the C_{17} saturated hydrocarbon as the major component, but only *Spirulina* sp. shows the presence of the C_{17} monoene in substantial amounts. All three strains also contain appreciable amounts of n-C_{15} and n-C_{16} alkanes, although they are not as abundant as reported by Gelpi and coworkers (14). Minor amounts of the n-alkanes C_{18} to C_{29} and traces of the branched alkanes from C_{17} to C_{28} are present. These compounds may tentatively be assigned to the series of 2-,

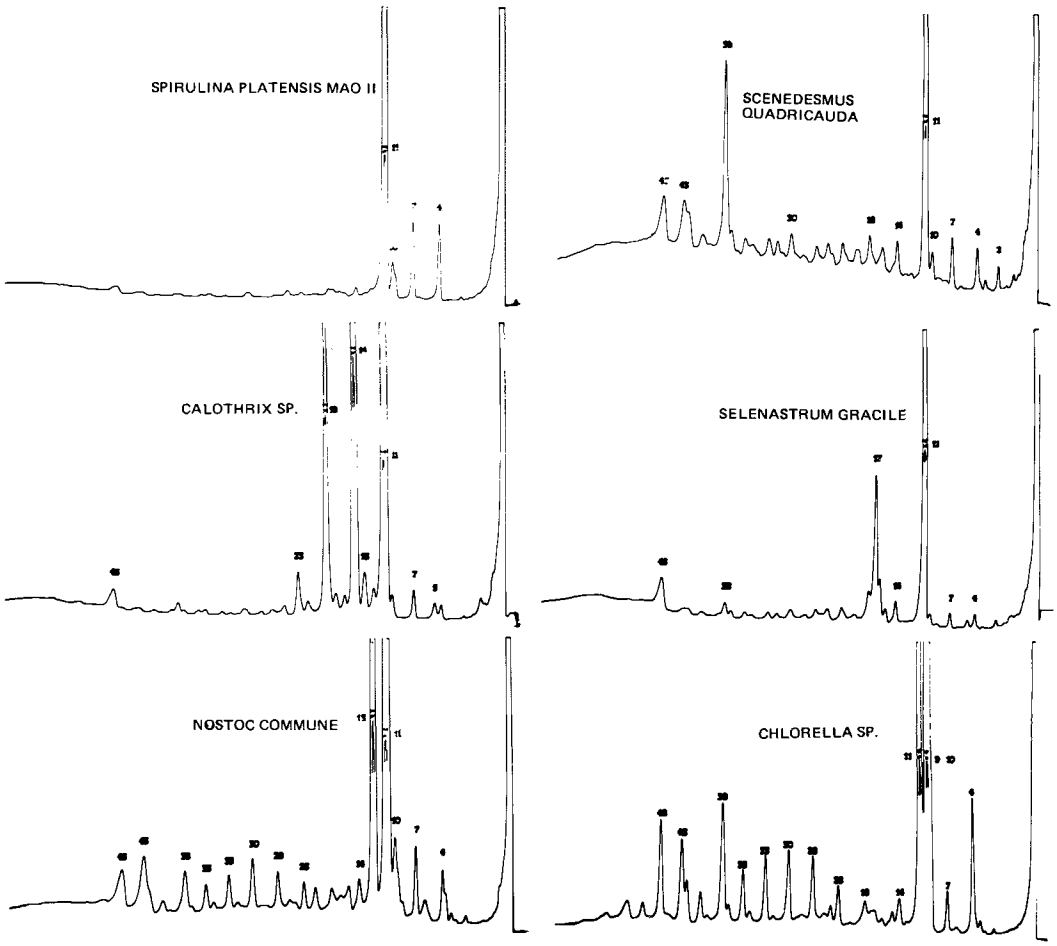

FIG. 3. Gas chromatograms of the hydrocarbon fraction of some algal species. Peak numbers: 2 = n-C 14, 4 = n-C-15, 5 (unidentified), 7 = n-C-16, 9 = Δ-C-17, 10 = iso-C-17, 11 = n-C-17, 13 = iso-C-18, 14 = n-C-18, 17 = iso-C-19, 18 = n-C-19, 23 = n-C-20, 25 (unidentified), 28 = n-C-21, 30 = n-C-22, 33 = n-C-23, 35 = n-C-24, 38 = n-C-25, 43 (unidentified), 45 = squalene.

7-, and 8-monomethyl alkanes, in agreement with Gelpi and coworkers (14). However, they are present in too small amounts to allow unequivocal identification of their structures by mass spectrometry.

The presence of unsaturated hydrocarbons is negligible, as can be shown after separating the unsaturated fraction by argentation TLC. The upper part of Figure 4 illustrates the gas chromatogram of the total hydrocarbon of the strain Mao I and the lower part, the gas chromatogram of the saturated hydrocarbon obtained after separation of the unsaturated fraction by TLC on Silica Gel G-$AgNO_3$. Only peaks 9 and 15 have disappeared, showing that they are due to C_{17} and C_{19} alkenes.

Three major components are present in *Calothrix* sp., the C_{17}, C_{18}, and C_{19}, n-alkanes, together with smaller amounts of C_{15}, C_{16}, and C_{20} n-alkanes. Traces of C_{17} and C_{18} branched alkanes are also present.

The last blue-green alga examined, *Nostoc commune*, besides the typical n-C_{17} peak, shows a prominent peak which, in agreement with the work of Gelpi and coworkers (14), can be identified as a mixture of 7-methyl and 8-methyl-heptadecanes. We were unable, on the other hand, to detect the presence of compound X, a polycyclic triterpenoid hydrocarbon reported by the previous authors.

Scenedesmus quadricauda has an n-C_{17}, along with substantial amounts of n-C_{25} alkane. The presence of a C_{27} monoene, previously reported by Gelpi and coworkers (14), could not be confirmed in this work.

In Figure 5, it is possible to observe the

TABLE IV

Quantitative Composition of Algae Hydrocarbons
(Percent of Total Hydrocarbon from Gas Chromatography Analysis)

Reference number	Hydrocarbon	*Spirulina platensis* Mao I	*Spirulina platensis* Mao II	*Spirulina* sp. (Mexico)	*Calothrix* sp.	*Nostoc commune*	*Scenedesmus quadricauda*	*Chlorella* sp.	*Uronema gigas*	*Uronema terrestre*	*Selenastrum gracile*
2	n-C-14	0.2	0.2		tr[a]	0.1	1.0	0.2	0.4	0.5	0.4
4	n-C-15	2.7	3.7	2.9	0.3	1.5	2.3	2.0	1.2	0.9	0.9
7	n-C-16	3.7	3.8	2.8	0.7	1.6	2.5	1.0	1.2	1.4	1.1
9	Δ-C-17	3.4	3.1	5.2	tr	3.2	tr	35.8	tr	0.3	tr
10	iso-C-17			tr	0.5		1.9		26.7	2.5	0.8
11	n-C-17	66.9	84.0	71.7	47.4	50.4	41.4	25.4	13.3	24.6	61.1
13	iso-C-18	tr	tr	tr	1.4	24.4	tr	0.9	0.5	tr	tr
14	n-C-18	0.6	0.5	0.7	30.9	1.0	2.0	tr	1.2	1.7	1.8
15	(unidentified)	0.8	tr	tr	0.7	1.0	2.2	3.8	1.1	1.1	1.3
16	(unidentified)		tr	tr	tr	tr	tr		5.8	0.7	
17	iso-C-19	0.4	1.3	2.9	0.9	tr	tr		3.6	10.1	15.1
18	n-C-19	0.9			10.4	0.6	2.5				
20	Δ-C-20	0.3	tr	-	tr	tr	2.3	tr	0.4	1.4	0.9
29	iso-C-22	tr	tr	tr	0.1	tr	0.7	tr	1.1	1.7	0.5
30	n-C-22	3.2	0.5	1.7	0.2	1.4	2.0	2.8	0.4	1.4	1.1
32	Δ-C-23	0.3	tr	tr	0.1	0.4	1.1	0.3	0.2	0.3	0.5
37	iso-C-25	0.4	-	tr	0.1	0.2	1.7	0.5	0.7	0.5	0.6
38	n-C-25	1.8	0.3	1.2	0.3	1.4	11.4	5.0	6.1	3.2	1.4
41	n-C-26	1.3	0.2	0.6	0.1	0.5	1.4	1.2	1.0	0.8	0.5
42	Δ-C-27	tr	0.4	1.0	0.1	3.5	7.2	0.4	9.7	10.9	1.0
43	(unidentified)	0.8						3.3	17.2		
45	squalene	0.8	0.9	1.3	0.8	2.2	5.7	3.9	3.7	24.7	4.7

[a]tr = Trace, <0.1.

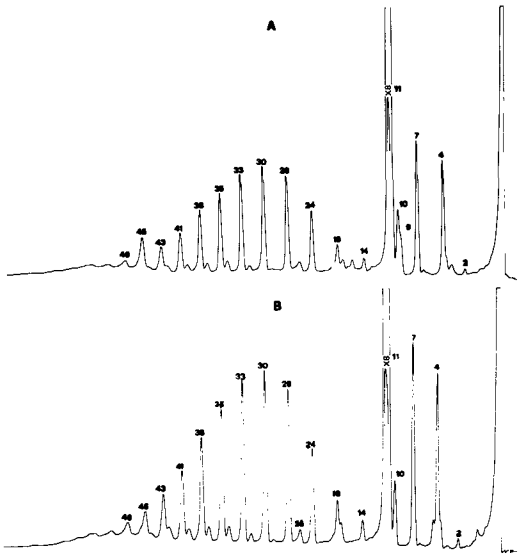

FIG. 4. Hydrocarbon gas chromatograms of *Spirulina platensis* Mao I. Trace A: total hydrocarbons; trace B: saturated hydrocarbons after separation by thin layer chromatography on Silica Gel-$AgNO_3$.

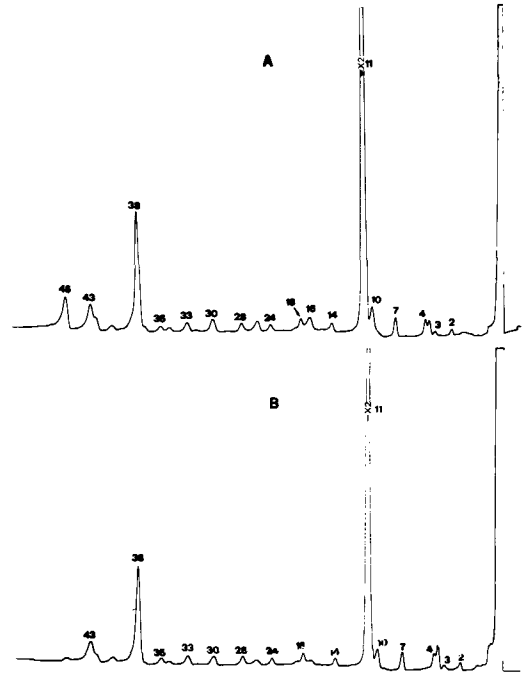

FIG. 5. Hydrocarbon gas chromatograms of *Scenedesmus quadricauda*. Trace A: total hydrocarbons; trace B: saturated hydrocarbons after separation by thin layer chromatography on Silica Gel-$AgNO_3$.

disappearance of peak 45 after argentation TLC. However, this component has a retention time corresponding to squalene, its TLC behavior is in agreement with the presence of at least four double bonds ($R_I = 0$), and the mass spectrum shows a molecular ion at m/e 410, as expected for a $C_{30}H_{50}$ hydrocarbon. The identification of peak 45 with squalene is therefore supported by the experimental evidence.

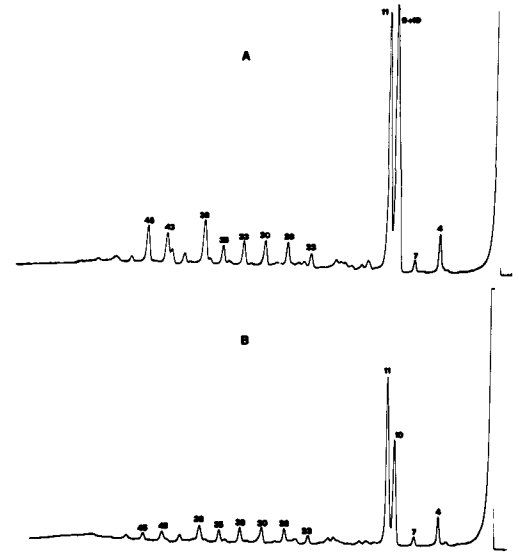

FIG 6. Hydrocarbon gas chromatograms of *Chlorella* sp. Trace A: total hydrocarbons; trace B: saturated hydrocarbons after separation by thin layer chromatography on Silica Gel-$AgNO_3$.

The hydrocarbon pattern of *Chlorella* sp. is rather simple, showing two major components, n-heptadecene and n-heptadecane, as previously reported for *Chlorella pyrenoidosa* (14). However, after separation of the unsaturated hydrocarbons on Silica Gel G-$AgNO_3$, it is possible to note (Fig. 6) that the unsaturated C_{17} is superimposed to a saturated hydrocarbon, very likely a branched heptadecane. Minor amounts of higher saturated hydrocarbons are also present.

Selenastrum gracile, besides the typical n-C_{17}, shows substantial amounts of n-C_{19}. Somewhat different is the hydrocarbon composition of both *Uronema gigas* and *U. terrestre*. The n-C_{17} alkane is not as abundant as in the other species studied, whereas substantial amounts of higher mol wt compounds (peaks 42, 43, and 45) are present. After argentation chromatography, peaks 42 and 45 are absent in the saturated fraction. Peak 42 is found in the monounsaturated band, very likely due to a C_{27} monoene, whereas peak 45 does not move from the start on the TLC plate and has been identified as squalene, as in *Scenedesmus quadricauda*.

In conclusion, the composition of the hydrocarbon fraction appears to be markedly dif-

ferent in the ten species examined. These differences, however, are not unexpected on the basis of earlier work performed on this fraction of algal lipids (14). All the species examined have n-heptadecane as a major component (peak 11 in the GC traces), and only *Scenedesmus* and *Uronema* (not shown in Fig. 3) contain substantial amounts of high mol wt hydrocarbons; i.e., *Scenedesmus* shows the presence of n-pentacosane, corresponding to peak 38, and *Uronema* contain a branched C_{19} saturated hydrocarbon. Unsaturated hydrocarbons are generally present in very limited quantities, with the exception of *Spirulina* sp. and *Chlorella* sp., which contain a C_{17} alkene. Furthermore, the green algae examined show an appreciable content of C_{27} monoene and of squalene. This triterpenoid hydrocarbon is particularly abundant in the strain of *Uronema terrestre* examined.

Work is in progress to determine the double bond position in the unsaturated hydrocarbons. The elongation-decarboxylation pathway, which at present seems to explain most of the experimental data for the biosynthesis of hydrocarbons in higher plants (27), might also prove correct for both saturated and unsaturated algae hydrocarbons. In the latter, the position of double bonds should afford further experimental evidence.

Notwithstanding some inconsistencies in distribution found in hydrocarbons (28-32), it seems justified to conclude that the prokaryotic blue-green algae show a simpler hydrocarbon pattern than the eukaryotic green algae. As can be noted from the data reported in Table V, all the green algae examined contain higher amounts of high mol wt and of higher unsaturated hydrocarbons (C_{20}-C_{27}) than the blue-green ones. However, caution against chemotaxonomic conclusion, drawn solely on the basis of hydrocarbon distribution, is suggested by the considerable effects of cultural conditions on this fraction, as shown by Patterson (33).

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Unsaponifiable Matter of Green and Blue-Green Algal Lipids as a Factor of Biochemical Differentiation of Their Biomasses: II. Terpenic Alcohol and Sterol Fractions

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ABSTRACT

The composition of the terpenic alcohol and sterol fractions of the unsaponifiables of ten algal species, five *Myxophyceae* and five *Chlorophyceae*, is discussed. The major component of the terpenic fraction is phytol, a diterpenic alcohol. Minor amounts of straight chain and triterpenic alcohols are also present. Practically all the species examined contain ten components in the sterol fraction: cholesterol, brassicasterol, Δ^5-ergostenol, poriferasterol, Δ^7-ergostenol, clionasterol, chondrillasterol, Δ^5-avenasterol, Δ^7-chondrillastenol, and an unidentified component. Identification of the sterols was made by gas chromatography-mass spectrometry, and a 24 S configuration was assumed. The prokaryotic blue-green algae are characterized by a higher content in cholesterol (3.5-14%) than the eucaryotic green algae (0-2.5). Also, brassicasterol, poriferasterol, clionasterol, and Δ^5-avenasterol are more abundant in blue-green algae. Δ^7-Ergostenol, chondrillasterol, and Δ^7-chondrillastenol predominate, on the contrary, in green algae.

INTRODUCTION

The sterol content of algae has been reported in recent reviews (1-3). Information on the distribution of these components in the different algal species is, however, still lacking, and even more scattered are the reports on the presence of terpenic alcohols, 4,4-dimethylsterols, and aliphatic alcohols.

Blue-green algae were believed not to contain sterols (4), and only recently cholesterol was shown to be present in *Anacystis nidulans, Fremyella diplosiphon* (5) and *Phormidium luridum* (6). *P. luridum* (6), besides cholesterol, also contained a mixture of unsaturated 24-ethyl-cholesterols possessing Δ^7-, $\Delta^{5,7}$-, Δ^5-bonds, together with their Δ^{22}-derivatives. The major component was 24-ethyl-cholesterol.

Cholesterol was reported to be the major sterol (80%) of *Spirulina platensis* Geitler by French workers (7). Brassicasterol, cholesterol, and 22-dihydrobrassicasterol were isolated from *Anabaena cylindrica* (8). In *Cyanidium caldarium* (9), cholesterol, ergosterol, 5,6-dihydroergosterol, campesterol, β-sitosterol, and 7-dehydrositosterol were identified, the predominant compounds being ergosterol, β-sitosterol, and campesterol.

Considerable data are available on the sterols of green algae, especially of *Chlorella*, although much of the early work might need reexamination. These algae contain a variable and complex mixture of C_{27}-, C_{28}-, and C_{29}- sterols (1,10-12).

When the configuration of the asymmetric carbon at C_{24} was rigorously established, green algae sterols were shown to have a 24 S configuration (10,11), in contrast with the sterols of higher plants, which are 24 R compounds.

In the present paper, the composition of both the terpenic alcohol and the sterol fraction is described. The composition of the hydrocarbon fraction was discussed in the preceding companion paper (13).

MATERIALS AND METHODS

Organisms and Culturing Conditions

The algal species investigated and the culturing conditions were described in the preceding paper (13).

Lipid Extraction and Separation of the Terpenic and Sterol Fractions

Lipid extraction, unsaponifiable preparation, and fractionation were previously reported (13). The terpenic and sterol fractions, obtained by thin layer chromatography (TLC) fractionation and visualized under UV light, were scraped from the preparative plates and recovered by extraction with ethyl ether.

Examination of Terpenic Fraction

The material recovered from the plate was acetylated twice with acetyl chloride and

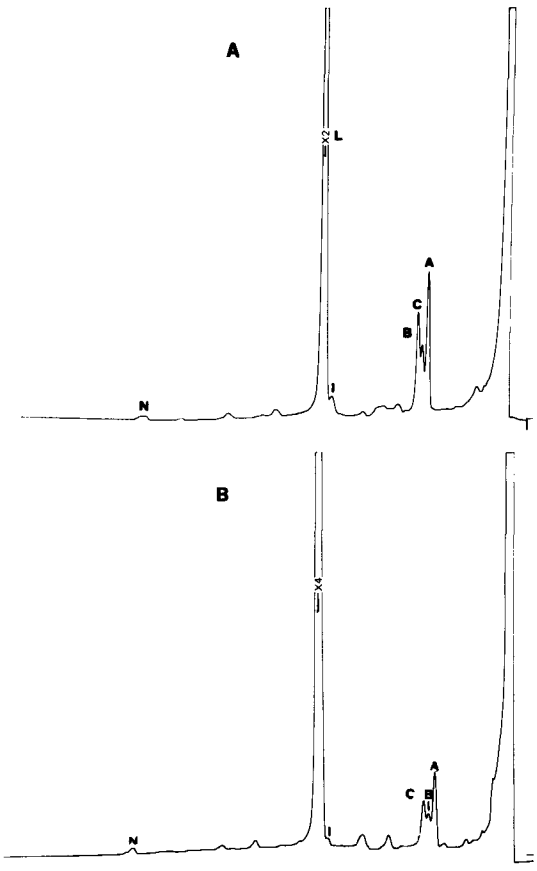

FIG. 1. Gas chromatograms of terpenic alcohol acetyl derivatives of *Spirulina platensis* Mao I. Trace A: total alcohols; trace B: saturated alcohols after separation by thin layer chromatography on Silica Gel-$AgNO_3$.

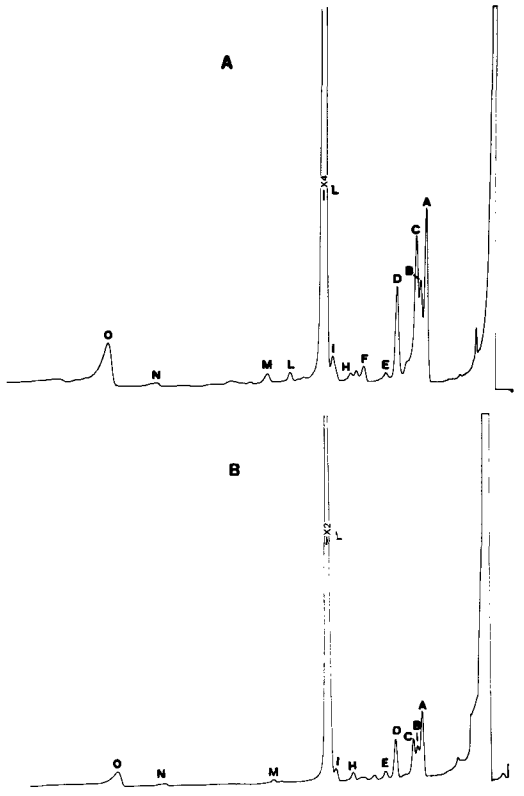

FIG. 2. Gas chromatograms of terpenic alcohol acetyl derivatives of *Scenedesmus quadricauda*. Trace A: total alcohols; trace B: saturated alcohols after separation by thin layer chromatography on Silica Gel-$AgNO_3$.

examined by gas liquid chromatography (GLC). A C. Erba model C gas chromatograph equipped with temperature programmer and flame ionization detector was used. A glass column (2m x 3mm inside diameter [ID]) packed with 3% JXR on 100-120 mesh acid washed and silanized Gas Chrom P was used. Temperature was programmed from 180 to 280 C at 5 C/min. Detector and injector temperatures were maintained at 280 C. Carrier gas was N_2, 35 ml/min.

TLC purification of the acetate mixtures was performed using 20 x 20 cm plates spread with a 0.3 mm layer of Silica Gel G containing 30% $AgNO_3$ (14). The developing solvent was an 8:2 (v/v) mixture of benzene:hexane.

Mass spectrometric analyses were done at Centro di Gascromatografia-Spettrometria di Massa of the University of Bologna. An LKB 9000 combination gas chromatograph-mass spectrometer equipped with a 2m x 4mm ID glass column packed with 1% SE 30 on acid washed and silanized 100-120 mesh Gas Chrom P was used. The gas chromatographic conditions were the same as those used for the conventional GLC. Helium was used as carrier gas at a flow rate of 20 ml/min. The He separator was maintained at 290 C. Ionizing potential was 70 eV and the ionizing current 60 μ A. Spectra were recorded on the apex of the gas chromatographic peaks.

Examination of the Sterol Fraction

The sterol fraction was converted into the corresponding trimethylsilyl ethers (15) and analyzed by GLC. A Perkin Elmer model 900 gas chromatograph equipped with a flame ionization detector was used. The column was 3m x 2mm ID packed with 1.5% OV 17 on 80-100 mesh Gas Chrom Z. The injector temperature was 270 C; detector temperature, 280 C; oven temperature, 250 C; carrier gas, N_2, 35-40 ml/min.

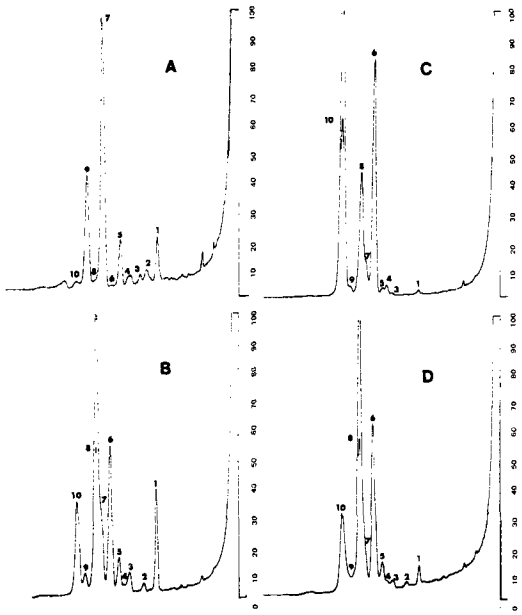

FIG. 3. Gas chromatograms of the sterol fraction (trimethylsilyl derivatives) of some algal species. Peak numbers: 1 = cholesterol, 2 = brassicasterol, 3 = Δ^5-ergostenol, 4 = unidentified component, 5 = poriferasterol, 6 = Δ^7-ergostenol, 7 = clionsterol, 8 = chondrillasterol, 9 = Δ^5-avenasterol (tentative), 10 = Δ^7-chondrillastenol.

The mass spectra were recorded on the LKB instrument of the Centro di Gascromatografia-Spettrometria di massa of the University of Bologna, adopting the same GLC conditions as for the conventional gas chromatographic analysis.

RESULTS AND DISCUSSION

The gas chromatographic analysis showed that all the algae examined present a very similar composition of the terpenic alcohol fraction. Figures 1 and 2 (Trace A) illustrate the GLC patterns of the terpenic fractions of *Spirulina platensis* strain Mao I and *Scenedesmus quadricauda*. One major component present shows the same retention time as phytol. The identity of this component with phytol was verified by comparison of its mass spectrum with the mass spectrum of an authentic sample of phytol. Purification on this fraction by TLC on Silica Gel G $AgNO_3$ did not afford a further separation of the compounds present (Fig. 1 and 2, Trace B).

Because the presence of appreciable amounts of phytol could be attributed to the degradation of chlorophyll during the saponification of the total lipids, we deemed it interesting to examine the composition of the terpenic fraction of algal lipids previously deprived of chlorophyll. For this purpose, the total lipids of both *Spirulina platensis* Mao I and *Scenedesmus quadricauda* were chromatographed on a column of Al_2O_3 (C. Erba, activity I sec. Brockmann) and recovered by elution with ethyl ether. After saponification and TLC separation, the terpenic fraction was converted to the corresponding trimethylsilyl derivatives and analyzed by GLC. The GLC peak of phytol was, in both cases, drastically reduced, confirming that its presence is largely due to the degradation of chlorophyll.

Minor amounts of other compounds are also present in this fraction. Peaks A, B, and C are probably due to aliphatic alcohols, but their identity has not yet been established unequivocally. Peaks N and O show a retention time similar to that of triterpenic alcohols. Due to the limited quantity of material available, identification of these components could not be completed in the present investigation.

The composition of the third unsaponifiable band studied, the sterol fraction, appears to be very interesting. The components of this fraction were converted into the corresponding trimethylsilyl ethers and analyzed by GLC. Some typical gas chromatograms of algal sterols are shown in Figure 3.

All the species examined show the presence of ten components in different proportions. The retention times of the compounds corresponding to chromatographic peaks 1, 2, 3, 5, 7, and 9 were identical to those of cholesterol, brassicasterol, campesterol, stigmasterol, β-sitosterol, and Δ^5-avenasterol, respectively. Furthermore, their mass spectra showed that they correspond to Δ^5-sterols. However, neither gas chromatography nor mass spectrometry, which was used to verify the sterol structure, can afford any information on the structure of the side chain. According to Patterson (1), the sterols of algae, especially green algae, differ from those of higher plants for the configuration of carbon 24, the alkyl groups at C-24 being in the 24 S configuration in algal sterols, in contrast with the 24 R configuration of higher plant sterols. Peaks 3, 5, and 7 could therefore be due to the optical isomers of campesterol, stigmasterol, and β-sitosterol; that is, Δ^5-ergostenol, poriferasterol and clionasterol, respectively.

The retention times of the sterols corresponding to peaks 6, 8, and 10 did not correspond with those of any available standard. Their mass spectra showed unequivocally that they are Δ^7-sterols. The component corresponding to peak 6 showed a molecular ion at m/e 472 and is concluded to be 24 methyl-Δ^7-

cholestenol. A molecular ion at m/e 484 showed that peak 8 is due to a C_{29} doubly unsaturated sterol, with one double bond being in the side chain, as shown by the ions at m/e 345 and 303 corresponding to M-side chain and M-side chain-42 (16). Therefore, this sterol must be 24-ethyl-$\Delta^{7,22}$-cholestadienol. Peak 10 shows a molecular ion at m/e 486 and the characteristic fragmentation pattern of Δ^7-sterols and is concluded to be 24-ethyl-Δ^7-cholestenol. Assuming that all these sterols have a 24 S configuration, they should correspond to Δ^7-ergostenol, chondrillasterol, and Δ^7-chondrillastenol, respectively. Work is in progress to ascertain the configuration of carbon 24. The sterols identified in the algae examined are reported in Table I.

The identification of Δ^5-avenasterol is only tentative, whereas compound 4 was not identified. It is of interest to note that both Δ^5- and Δ^7- sterols have been found in all the algae examined.

Table II shows the sterol composition of the ten algal species. Practically all the species examined contain the same sterols, but their amount varies considerably, and a clear difference can be noted between green and blue-green algae. Cholesterol is contained in both classes but is absent in *Scenedesmus quadricauda*. The cholesterol content of the blue-green algae studied in the present investigation is higher than that of the green ones but never attains the levels previously reported by Forin et al. (80%) (7). Also, brassicasterol is apparently contained in higher quantities in blue-green algae, and its content is particularly high in *Calothrix* sp.

The content of 24-methyl-Δ^5-cholestenol (Δ^5-ergostenol) is notable in *Chlorella* sp. but does not seem to differentiate the two algal classes.

With the exception of *Chlorella* sp., 24-ethyl-$\Delta^{5,22}$-cholestadienol (poriferasterol) seems to be characteristic of blue-green algae, whereas 24-methyl-Δ^7-cholestenol or Δ^7-ergostenol is always more abundant in green algae, with the possible exception of *Nostoc commune*.

Clionasterol (24-ethyl-Δ^5-cholestenol) is contained only in trace amounts in the green algae, except *Chlorella*, and is present in very high quantities in the three strains of *Spirulina*.

Chondrillasterol (24-ethyl-$\Delta^{7,22}$-cholestadienol) is present in notable percentage in all the green algae analyzed, and particularly in *Scenedesmus quadricauda*, whereas, among the blue-green algae, only *Nostoc* and *Calothrix* contain this sterol in comparable amounts. The sterol tentatively identified as Δ^5-avenasterol is

TABLE I

Sterols of Algae Examined

Peak number	RRT[a]	Identification	24 S		24 R
1	1.00	Cholesterol			
2	1.14	24-Methylen-Δ^5,24-28-cholestadienol		(Brassicasterol)	
3	1.32	24-Methyl-Δ^5-cholestenol	(Campesterol	or	Δ^5-Ergostenol)
4	1.39	(unidentified)			
5	1.44	24-Ethyl-$\Delta^{5,22}$-cholestadienol	(Stigmasterol	or	Poriferasterol)
6	1.54	24-Methyl-Δ^7-cholestenol		(Δ^7-Ergostenol)	
7	1.66	24-Ethyl-Δ^5-cholestenol	(β-Sitosterol)	or	Clionasterol)
8	1.71	24-Ethyl-$\Delta^{7,22}$-cholestadienol	(α-Spinasterol	or	Chondrillasterol)
9	1.87	Δ^5-Avenasterol (tentative)			
10	1.96	24-Ethyl-Δ^7-cholestenol	(Δ^7-Stigmastenol	or	Δ^7-Chondrillastenol)

[a]RRT = relative retention time.

TABLE II

Quantitative Composition of Algae Sterols[a]

Peak number	RRT[b]	Identification	*Spirulina platensis* Mao I	*Spirulina platensis* Mao II	*Spirulina* sp. (Mexico)	*Calothrix* sp.	*Nostoc commune*	*Scenedesmus quadricauda*	*Chlorella* sp.	*Uronema gigas*	*Uronema terrestre*	*Selenastrum gracile*
1	1.00	Cholesterol	6.5	3.5	14.6	10.0	10.1	-	2.5	1.8	tr[c]	0.1
2	1.14	Brassicasterol	2.7	2.5	1.5	20.4	0.9	-	1.0	0.7	-	-
3	1.32	Δ^5-Ergostenol	1.3	2.2	3.6	7.0	2.9	0.3	16.3	1.3	1.4	0.3
4	1.39	(Unidentified)	1.1	tr	6.6	tr	tr	0.2	-	1.0	tr	1.2
5	1.44	Poriferasterol	7.0	3.9	8.0	9.9	4.5	0.3	11.7	3.0	0.7	0.9
6	1.54	Δ^7-Ergostenol	tr	7.7	7.4	6.5	18.8	20.4	23.9	24.5	23.9	27.0
7	1.66	Clionasterol	50.8	45.6	32.7	7.9	4.4	tr	6.4	tr	tr	tr
8	1.71	Chondrillasterol	0.8	6.5	7.0	24.3	42.2	70.4	23.5	49.0	64.2	20.3
9	1.87	Δ^5-Avenasterol (tentative)	22.4	7.1	8.4	11.4	3.5	tr	1.2	2.1	tr	1.2
10	1.96	Δ^7-Chondrillastenol	1.6	15.2	8.9	2.6	12.6	8.4	3.4	16.6	8.3	49.0
		Other peaks	5.8	5.8	1.3	-	-	-	10.1	-	1.5	-

[a]Quantitation was done with a gas chromatography data system Perkin Elmer model PEP-1.
[b]RRT = relative retention time.
[c]tr = trace, <0.1.

typical of blue-green algae and is particularly abundant in one strain of *Spirulina platensis*.

Δ^7-Chondrillastenol, which represents almost 50% of the sterol fraction of *Selenastrum gracile*, is present in all species, but its content varies appreciably without any apparent rule.

The presence of small quantities of 24-desalkyl-sterols (cholesterol) together with higher amounts of 24-alkyl-sterols in the procaryotic blue-green algae is in agreement with their biochemical evolution. The origin of the two types of sterols resides, in fact, in a biosynthetic bifurcation in which the $\Delta^{24(25)}$ bond alternatively is reduced or alkylated (17). In higher photosynthetic organisms, the alkylation mechanism is predominant, whereas the reduction mechanism is operating in the non-photosynthetic ones. Accordingly, the Δ^{24} reduction mechanism seems to be inhibited or even lost in the eucaryotic green algae; the cholesterol production in this case is very low.

The relative amounts of the two types of sterols could, however, be influenced by the culturing conditions and could change in eterotrophic metabolism. This appears to be a very interesting topic which will be investigated in the future.

Although it seems desirable to survey as many algae as possible before drawing any conclusion regarding the taxonomic and evolutionary significance of the unsaponifiable components, the present work shows that both the hydrocarbon and the sterol fractions are very promising for a better understanding of the algal biochemical evolution.

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Effects of Dietary Saturated and *Trans* Fatty Acids on Tissue Lipid Composition and Serum LCAT Activity in the Rat

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ABSTRACT

Groups of rats were fed from weaning with diets containing 5% by wt of hydrogenated coconut oil (HCO), safflower oil, or a concentrate of ethyl elaidate and linolelaidate (TRANS) as the sole source of dietary fat. Fatty acid composition of the lipid classes from serum, liver, heart, and kidney was determined, and the serum lecithin:cholesterol acyl transferase (LCAT) activities were assayed for each animal. Serum LCAT activity was increased by both the HCO and TRANS diets in the early stages of the development of an essential fatty acid (EFA) deficiency but was suppressed in the animals of the TRANS group as they became older. The HCO and TRANS groups exhibited changes in tissue lipid fatty acid composition, as well as reduced growth, characteristic of an EFA deficiency. Conversion of oleic acid to eicosatrienoic acid was impaired in the animals fed the TRANS diet, greatly increasing the octadecenoic acid content of the tissue lipids at the expense of eicosatrienoic acid. The TRANS diet also suppressed incorporation of eicosatrienoic acid into cholesteryl esters of tissue and serum, indicating that, when fed as the sole source of unsaturated fat, *trans* fatty acids influenced the metabolism of unsaturated fatty acids and cholesterol.

INTRODUCTION

Trans fatty acids are known to be deposited in the tissues of rats (1-4) and are readily catabolized (5,6). When fed with adequate amounts of essential fatty acids (EFA), they have little effect on growth (7) but, when fed as the sole source of fat in the diet, they intensify the symptoms of an EFA deficiency (8). *Trans* unsaturated fatty acids differ from the naturally occurring *cis* isomers and resemble saturated fatty acids in acyl transfer reactions (9), incorporation into phosphatidyl choline and triglycerides (9,10), cholesterol esterifying activity (11-13), enzyme activation in liver (14,15), absorption (16-19), and β-oxidation (5,6,20). *Trans* acids and *cis* acids impart different physical properties to phospholipids which are important constituents of biological membranes (21). These and other aspects of the metabolism of *trans* fatty acids have been reviewed recently by Kummerow (22). *Trans, trans* and *cis, trans* isomers of linoleic acid are devoid of EFA activity (23-25), and only minor amounts of these acids are converted to higher polyunsaturated fatty acids (2,26,27). Hence, one of the problems in studies of the nutritional effects of *trans* fatty acids has been to distinguish their effects from those of an EFA deficiency. We report here a comparison of the effects of diets containing a mixture of elaidate and linolelaidate (TRANS), hydrogenated coconut oil (HCO), or safflower oil (SAFF) upon the fatty acid compositions of lipid classes of serum, liver, kidney, and heart, and upon serum lecithin:cholesterol acyl transferase (LCAT) activity.

MATERIALS AND METHODS

Animals

Weanling male rats of the Sprague-Dawley strain, 50-60 g (ARS Sprague-Dawley Corp., Madison, WI), were housed in individual cages and fed ad libitum a basic fat-free diet to which was added 5% by wt of HCO, SAFF, or TRANS. The fat-free diet consisted of 25% vitamin test casein; 64% sucrose; 4.4% salt mixture Wesson modified, Osborne-Mendel salt mix (General Biochemicals, Chagrin Falls, OH); 4.4% nonnutritive cellulose, Alphacel (Nutritional Biochemical Corporation, Cleveland, OH); 1.1% choline mixture (22% choline dihydrogen citrate, 78% casein); and 1.1% vitamin mixture (28). The TRANS fat was prepared from safflower oil fatty acids according to the procedure described by McCutcheon et al. (29) and fractionated by low temperature fractional crystallization and finally high vacuum distillation of ethyl esters. The composition of the final preparation was 21.3% palmitate, 9.1% stearate, 20.8% elaidate, and 47.5% linolelaidate. The diets were stored at -20 C and were fed fresh daily.

The HCO and SAFF groups contained 15

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TABLE I

Effect of Dietary Fat[a] on Unsaturated Fatty Acids of Serum and Tissue Lipids of Rats 14 and 43 Weeks[b] after Weaning (% by wt of total fatty acids)

Diet[a]	16:1	18:1	Linolelaidate	18:2	ω-9 20:3	ω-6 20:4
			Serum			
SAFF	1.9 ± 0.1[c]	9.9 ± 1.0	-	22.9 ± 3.2	-	39.7 ± 4.0
(")[b]	0.3 ± 0.0	3.3 ± 0.1	-	26.6 ± 1.0	-	38.5 ± 1.2
HCO	8.8 ± 2.6	41.4 ± 2.8	-	1.8 ± 0.3	17.0 ± 1.3	4.2 ± 0.7
(")	8.1 ± 1.4	48.5 ± 2.6	-	-	14.6 ± 2.2	-
TRANS	10.5 ± 1.4	58.7 ± 1.0	3.5 ± 0.4	2.1 ± 0.3	1.4 ± 0.5	-
S-TRANS	9.0 ± 0.8	54.6 ± 2.3	3.6 ± 0.4	2.1 ± 0.2	5.2 ± 2.6	-
			Liver			
SAFF	1.7 ± 0.5	11.3 ± 2.9	-	23.6 ± 1.5	-	19.7 ± 2.9
(")	0.7 ± 0.4	5.9 ± 1.1	-	28.2 ± 2.8	-	23.6 ± 1.6
HCO	8.8 ± 2.6	41.4 ± 2.8	-	1.8 ± 0.3	17.0 ± 1.3	4.2 ± 0.7
(")	7.0 ± 0.9	35.8 ± 3.1	-	-	17.3 ± 3.1	3.4 ± 0.9
TRANS	9.0 ± 2.1	40.0 ± 2.3	2.1 ± 0.7	1.7 ± 0.5	7.2 ± 1.4	3.0 ± 0.4
			Kidney			
SAFF	2.0 ± 1.0	13.5 ± 2.1	-	17.5 ± 1.8	-	23.2 ± 3.5
(")	1.3 ± 0.7	13.8 ± 0.9	-	37.3 ± 3.8	-	15.2 ± 1.8
HCO	5.9 ± 1.5	33.3 ± 4.0	-	1.4 ± 0.3	10.1 ± 2.6	9.2 ± 1.4
(")	7.1 ± 0.4	40.5 ± 4.2	-	-	11.5 ± 2.6	4.7 ± 2.4
TRANS	7.2 ± 1.1	37.7 ± 2.7	2.6 ± 0.4	1.9 ± 0.3	5.6 ± 0.6	7.8 ± 1.5
			Heart			
SAFF	0.9 ± 0.3	9.9 ± 0.9	-	25.8 ± 1.3	-	21.2 ± 1.6
(")	0.3 ± 0.2	7.3 ± 1.0	-	32.3 ± 5.0	-	20.8 ± 2.5
HCO	4.2 ± 0.4	28.0 ± 0.6	-	3.3 ± 0.9	23.6 ± 1.1	7.7 ± 1.1
(")	4.0 ± 0.5	32.5 ± 3.3	-	-	26.8 ± 2.1	2.4 ± 2.7
TRANS	6.5 ± 0.4	39.1 ± 1.6	7.3 ± 0.8	3.9 ± 1.4	11.0 ± 0.9	7.3 ± 1.2

[a]SAFF = safflower oil, HCO = hydrogenated coconut oil, TRANS = elaidinized safflower oil, S-TRANS = four animals shifted from HCO to TRANS diet from 12th to 14th week.

[b]Diet in parentheses = 43 weeks after weaning.

[c]Mean ± SD.

animals each and the TRANS group 10 animals. The wts of the animals were recorded weekly. At the end of 12 weeks, 4 animals of the HCO group were switched to the TRANS diet for an accessory experiment. At 14 weeks these animals, 6 animals of the HCO group, 10 animals of the SAFF group, and the 10 animals of the TRANS group were sacrificed. The remaining 5 animals in each of the HCO and SAFF groups were continued on their respective diets for an additional 6½ months to develop the long-term effects of an EFA deficiency. The supply of the TRANS fat was sufficient only to continue the animals of this group through the 14th week.

Ca. 2 ml of blood was withdrawn from the retro-ocular plexes of each animal in each group at 39, 70, and 99 days, and at 300 days for the animals continued on the HCO and SAFF diets. LCAT activity was determined on an aliquot of serum; the remainder of the serum was frozen and stored at -20 C for subsequent analysis of lipid class and fatty acid composition. The animals were killed by exsanguination under light ether anesthesia, and the livers, hearts, testes, and kidneys were excised, weighed, quickly frozen on dry ice, and stored at -20 C for lipid analysis. Studies of the effect of the dietary fats on lipid metabolism in the testes will be reported in a separate communication.

LCAT Activity

A modification (30) of the procedure of Stokke and Norum (31) was used with an albumin, cholesterol-4-^{14}C substrate prepared as described by Porte and Havel (32).

Lipid Class Isolation and Analysis

The lipid was extracted from the tissues or serum with chloroform:methanol (2:1), as previously described (33). The content of cholesteryl esters, triglyceride, and cholesterol was determined by thin layer chromatography (TLC) using the charring-densitometry technique (33,34), with appropriate authentic standards of highly purified cholesterol, cholesteryl

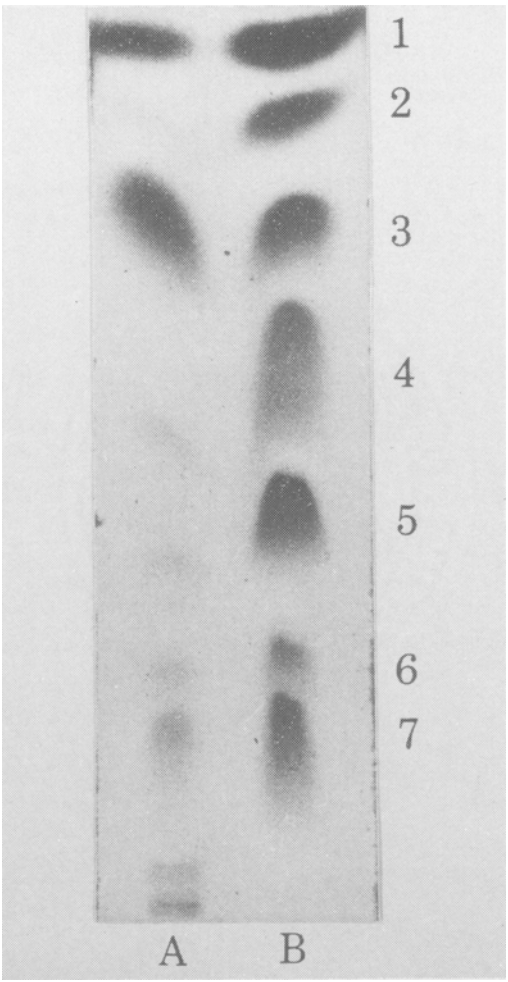

FIG. 1. Fractionation of liver fatty acid methyl esters of the TRANS group of animals by argentation-thin layer chromatography. A, sample; B, reference methyl esters; 1, methyl stearate; 2, methyl elaidate; 3, methyl oleate; 4, methyl linolelaidate; 5, methyl linoleate; 6, *cis-trans* isomers of methyl linolenate; 7, methyl linolenate.

oleate, and triolein obtained from the Lipid Preparation Laboratory of The Hormel Institute. The cholesteryl esters were separated first with petroleum ether:ethyl ether (90:10) near the top of the plate; the plate was dried for ca. 5 min under nitrogen and redeveloped to a line ca. 5 cm below the first solvent front using petroleum ether:ethyl ether:acetic acid (80:20:0.2). This system facilitated the analysis by providing a complete separation of all components. Each analysis was made in quadruplicate; the relative error was ca. ± 2% for major components and ± 10% for minor components. Total amount of polar lipid was calculated by difference from the sum of the neutral lipids and the total lipid which was determined gravimetrically on an aliquot of the chloroform:methanol extract. Cholesteryl esters and triglycerides were isolated for fatty acid analysis by TLC using the same solvent system. In these experiments, the spots were made visible by spraying the plates with a methanol solution of 0.2% 2,4-dichlorofluorescene and scraped directly into vials for conversion to methyl esters for analysis by gas-liquid chromatography (GLC) as described below. Phosphatidyl choline was isolated by TLC for fatty acid analysis because of its relationship to LCAT activity in serum. The solvent system used for the isolation of these compounds was chloroform: methanol:and concentrated ammonium hydroxide (65:35:5).

The total lipids and individual lipid classes were converted to methyl esters using HCl as a catalyst as previously described (35) and analyzed by GLC. The GLC was carried out at 180 C with a Barber Colman instrument, Selectra System Series 5000, equipped with a hydrogen flame detector and a 180 x 0.4 cm column packed with 15% ethylene glycol succinate on Gas-Chrom P mesh 100-120 (Applied Science Laboratories, Inc., State College, PA). Nitrogen was used as the carrier gas, and the percentage distribution of fatty acids was determined by proportion from the peak areas. Identification of the peaks was made on the basis of the retention times of known methyl esters. Analysis of methyl esters of *trans* fatty acids was made using a SILAR 10 C column, 10% on Gas Chrom Q mesh 100-120, 180 x 2 cm (Applied Science Laboratories), operated at 160 C with nitrogen as a carrier gas.

For the separation of methyl esters of *trans* fatty acids, plates coated with Silica Gel H (E.M. Laboratories, Inc., Elmsford, NY) were used. The samples were chromatographed with a solvent system of petroleum ether:ethyl ether:acetic acid (90:10:1).

RESULTS

The animals of the HCO and TRANS group developed dermal symptoms on feet and tail, characteristic of an EFA deficiency, and growth was suppressed. The wts of the animals of the TRANS group were significantly ($P<0.010$) lower than those of the HCO group, which in turn were significantly ($P<0.001$) lower than those of the SAFF group, at ca. the 5th week after weaning. At the 14th week, the wts of the animals in the three groups (TRANS, HCO, and SAFF) were 263 ± 16, 320 ± 28, and 390 ± 26 g (M ± SD), respectively.

TABLE II

Effect of Dietary Fat[a] on Unsaturated Fatty Acids of Cholesteryl Esters in Tissue of Rats 14 and 43 Weeks after Weaning (% by wt of total fatty acids)

Diet[a]	16:1	18:1	Linolelaidate	18:2	ω-9 20:3	ω-6 20:4
			Serum			
SAFF	1.1 ± 0.5[c]	2.1 ± 1.5	-	14.5 ± 2.0	-	78.3 ± 4.1
(")	0.4 ± 0.2	3.2 ± 0.6	-	15.9 ± 1.6	-	74.4 ± 2.7
HCO	12.3 ± 2.7	33.2 ± 4.4	-	1.9 ± 0.6	28.4 ± 2.9	9.0 ± 1.8
(")	12.7 ± 0.6	52.3 ± 1.3	-	-	19.1 ± 0.8	3.0 ± 1.4
TRANS	12.0 ± 1.2	62.8 ± 2.4	2.9 ± 0.5	1.3 ± 0.2	1.3 ± 0.6	0.4 ± 0.3
S-TRANS	13.0 ± 1.8	61.0 ± 2.9	1.3 ± 1.0	1.2 ± 1.0	3.0 ± 2.0	1.3 ± 0.4
			Liver			
SAFF	3.3 ± 0.8	16.8 ± 2.4	-	17.2 ± 0.8	-	10.0 ± 2.4
(")	1.9 ± 0.5	20.5 ± 2.0	-	39.6 ± 2.2	-	22.6 ± 3.8
HCO	11.2 ± 2.3	55.9 ± 3.8	-	0.7 ± 0.7	1.9 ± 1.0	trace
(")	12.4 ± 1.6	58.9 ± 0.6	-	-	2.0 ± 0.2	-
TRANS	12.6 ± 3.2	58.2 ± 2.6	5.2 ± 0.6	1.4 ± 0.2	trace	trace
			Kidney			
SAFF	4.6 ± 1.2	10.6 ± 0.8	-	15.1 ± 2.0	-	38.5 ± 2.7
(")	0.6 ± 0.1	13.6 ± 1.3	-	24.2 ± 1.2	-	39.2 ± 3.8
HCO	4.6 ± 0.1	54.2 ± 1.2	-	trace	7.1 ± 0.8	3.1 ± 0.8
(")	8.0 ± 0.9	57.1 ± 0.5	-	-	7.7 ± 0.4	2.5 ± 1.0
TRANS	6.0 ± 1.0	57.6 ± 1.3	10.0 ± 1.1	1.0 ± 0.2	1.4 ± 0.6	1.7 ± 0.5
			Heart			
SAFF	1.8 ± 1.3	7.7 ± 1.1	-	30.1 ± 1.0	-	48.6 ± 2.5
(")	1.0 ± 0.6	9.4 ± 3.7	-	19.4 ± 1.9	-	52.6 ± 1.9
HCO	6.3 ± 0.3	34.1 ± 2.6	-	3.9 ± 1.6	23.2 ± 1.3	8.6 ± 0.3
(")	8.8 ± 1.5	51.2 ± 0.9	-	trace	12.6 ± 0.9	-
TRANS	6.9 ± 1.1	42.9 ± 2.9	-	8.9 ± 0.9	5.2 ± 2.1	5.9 ± 1.7

[a]SAFF = safflower oil, HCO = hydrogenated coconut oil, TRANS = elaidinized safflower oil, S-TRANS = four animals shifted from HCO to TRANS diet from 12th to 14th week.

[b]43 weeks after weaning = (").

[c]Mean ± SD.

Fatty Acid Composition

The pattern of unsaturated fatty acids of the total lipids of sera, livers, hearts, and kidneys of the animals of the HCO and TRANS groups were typical of an EFA deficiency after 14 weeks, namely low linoleic and arachidonic acid contents and high contents of monoenoic and eicosatrienoic acids (Table I). However, the octadecenoic acid fraction (18:1) was generally higher and the eicosatrienoic acid fraction (20:3) lower in the tissue lipids and sera of the animals of the TRANS group than in those of the HCO group.

Only the serum lipids of the four animals switched from the HCO to the TRANS diet were analyzed. However, the fatty acid composition of these lipids had become almost the same as that of the TRANS group in the short time of 2 weeks, showing further a difference between the effects of these diets. The higher level of monoenoic acid in the TRANS group was not due to elaidic acid, as indicated by GLC and argentation-TLC (Fig. 1). Linolelaidic acid content of the diet was only ca. 2.5% by wt, but small amounts of this acid were detected in some of the tissue lipids (Table I). Little, if any, of the dietary linolelaidic or elaidic acids were converted to higher polyunsaturated fatty acids, as shown by argentation-TLC as well as by GLC analysis. The results in Figure 1 were obtained with liver; virtually identical results were obtained with serum.

The unsaturated fatty acid composition of individual lipid classes of serum and tissues of the animals of all groups after 14 weeks are presented in Tables II-IV. The analyses of saturated fatty acids are not reported because there is virtually no difference in the percentage composition of these acids between the HCO and TRANS groups and effects of an EFA deficiency on these acids are well documented. These analyses (Tables II-IV) showed a striking difference between the effects of the TRANS and HCO diets on the fatty acid composition of serum and the tissue lipids, except for liver, namely the virtual absence of 20:3 and a cor-

TABLE III

Effect of Dietary Fat[a] on Unsaturated Fatty Acids of Triglycerides in Tissues of Rats 14 and 43 Weeks after Weaning (% by wt of total fatty acids)

Diet[a]	16:1	18:1	Linolelaidate	18:2	ω-9 20:3	ω-6 20:4
			Serum			
SAFF	1.7 ± 0.4[c]	13.4 ± 1.5	-	31.3 ± 5.2	-	10.3 ± 1.0
(")	-	10.2	-	51.4	-	13.8
HCO	5.7 ± 1.4	55.7 ± 1.9	-	trace	4.5 ± 1.2	trace
(")	5.6	59.7	-	-	4.5	-
TRANS	4.9 ± 0.5	61.8 ± 4.5	trace	3.3 ± 0.7	trace	trace
S-TRANS	3.2 ± 1.0	58.3 ± 4.1	trace	trace	2.0 ± 0.9	1.0 ± 0.5
			Liver			
SAFF	3.1 ± 0.2	14.8 ± 0.8	-	28.4 ± 2.3	-	18.2 ± 2.1
(")	2.3	13.6	-	53.9	-	4.8
HCO	8.4 ± 2.7	52.3 ± 0.8	-	-	1.0 ± 0.1	trace
(")	10.0	60.4	-	-	1.1	-
TRANS	7.8 ± 2.2	52.9 ± 2.5	trace	1.4 ± 0.8	trace	trace
			Kidney			
SAFF	5.5 ± 1.3	27.9 ± 2.0	-	30.0 ± 2.3	-	0.7 ± 0.2
(")	2.4	19.4	-	52.2	-	1.2
HCO	9.9 ± 2.7	49.7 ± 2.1	-	-	0.4 ± 0.3	trace
(")	11.2	53.5	-	-	0.7	-
TRANS	8.9 ± 1.8	49.7 ± 1.9	4.4 ± 0.3	trace	trace	trace
			Heart			
SAFF	3.3 ± 0.6	25.4 ± 1.6	-	33.7 ± 2.3	-	0.9 ± 0.1
(")	1.7	18.4	-	52.7	-	2.5
HCO	5.4 ± 0.8	48.1 ± 2.3	-	trace	trace	trace
(")	7.2	59.8	-	-	2.4	-
TRANS	7.7 ± 1.2	49.1 ± 2.3	5.5 ± 0.7	trace	trace	trace

[a]SAFF = safflower oil, HCO = hydrogenated coconut oil, TRANS = elaidinized safflower oil, S-TRANS = four animals shifted from HCO to TRANS diet from 12th to 14th week.

[b]43 weeks after weaning = (").

[c]Mean ± SD; where no standard deviations are shown, samples were pooled for analysis.

responding increase in the level of 18:1 in the cholesteryl esters. This effect was dramatically demonstrated in the analysis of the serum cholesteryl esters of the four animals switched from the HCO to the TRANS diet. In <2 weeks, the 20:3 content of the cholesteryl esters of the serum of these animals decreased from ca. 28 to 3% and the 18:1 increased from 33 to 61%. Similar differences were observed in the 20:3 content of the cholesteryl esters of the kidney and heart of the animals of the HCO and TRANS groups. On the other hand, the cholesteryl esters of the liver of both the TRANS and HCO groups contained only minor amounts of 20:3, in spite of the fact that it was a major constituent of the total lipid.

Linolelaidic acid accumulated preferentially in cholesteryl esters and triglycerides, although traces were detected in phosphatidyl choline. In general, the changes in fatty acid composition of the triglycerides and phosphatidyl choline of each group resembled that of the total lipid, differing mainly in degree of the change, as might be expected from the characteristic compositions of these lipid classes.

The fatty acid compositions of the tissues and serum lipids of the SAFF group changed little between 14 and 43 weeks (Table II-IV). The major effect of the prolonged feeding of the HCO diet on the fatty acid composition of the serum and tissue lipids was a further depletion of arachidonic and linoleic acids. The 20:3 content of the cholesteryl esters of serum and heart lipids was lower and that of the 18:1 higher than in the animals of this group sacrificed at the 14th week in the experiment. However, the concentration of the 20:3 in the cholesteryl esters of these lipids was relatively high (19.1% and 12.6%, respectively) and unchanged in the kidney lipids (7.7%).

Lipid Composition

The concentration of lipid in the serum of the animals of both the TRANS and HCO groups was lower than for the SAFF group after 14 weeks, generally characteristic of an

TABLE IV

Effect of Dietary Fat[a] on Unsaturated Fatty Acids of Phosphatidyl Choline in Tissues of Rats 14 and 43 Weeks after Weaning (% by wt of total fatty acids)

Diet[a]	16:1	18:1	Linolelaidate	18:2	ω-9 20:3	ω-6 20:4
			Serum			
SAFF	trace	4.7 ± 1.1[c]	-	13.1 ± 0.6	-	30.1 ± 2.3
(")[b]	-	3.5	-	15.3	-	28.6
HCO	1.4 ± 0.5	17.8 ± 1.2	-	1.7 ± 0.8	26.3 ± 1.4	3.6 ± 0.8
(")	1.4	25.0	-	trace	29.6	-
TRANS	2.9 ± 1.0	39.3 ± 0.4	trace	3.4 ± 0.3	9.1 ± 1.0	trace
S-TRANS	1.8 ± 0.7	34.1 ± 3.7	trace	3.4 ± 1.0	13.4 ± 2.7	2.3 ± 0.4
			Liver			
SAFF	1.0 ± 0.1	5.4 ± 0.2	-	17.5 ± 2.2	-	28.3 ± 1.5
(")	-	5.1	-	11.3	-	36.7
HCO	3.6 ± 1.0	22.3 ± 0.5	-	0.9 ± 0.3	21.6 ± 2.1	4.1 ± 0.1
(")	5.7	27.3	-	-	24.8	3.4
TRANS	5.8 ± 1.4	32.9 ± 1.9	trace	3.6 ± 0.8	10.7 ± 1.8	2.1 ± 0.2
			Kidney			
SAFF	0.6 ± 0.2	8.1 ± 0.5	-	9.3 ± 0.6	-	30.8 ± 1.0
(")	-	8.7	-	12.6	-	29.8
HCO	4.1 ± 0.7	28.6 ± 1.7	-	1.4 ± 0.6	15.8 ± 0.2	7.5 ± 1.4
(")	5.2	34.7	-	-	16.1	-
TRANS	6.4 ± 0.4	41.3 ± 0.7	2.7 ± 0.2	2.5 ± 0.2	4.2 ± 0.8	2.9 ± 0.3
			Heart			
SAFF	trace	9.8 ± 0.3	-	26.7 ± 4.3	-	22.3 ± 2.6
(")	-	7.2	-	12.0	-	33.0
HCO	2.2 ± 0.4	22.5 ± 0.7	-	2.1 ± 0.8	28.2 ± 1.6	4.3 ± 0.8
(")	3.2	26.0	-	-	29.7	-
TRANS	5.5 ± 0.4	42.1 ± 2.4	1.4 ± 0.2	5.2 ± 0.6	6.6 ± 1.1	2.1 ± 0.4

[a]SAFF = safflower oil, HCO = hydrogenated coconut oil, TRANS = elaidinized safflower oil, S-TRANS = four animals shifted from HCO to TRANS diet from 12th to 14th week.

[b]43 weeks after weaning = (").

[c]Mean ± SD; where no standard deviations are shown, samples were pooled for analysis.

EFA deficiency (Table V). There also were differences in the lipid class composition of the serum lipids among the three groups. The greatest differences occurred in the TRANS group, especially in the polar lipid content which was much lower than in the other groups. Differences also occurred in the liver lipids among the three groups, but these were not so pronounced as in serum. In contrast to liver and serum, there were only minor differences in the level and composition of the lipid classes of the heart and kidneys of the three groups, in spite of large differences in fatty acid composition.

The changes in the serum and liver lipids of the TRANS group could well have been caused by effects on enzyme activities in the conversion of 18:1 to 20:3 and on incorporation of the latter acid into cholesteryl esters. To study this possibility, the effects of the diets on serum LCAT activity were investigated.

LCAT Activity

By 70 days, LCAT activity had increased in the HCO group to a significantly ($P<0.001$) higher level than in the SAFF group and generally remained higher (Table VI). In contrast to the HCO group, the LCAT activity of the TRANS group was increased at 39 days and then decreased to ca. 50% of that of the SAFF group at 14 weeks.

DISCUSSION

Depletion of linoleic and arachidonic acid in sera and livers together with a depression in growth indicated that an EFA deficiency developed in the animals of both the HCO and TRANS groups at the end of 14 weeks. The decrease in the level of serum lipids is also characteristic of an EFA deficiency. As might be expected from previous studies (2), the TRANS diet suppressed the growth of the animals to a greater extent than did the HCO

TABLE V

Effect of Dietary Fat[a] on Lipid Class Composition in Tissues of Rats 14 and 43 Weeks after Weaning

Tissue		Diet[a] SAFF 14 weeks	SAFF 43 weeks	HCO 14 weeks	HCO 43 weeks	TRANS 14 weeks
Serum (mg/dl)	TL[b]	455 ± 110[c]	423 ± 40	311 ± 31	300 ± 76	231 ± 44
	CE	128 ± 20.7	189.0	90.7 ± 2.3	151.0	102.7 ± 33.3
	TG	39.5 ± 5.6	25.0	60.8 ± 11.2	35.0	29.9 ± 7.0
	C	30.8 ± 4.8	47.0	18.9 ± 1.2	21.0	17.2 ± 5.3
	PL	256.2 ± 79.1	162.0	140.6 ± 37.9	93.0	81.2 ± 47.6
Liver (mg/gm wet wt)	TL	31.6 ± 1.8	58.8 ± 0.3	55.4 ± 3.4	58.9 ± 2.9	46.3 ± 33.8
	CE	trace	1.0	1.8 ± 0.3	5.3	4.3 ± 0.3
	TG	9.5 ± 1.8	12.1	23.4 ± 7.0	10.4	9.6 ± 3.0
	C	1.6 ± 0.3	3.2	2.6 ± 0.4	3.1	2.6 ± 0.8
	PL	20.5 ± 2.6	42.4	27.7 ± 6.8	40.1	29.1 ± 5.5
Kidney (mg/gm wet wt)	TL	42.9 ± 4.6	54.5 ± 4.5	42.6 ± 6.0	49.0 ± 2.3	42.3 ± 4.4
	CE	0.2	0.3	0.6	1.5	0.7
	TG	8.1	20.4	7.6	12.5	7.5
	C	3.4	4.3	3.0	4.4	3.9
	PL	31.2	29.5	31.3	30.6	30.2
Heart (mg/gm wet wt)	TL	31.1 ± 2.0	27.5 ± 0.9	31.7 ± 0.7	29.6 ± 0.7	30.5 ± 3.0
	CE	0.2	trace	0.2	trace	0.2
	TG	8.4	3.8	5.1	3.6	9.8
	C	1.2	1.6	1.1	1.6	1.5
	PL	21.3	22.2	25.4	24.4	19.1

[a]SAFF = safflower oil, HCO = hydrogenated coconut oil, TRANS = elaidinized safflower oil ethyl esters.
[b]TL = total lipid extract, CE = cholesteryl esters, TG = triglycerides, C = cholesterol, PL = polar lipids.
[c]Mean ± SD; where no standard deviations are shown, samples were pooled for analysis.

TABLE VI

Effect of Dietary Fat on Serum Lecithin:Cholesterol Acyl-Transferase (LCAT) Activity (nmol cholesterol esterified/min/liter)

Diet[a]	Days after weaning 39	70	99	300
SAFF	1327 ± 125[b]	1285 ± 146	1241 ± 117	1429 ± 87
HCO	1390 ± 227	1766 ± 148	1170 ± 80	1791 ± 130
TRANS	1650 ± 223	1041 ± 328	556 ± 194	

[a]SAFF = safflower oil, HCO = hydrogenated coconut oil, TRANS = elaidinized safflower oil.

[b]Mean ± SD. Difference between LCAT activity of SAFF and HCO groups was significant at 70 days ($P<0.001$) and at 300 days ($P<0.025$). Difference between LCAT activity of SAFF and TRANS groups was significant at 39 days ($P<0.05$) and 99 days ($P<0.001$). Difference between LCAT activity of HCO and TRANS groups was significant at 39 days ($P<0.05$), 70 days ($P<0.001$), and at 99 days ($P<0.001$).

diet, suggesting that this diet increased the severity of the EFA deficiency, as observed by Holman and Aaes-Jorgenson (8). Although the animals of the TRANS group were more severely affected than those of the HCO group, the TRANS diet appeared to exert a specific effect on the interconversion of 18:1 to 20:3 and the incorporation of fatty acids into cholesteryl esters. These effects did not appear to be related directly to the accumulation of the *trans* fatty acids in the lipids because only minor amounts of these acids were deposited in the tissues at the levels they were fed.

In spite of the higher content of 18:1 in the lipids of the TRANS group than of the HCO group, little of the 18:1 consisted of elaidate, as might be expected had it been fed at a higher level (22). The increase in 18:1 may be due to a lower rate of conversion to 20:3. The 18:1 could also increase as a result of increased synthesis but, because the concentration of 20:3 is correspondingly lower in the TRANS than the HCO group, it appears that the *trans* fatty acids have an inhibitory effect on the

enzyme system involved in the chain elongation and desaturation of unsaturated fatty acids. A similar effect was observed in previous work with linoleate (2). Whether this effect is due to either the dietary elaidate or linolelaidate, or both, was not determined in the present study.

In accord with the studies of Sugano and Portman (36), the present study shows that LCAT activity is increased by an EFA deficiency. The reason for an increase in LCAT activity is not clear, although these investigators suggested that it may be related to an effect on the lipoprotein substrate. LCAT appears to have a specificity for highly unsaturated fatty acids, especially arachidonic acid (37), which are usually concentrated in the 2-position of phosphatidyl choline. Hence, the high concentration of 20:3 in cholesteryl esters of the serum lipids of the animals of the HCO group is not unexpected, inasmuch as this fatty acid is also esterified preferentially in the 2-position of phosphatidyl choline in EFA deficient animals (10,38). Previous studies (10) on liver lipids indicate that the 20:3 also occurs largely in the 2-position of phosphatidyl choline in animals fed diets containing *trans* fatty acids. Thus, it appears that the specificity of the LCAT enzyme is affected by the TRANS diet inasmuch as changes in fatty acid composition of the cholesterol esters occurred much faster than an effect on the rate of the acyl transferase reaction.

The fact that little cholesteryl eicosatrienoate is present in the livers of the animals of the HCO group indicates that, in serum, this ester arises largely from the action of LCAT activity. However, liver is a primary source of serum cholesteryl ester, particularly for the low density (LDL) and very low density lipoprotein (VLDL) fractions (39). Hence, the major source of the saturated and monoenoic cholesteryl esters of the serum of the animals of the HCO group is probably the liver. Some of these esters could arise from LCAT activity in that cholesteryl esters of the high density liproteins exchange with those of LDL and VLDL (40). Since no cholesteryl eicosatrienoate appears to be produced in the liver, the appearnce of this ester in serum LDL and VLDL might serve as an indication of the extent of these exchange reactions. The low level of total serum cholesterol and the composition of the serum cholesteryl esters of the HCO group may be explained on the basis of impaired secretion of cholesterol and cholesteryl esters of saturated and monoenoic acids from liver, together with an increase in serum LCAT activity.

Although the cholesteryl esters of the liver lipids of the animals fed the HCO diet contained little 20:3, it was present in the cholesteryl esters of the hearts and kidneys of these animals. In contrast, little 20:3 was found in the cholesteryl esters of the lipids of the hearts and kidneys or the livers of the animals fed the TRANS diet. The absence of 20:3 in the cholesteryl esters of the lipids of the kidneys and hearts of the animals fed the TRANS diet may be due to a specific effect of the *trans* fatty acids on cholesteryl esterifying activity. The reports of others (11-13) also indicate that the activation of fatty acids is influenced by *trans* fatty acids.

At present, it is now known if the effect of the TRANS diet is due to either elaidic or linolelaidic acids, or both. Regardless, the influence of these acids appears to be far out of proportion to their concentration in the diet, for it seems unlikely that the small amount accumulated in the tissues could be responsible for their metabolic effects.

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Sterol Synthesis in the Liver, Intestine, and Lung of the Guinea Pig

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ABSTRACT

The relative rates of sterol synthesis in the liver, ileum, and lung of the guinea pig have been studied by measuring the incorporation by tissue slices of ^{14}C-labeled acetate into digitonin-precipitable sterols. The liver showed maximum incorporation of acetate at pH 6.5, the ileum at pH 7.5, and the lung at pH 6.0. The incorporation of acetate approached the maximum rate at a concentration of 10 mM with the liver and lung and 5 mM with the ileum. Using these conditions of assay, sterol synthesis was measured in the liver, ileum, and lung of four groups of guinea pigs killed at 6-hourly intervals. Depending on the time of day, the rate of sterol synthesis in the ileum was from 6 to 14 times that in the liver, while in the lung the rate was up to 3 times that shown by the liver. Additional studies showed that all regions of the small intestine synthesized sterol at a higher rate than the liver, with the highest rate of synthesis occurring in the ileum. The rates observed in the adrenal, testis, muscle, adipose tissue, and skin indicated that these tissues are not quantitatively important sites of sterol synthesis in the guinea pig.

INTRODUCTION

Considerable information now exists concerning the relative rates of sterologenesis and, more particularly, of cholesterogenesis in various tissues of a number of species. In the rat, the liver and ileum show the highest rates of cholesterol synthesis, the liver being quantitatively a more important site of synthesis than the intestine (1). In monkeys, the relative rates of hepatic and intestinal cholesterol synthesis varies markedly with the species, although the liver consistently shows a higher rate of cholesterol synthesis than does the intestine (2,3). The relatively lower rates of cholesterogenesis in the intestine of the rat and monkey are in marked contrast to those in the human, in which the rate in the ileum is ca. 4 times that in the liver (4).

The results of studies on the relative rates of sterol synthesis in the guinea pig suggest that this species may show similar tissue differences in the rates of sterol synthesis to those in the human. Thus, Schwenk et al. (5), in a study with guinea pigs, found that the incorporation rate of labeled acetate into sterols in vivo by the gastrointestinal tract was ca. 10 times higher than that shown by the liver. A more recent study by Swann and Siperstein (6) demonstrated that, in the guinea pig, the rate of sterol synthesis in vitro in the liver was very low relative to that in the intestine. The lung was also found to have a higher rate of sterologenesis than the liver. However, these studies with guinea pigs did not take into account either the different rates of sterol synthesis in various regions of the intestine which occur in the rat, squirrel monkey, and man (1,2,4) or the diurnal variations in sterologenic activity which have been demonstrated in rat liver (7,8) and intestine (9).

In this paper we report the relative rates of sterol synthesis in the liver, ileum, and lung of the guinea pig as measured in vitro at regular intervals throughout the day. We also describe the relative rates of sterol synthesis in various regions of the gastrointestinal tract and in other tissues of the guinea pig. The results of comparative studies on sterologenesis in the liver and ileum of the rat are included.

MATERIALS AND METHODS

Source and Housing of Animals

All animals were bred in the Animal Breeding Establishment of the Australian National University. Adult male and female guinea pigs of an outbred albino strain and male outbred albino Wistar-derived rats were used. At the time of study, the guinea pigs were aged from 10 to 26 weeks while the rats were aged ca. 10 weeks. The guinea pigs were maintained in groups of from 3 to 5 under controlled lighting with equal periods of light and dark for at least 18 days before the experiments were carried out. Their diet and drinking water were available ad libitum. The composition of the diet, which contained no added cholesterol, was identical to that used in our earlier studies with rabbits (10). The drinking water contained 0.010% w/v L-ascorbic acid (pro analysi grade,

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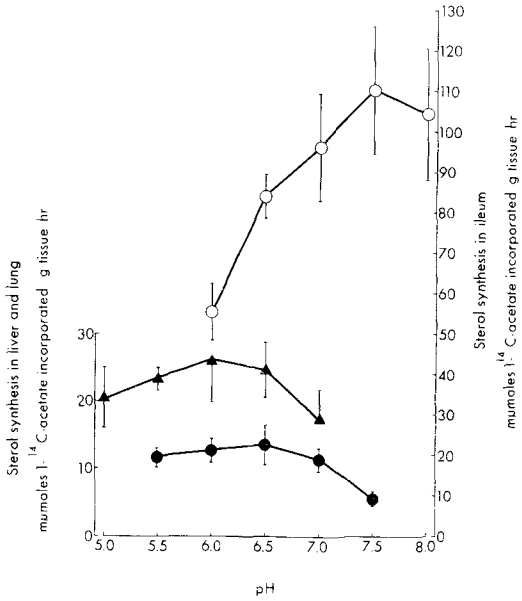

FIG. 1. Effect of pH on incorporation of 1-^{14}C-acetate into total digitonin-precipitable sterols by slices of guinea pig liver (●——●), ileum (○——○), and lung (▲——▲). Tissue slices were incubated in triplicate in Krebs-Ringer phosphate buffer containing 1-^{14}C-acetate as described in Materials and Methods. Values are the mean ± SE of determinations on tissues from three animals. Studies were made 3.5-5 hr after commencement of the dark period.

E. Merck, 61 Darmstadt, West Germany). All other conditions of maintenance were as described previously (10,11). The rats were maintained in groups of five under conditions similar to those described previously (10), except that they were under a natural lighting cycle in which the period of darkness lasted ca. 10 hr.

Sampling of Tissues and Preparation of Tissue Slices

Animals were killed by stunning and then exsanguinated. All guinea pigs were killed between 3.5 and 5 hr after the commencement of the dark period, except in the study on diurnal variations in sterol synthesis, in which groups of animals were killed at 6-hourly intervals. In the study with rats, the animals were killed at midnight and midday. The tissues were quickly excised and placed in ice-cold 0.9% saline. The gallbladders of guinea pigs were removed from the liver after its excision. All regions of the gastrointestinal tract, after being freed from surrounding mesentery, were opened longitudinally and washed thoroughly in ice-cold saline. Skin was obtained from the backs of guinea pigs after the hair had been removed. Tissue slices were prepared freehand with a razor blade to a thickness of ca. 0.8-1.0 mm. The adrenals were not sliced.

Assay of Sterol Synthesis

The assay of sterol synthesis was carried out essentially as described by Sabine et al. (12). Tissue slices (200 mg) were incubated in triplicate in Krebs-Ringer phosphate buffer containing sodium 1-^{14}C-acetate (100 μCi/mmol; The Radiochemical Centre, Amersham, Bucks, England). In the experiments with guinea pigs, the liver was incubated at pH 6.5 and the lung at pH 6.0, both in buffer containing 10 mM acetate, while the ileum and all other tissues were incubated at pH 7.5 in buffer containing 5 mM acetate. Rat liver and ileum were incubated at pH 7.4 in the presence of 10 mM acetate. All incubations were carried out in an atmosphere of oxygen at 37 C for 2 hr. In all tissues, incorporation of acetate into sterol was linear over this time period. During the incubation, flasks were shaken in a reciprocating water bath at a rate of 100 oscillations/min. The preparation and counting of the digitonin-precipitable sterol fractions were carried out as described by Sabine et al. (12). Radioactivity was measured in 0.5% w/v 2,5-diphenyloxazole in toluene (10 ml) using a liquid scintillation counter (Model LS 100, Beckman Instruments Inc., Palo Alto, CA 94304). Counting errors due to quenching were negligible. Sterol synthesis was expressed as mμmol of 1-^{14}C-acetate incorporated into total digitonin-precipitable sterols/g wet tissue/hr.

RESULTS

The first series of experiments was concerned with defining the optimal pH and acetate concentration of the incubation medium for the assay of sterol synthesis in the liver, ileum, and lung. Figure 1 shows the effect of pH of the incubation medium on sterol synthesis in these tissues. Although the liver showed maximum sterol synthesis at pH 6.5, there was little difference in the rates between pH 5.5 and 7.0. This differed markedly from the ileum, which showed a distinct optimum at pH 7.5, and to a lesser extent from the lung, which showed a less pronounced optimum at pH 6.0. These differences in pH optima may reflect the differences in the pH of the tissues. It has been reported that, in the guinea pig, the pH of the liver and the ileum are 6.9 and 7.4-7.6, respectively (13). Apart from the tissue differences in pH optima, there was also marked variation in the overall rates of sterologenesis, particularly between the liver and ileum. Irrespective of the pH of the incubation medium, the rate of sterol synthesis

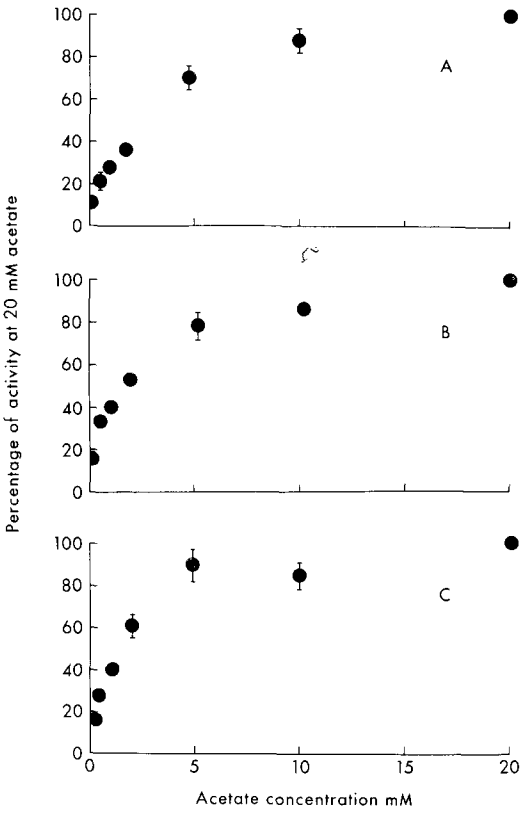

FIG. 2. Effect of acetate concentration on incorporation of 1-^{14}C-acetate into total digitonin-precipitable sterols by slices of guinea pig liver (A), lung (B), and ileum (C). Tissue slices were incubated in triplicate in Krebs-Ringer phosphate buffer containing 1-^{14}C-acetate at varying concentrations of acetate but at constant sp act. Details of the procedure are described in Materials and Methods. Values are the mean ± SE of determinations from four animals. Separate groups of animals were used for each tissue. Studies were made 3.5-5 hr after commencement of the dark period.

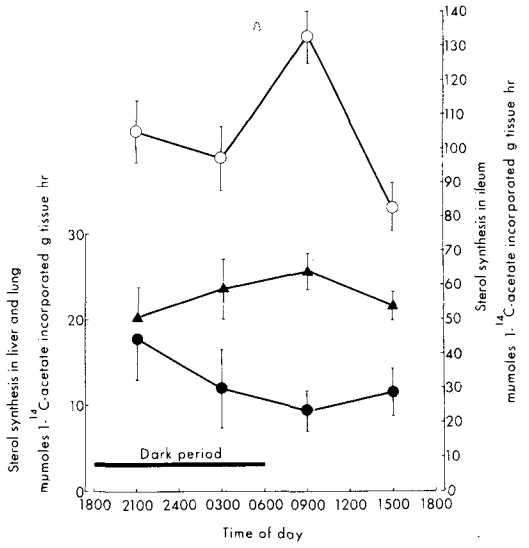

FIG. 3. Diurnal variation in incorporation of 1-^{14}C-acetate into total digitonin-precipitable sterols by slices of guinea pig liver (●——●), ileum (○——○), and lung (▲——▲). Groups of animals were killed at 6-hourly intervals and tissue slices incubated in triplicate in Krebs-Ringer phosphate buffer containing 1-^{14}C-acetate as described in Materials and Methods. At both 2100 and 0900 hr, 10 animals were used, while at 0300 and 1500 hr there were 7 animals. Values are the mean ± SE.

in the ileum was much higher than that of the liver. Activity in the lung was also higher than in the liver, but much less than that found in the ileum.

The effect of acetate concentration on the rate of sterol synthesis in the liver, ileum, and lung was investigated by varying the acetate concentration of the incubation medium over the range 0.2-20 mM while simultaneously maintaining a constant sp act of the labeled substrate (Fig. 2). Although the absolute rates of sterol synthesis for each tissue varied in the same way with changes in the acetate concentration, there was marked variation between animals in the rate of synthesis at any particular concentration of acetate. This was particularly so with the liver. The rate of synthesis at each acetate concentration was, therefore, expressed as a percentage of the activity observed at 20 mM acetate. The mean absolute rates of synthesis (expressed as mμmol/g/hr) for each tissue at 20 mM acetate were: liver (65.0 ± 22.99), ileum 102.8 ± 22.65), and lung (22.3 ± 2.65). As shown in Figure 2A, 88% of the maximum sterologenic activity in the liver was obtained at 10 mM acetate. The same result was found for the lung (Fig. 2B). In the ileum (Fig. 2C), however, a similar percentate of maximum activity was observed at an acetate concentration of 5 mM.

The results of the experiments in which sterol synthesis was measured in the liver, ileum, and lung of male guinea pigs killed at 6-hourly intervals over an 18 hr period are shown in Figure 3. At all four time points, the rate of sterol synthesis in the ileum was much higher than that in the liver and lung. The lung, in turn, consistently showed a higher rate of sterologenesis than the liver. These findings agree with those of Swann and Siperstein (6). However, the extent of difference between the three tissues in their rates of sterol synthesis varied markedly with the time of day. This was particularly so with the ileum and liver. At 2100 hr, the rate of synthesis in the ileum was

TABLE I

Rates of Sterol Synthesis in Liver and Gastrointestinal Tract of the Guinea Pig[a]

	Sterol synthesis[b]					
	Liver	Stomach	Duodenum	Jejunum	Ileum	Transverse colon
Male (4)[c]	31.0 ± 12.62[d]	29.3 ± 3.28	55.7 ± 6.02	52.3 ± 5.80	114.0 ± 13.88[e]	15.3 ± 1.78
Female (6)	21.3 ± 13.85	25.3 ± 2.83	52.0 ± 8.87	41.0 ± 7.37	65.7 ± 8.03[f]	6.0 ± 0.92

[a]Determined in the same animals, 3.5-5 hr after the commencement of the dark period.

[b]Sterol synthesis is expressed as mμmol of 1-^{14}C-acetate incorporated into total digitonin-precipitable sterols/g wet tissue/hr. Tissue slices were incubated in triplicate in Krebs-Ringer phosphate buffer containing 1-^{14}C-acetate as described in Materials and Methods.

[c]Number of animals studied.

[d]Values are mean ± SE. Comparisons by Student's t-test with corresponding values for liver.

[e]$P<0.01$.

[f]$P<0.05$.

6 times that in the liver, while 12 hr later it was 14 times higher than the rate observed in the liver. A similar but less pronounced trend was found in the differences in the rates of sterologenesis for the liver and lung.

Sterol synthesis in the liver occurred at a maximal rate 3 hr after the onset of the dark period but, as the variation between animals at each time point was high, the rate did not differ significantly with the time of day. Similarly, sterologenesis in the lung did not show a significant diurnal variation. However, in the ileum, maximum sterol synthesis occurred 3 hr after the onset of the light period; this was significantly higher than the rate observed 12 hr earlier ($P<0.05$) and corresponded to the time when liver sterol synthesis was lowest.

In the rat, the diurnal rhythms of cholesterol synthesis in the liver and ileum are the same, except that the amplitude of the rhythm in the ileum is less than that in the liver (9). Such rhythmic changes in cholesterol synthesis from acetate are associated with parallel changes in the activity of β-hydroxy-β-methylglutaryl (HMG) Coenzyme A reductase (14), the rate-limiting enzyme of cholesterol synthesis. In addition, the cyclic patterns of cholesterol synthesis (9) and HMG CoA reductase activity (15) in rats are closely linked to the diurnal pattern of feeding. The differences between the rat and guinea pig in the diurnal patterns of hepatic and intestinal sterologenesis may thus be explained by the marked difference in the feeding patterns of the two species. Unlike the rat, which has only one peak of eating activity during a 24 hr cycle and consumes at least 70% of its total daily intake during the dark period (10), the guinea pig shows three periods of increased eating activity during a 24 hr cycle, although the total feed consumption in the light and dark periods is not different (11).

To quantitate sterol synthesis in the liver, ileum, and lung, differences in organ wts must be taken into account. The ileum, which was taken to be the third of the small intestine proximal to the ileocaecal junction, weighed ca. 1/6 that of the liver. From the data at the various time points in Figure 3, the total sterol synthesis in each organ, expressed as μmol/organ/24 hr, was calculated to be: liver, 9.1; lung, 2.1; and ileum, 12.8. The ileum alone, therefore, produces more sterol than does the whole liver. This contrasts greatly to the rat and particularly to the squirrel monkey, in which the liver is quantitatively a much more important site of sterologenesis than the ileum (1,2). Although total sterol synthesis in the lung was much less than that in the liver and ileum, the lung of the guinea pig is a much more active region of sterologenesis than it is in either the rat or squirrel monkey (1,2).

Table I describes the relative rates of sterol synthesis in the liver and various regions of the gastrointestinal tract of male and female guinea pigs. In both sexes, the rates in the liver showed high variation between animals. For example, of the six females studied, five showed a rate of hepatic sterol synthesis which was < 15 mμmol/g/hr while, in one of the females, the rate in the liver was 90 mμmol/g/hr. An analysis of variance confirmed that the variation between animals was greater than that between the replicates for each animal (females, $P < 0.005$; males $P < 0.025$). As the guinea pigs were killed at the time in the lighting cycle when the rate of sterol synthesis in the liver was maximal and that in the ileum near minimal, the rates for the various regions of gastroin-

testinal tract probably represent an underestimation of their average daily rate of sterol synthesis relative to that in the liver. Despite this, all regions of the small intestine, particularly the ileum, exhibited higher rates of sterol synthesis than the liver. The stomach was found to synthesize sterol at rates comparable to those of the liver. However, the wt of the liver was, on average, 8 times that of the stomach, making the latter quantitatively less important. In both males and females, the rate of sterol synthesis in the transverse colon was much less than that in the other regions of the gastrointestinal tract examined and also less than that in the liver. It is clear, however, from the data given in Figure 3 and in Table I, that in the guinea pig the gastrointestinal tract makes a much greater contribution to total body sterol synthesis than does the liver.

To examine the possibility that the higher intestinal rates of sterol synthesis in the guinea pig may have been the result of the assay conditions applied, an additional experiment was carried out to measure sterol synthesis in rat tissues using a similar procedure to that described for the guinea pigs. Two groups of rats which had been maintained under natural lighting conditions were killed at midnight and midday, respectively, and sterol synthesis measured in the liver and ileum. These time points were selected because they are known to represent the high and low points, respectively, in the diurnal rhythm of cholesterol synthesis in rat liver and ileum (9). The results of this experiment are described in Table II. At both midnight and midday, the rate of hepatic sterol synthesis was higher than that in the ileum, the difference being significant for the midnight values ($P<0.01$). Both tissues showed their highest rates of synthesis at midnight. In the liver, there was a 3.8-fold increase in sterol synthesis at midnight, while in the ileum a 1.7-fold increase was observed. These results confirm those of others (9) and demonstrate that the guinea pig and rat show a genuine species difference in their relative rates of intestinal and hepatic sterologenesis.

Preliminary measurements of sterol synthesis in several other tissues of the guinea pig were also made. The observed rates in mμmol/g/hr for these tissues were: skin (2.1), thigh muscle (<0.1), epididymal adipose tissue (0.1), adrenal (27.9), and testis (9.3). These data, which are based on determinations in two or three animals, suggest that none of these tissues are quantitatively important sites of sterologenesis in the guinea pig. However, more animals would need to be studied to substantiate these results.

TABLE II

Rates of Sterol Synthesis in the Liver and Ileum of the Rat at Midnight and Midday

	Sterol synthesis[a]	
Time of day	Liver	Ileum
Midnight (5)[b]	589.8 ± 78.93[c]	143.1 ± 36.64[d]
Midday (5)	156.7 ± 33.20[d]	84.5 ± 17.21

[a]Sterol synthesis is expressed as mμmol 1-^{14}C-acetate incorporated into total digitonin-precipitable sterols/g wet tissue/hr. Tissue slices were incubated in triplicate in Krebs-Ringer phosphate buffer containing 1-^{14}C-acetate as described in Materials and Methods.

[b]Number of animals studied.

[c]Values are mean ± SE. Comparisons by Student's t-test with the midnight value for liver.

[d]$P< 0.01$.

DISCUSSION

In the studies described here, the rate of acetate incorporation into the digitonin-precipitable sterol fraction of each tissue was taken as a measure of the inherent rate of sterol synthesis of that tissue. No attempt was made to determine the possible differences between tissues in either the rate of entry of acetate into cells or the rate of acetyl CoA formation. However, it is unlikely that such effects could account for the marked differences in the rates of hepatic and intestinal sterologenesis in guinea pigs because, when similar conditions of assay were used in rats, the rate of hepatic sterol synthesis was higher than that in the intestine, this being the reverse of differences found in guinea pigs. The high variation in the rates of sterologenesis in guinea pig tissues, particularly in the liver, genuinely reflected marked individual variation between animals as there was good agreement between replicates from the same animal. High individual variation in hepatic cholesterol synthesis in the Cebus monkey has also been reported (16). Such variation is not found in the laboratory rat, possibly because of its prolonged selective breeding.

The low rate of hepatic sterol synthesis in the guinea pig appears to result from marked feedback inhibition which is probably exerted by both bile acids and cholesterol. More recent work in this laboratory has shown that the feeding to guinea pigs of cholestyramine, a resin which sequesters bile acids and thus reduces cholesterol absorption, results in a 13-fold increase in the rate of hepatic sterol synthesis but does not significantly increase the rate of intestinal sterologenesis. Under these conditions, the liver is quantitatively the most important site of sterol synthesis (Turley and West, unpublished observations).

In the rat, squirrel monkey, and man (1,2,4), sterologenesis in the liver, intestine, and various other tissues is considered a direct measure of cholesterogenesis because the principal sterol produced is cholesterol. While this also applies to sterol synthesis in the liver of the guinea pig, it cannot be applied to intestinal sterologenesis in this species because the principal sterols produced are lathosterol and 7-dehydrocholesterol (17). Thus, although the sterols synthesized in the ileum of the human and guinea pig are different, the level of sterologenic activity in the ileum greatly exceeds that in the liver in both species. This is in contrast to the relative rates of hepatic and intestinal sterologenesis in the rat and squirrel monkey (1,2). Thus, the guinea pig may often be more appropriate than the rat or squirrel monkey for studying factors involved in the control of sterologenesis. For example, it may be useful in studying the effect of the pattern of feed intake and, more particularly, the effect of dietary inadequacy of vitamin C. There are several reports on the effect of vitamin C deficiency on liver sterol synthesis (18-20), but none of these included measurements on intestinal sterologenesis. On the basis of the present study, future measurements of sterol synthesis in scorbutic guinea pigs should focus as much on the intestine as on the liver.

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Myocardial Lipids and Nucleotides of Rats Fed Olive Oil or Rapeseed Oil

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ABSTRACT

After 1 week, the level of myocardial fatty acids was 4 times greater in young rats fed high erucic rapeseed oil than in those fed olive oil. The proportion of erucic acid was 5.6% in the mitochondrial fraction, 15.1% in the microsomal fraction, and 34.8% in the floating fat fraction. This incorporation of erucic acid into triglycerides of the floating fat was evidence of esterification. The changes in the mitochondrial lipids did not alter the content of adenine nucleotides of the myocardium nor its apparent capacity to oxidize substrates.

INTRODUCTION

Cardiac phospholipids of rats fed erucic acid contain low levels of this fatty acid (1-5). Although there was agreement about an alteration in the composition of cardiac membranes, there was conflict about changes in the function of the mitochondria. Houtsmuller et al. (6) found a decreased level of oxygen consumption with substrates of the tricarboxylic acid cycle; Dow-Walsh et al. (7) observed no change in the presence of heparin but a reduced rate of oxidation without it; Kramer et al. (8) and Cheng and Pande (9) detected no change with fatty acid substrates. It was, therefore, decided to investigate the composition of myocardial organelles, the capacity of the mitochondria to utilize substrates (pyruvate plus malate), and the level of adenine nucleotides.

MATERIALS AND METHODS

Male Sprague-Dawley rats CD® obtained at weaning from Canadian Breeding Farms (St. Constant, Quebec) were randomly divided into two groups and housed in individual cages. They received ad libitum for 1 week a basal diet containing in % by wt: casein, 25; sucrose, 15; cornstarch, 30; vitamin mixture (1), 1; salt mixture (10), 3; alphacel (nonnutritive cellulose), 6; and either olive oil or rapeseed oil, 20. The latter oil contained 12.8% eicosenoic acid and 38.1% erucic acid.

In the first set of experiments, rats were fasted for 5 hr, decapitated, the thorax opened, and a portion of the ventricular tissue dropped immediately into liquid nitrogen for nucleotide determinations. The remainder of the myocardium was stored in saline at -20 C and later utilized for lipid analyses. In another set of experiments, the organelles of the myocardium were separated by differential centrifugation.

Mitochondrial and Microsomal Preparations

Cardiac mitochondria were isolated according to Tyler and Gonze (11). Care was taken to avoid contamination of the preparation with lipid. The 8,000 g supernatant was used to isolate microsomes at 105,000 g for 1 hr. The microsomal pellet was washed with the isolation medium, resuspended, and centrifuged again at 105,000 g. A floating fat layer was observed only on the first microsomal supernatant in preparations from rats fed rapeseed oil.

Analytical Procedures

The lipids were extracted by the procedure of Bligh and Dyer (12) as modified by Hanson and Olley (13). The fatty acid composition was determined by gas-liquid chromatography of the methyl esters on a Hewlett-Packard instrument, model 7620A, equipped with a 6 ft 1/8 in. outside diameter stainless steel column, packed with 10% butane diolsuccinate on

TABLE I

Principal Fatty Acids of Myocardium of Rats Fed Olive Oil or Rapeseed Oil

Fatty acid (%)	Olive oil	Rapeseed oil
	(10)[a]	(9)
16:0	12.2 ± 0.2[b]	5.0 ± 0.2
18:0	22.1 ± 0.1	6.3 ± 0.3
18:1	18.3 ± 0.7	19.5 ± 0.6
18:2(n-6)	14.4 ± 0.3	11.4 ± 0.2
20:1	0.2 ± 0.1	12.5 ± 0.4
20:4(n-6)	17.4 ± 0.4	5.2 ± 0.2
22:1		31.5 ± 0.7
22:5(n-6)	0.7 ± 0.1	0.1 ± 0.1
22:5(n-3)	1.4 ± 0.1	0.8 ± 0.1
22:6(n-3)	9.5 ± 0.2	3.4 ± 0.1
Total (mg/g)	15.4 ± 1.1	58.5 ± 5.9

[a]Number of rats in parentheses.

[b]Mean ± standard error of mean.

TABLE II

Fatty Acid Composition of Subcellular Components

	Subcellular distribution (% total fatty acids)				
	Mitochondrial		Microsomal		Floating
	OO[a]	RSO[a]	OO	RSO	RSO
16:0	9.8 ± 0.3[b]	6.3 ± 0.3	19.1 ± 0.7	11.8 ± 0.3	5.8 ± 0.1
18:0	22.5 ± 0.5	18.9 ± 0.4	31.6 ± 1.5	23.9 ± 1.2	tr
18:1	15.2 ± 0.6	13.1 ± 0.3	23.1 ± 0.5	17.8 ± 0.6	25.9 ± 1.5
18:2(n-6)	16.9 ± 0.5	20.7 ± 0.3	7.5 ± 0.8	8.0 ± 0.8	10.2 ± 0.3
20:1	0.3 ± 0.1	5.2 ± 0.2	1.1 ± 0.2	9.5 ± 0.4	15.3 ± 0.4
20:4(n-6)	20.4 ± 0.4	19.3 ± 0.4	12.2 ± 1.1	8.4 ± 1.4	1.1 ± 0.2
22:1		5.6 ± 0.5		15.1 ± 0.9	34.8 ± 0.8
22:5(n-6)	0.6 ± 0.1				0.4 ± 0.1
22:5(n-3)	1.4 ± 0.1	1.0 ± 0.2	0.3 ± 0.2	1.0 ± 0.3	0.9 ± 0.1
22:6(n-3)	10.1 ± 1.0	6.8 ± 0.7	3.0 ± 0.4	2.4 ± 0.4	1.2 ± 0.1

[a]OO = olive oil, RSO = rapeseed oil.

[b]Mean of four to six rats ± standard error of mean.

Anakrom ABS (80/90 mesh), and operated at 185 C. Methyl lignocerate was used as an internal standard for quantitation. Lipid classes were separated by thin layer chromatography (14); the fatty acid composition was determined for each by gas chromatography.

The frozen tissue was weighed, homogenized in 0.6N perchloric acid (1:5 w/v), and centrifuged. The supernatant was neutralized with 2.2M K_2HPO_4 and utilized to determine the level of adenosine triphosphate (ATP) by the procedure of Greengaard (15), and the levels of adenosine diphosphate (ADP) and adenosine monophosphate (AMP) by the procedure of Adams (16).

Mitochondrial oxidation was measured polarographically with a Clark oxygen electrode. A 3 ml reaction mixture contained 0.225M mannitol, 0.70M sucrose, 1mM ethylenediaminetetraacetic acid, K phosphate 0.01 M buffer (pH 7.2), and 0.2-0.4 mg protein.

Adenine nucleotide translocase activity was measured by the procedure of Wojtczak and Zaluska (17). The reaction mixture contained 40 mM Tris-HCl (pH 7.4), 100 mM KCl, 1.0mM $MgCl_2$, 0.5 to 3.0 mg mitochondrial protein, and 60 μM ^{14}C ADP. Data were normalized to account for varying amounts of added radioactivity.

RESULTS AND DISCUSSION

The level of myocardial fatty acids was 4 times greater in rats fed rapeseed oil than in those fed olive oil (Table I). In the fatty hearts, erucic was ca. 1/3 of the fatty acids. The composition of the original rapeseed oil also accounted for some of the other differences observed between the two groups. The C_{22}-polyenoic acids constituted a higher proportion of the cardiac fatty acid in the control group, but their dilution by the accumulated fatty acids indicated no actual loss in the rats receiving rapeseed oil.

In the mitochondria, where triglycerides were absent, the pattern of fatty acids again reflected the dietary differences (Table II). As expected, eicosenoic and erucic acids were present in rats fed rapeseed oil. The C_{22}-polyenoic acids did differ between the two dietary groups, and the total (n-6) fatty acids exceeded the (n-3) fatty acids to a greater extent with rapeseed oil than with olive oil.

The microsomal fatty acids reflected the dietary differences in fatty acids and were distinguished by 3 times the concentration of erucic acid found in the mitochondria. Lipid class separations indicated no triglycerides in the microsomal fraction of rats fed olive oil. In contrast, 15% of the fatty acids in the microsomal fraction of those fed rapeseed oil occurred as triglycerides. Floating fat was not apparent in the supernatant from rats fed olive oil but was appreciable from those fed rapeseed oil. The fatty acid composition of this fraction resembled that of the diet.

The dietary rapeseed oil had no effect on the myocardial nucleotide levels (Table III), nor was any change noted in the ability of the mitochondria to oxidize the substrates tested or in their capacity for oxidative phosphorylation (Table IV).

The characteristic lipidosis was observed in rats fed high erucic rapeseed oil for 1 week. At this time, eicosenoic and erucic acids occurred in the cardiac mitochondrial and microsomal preparations. Blomstrand and Svensson (5) observed this incorporation of erucic acid into the mitochondrial phospholipids and particularly into cardiolipin. Here, where the microsomal

TABLE III

Myocardial Nucleotide Levels[a]

Dietary fat	ATP μ mol/g	ADP μ mol/g	AMP μ mol/g
Olive oil	2.78 ± 0.15[b] (9)[c]	0.81 ± 0.07 (5)	0.20 ± 0.01 (5)
Rapeseed oil	2.70 ± 0.13 (9)	0.82 ± 0.10 (5)	0.24 ± 0.03 (5)

[a]ATP = adenosine triphosphate, ADP = adenosine diphosphate, AMP = adenosine monophosphate.

[b]Mean ± standard of mean.

[c]Number of determinations in parentheses.

TABLE IV

Cardiac Mitochondrial Oxidation

Substrates	Dietary fat: Olive oil (nmol O_2/min/mg protein)	Dietary fat: Rapeseed oil (nmol O_2/min/mg protein)
Malate[a] + pyruvate[b]	48.6 ± 3.8[d]	42.9 ± 3.0
Malate + pyruvate + ADP[c]	113.9 ± 11.5	91.4 ± 6.0
Adenine nucleotide translocase activity[e]	100	117 ± 10

[a]1.0mM

[b]5.0mM

[c]0.26mM adenosine diphosphate.

[d]Mean of six determinations ± standard error of mean.

[e]Expressed as dpm mg protein and normalized by assigning 100 to the controls.

fraction was also examined, erucic acid was more pronounced in it than in the mitochondrial preparation.

Endoplasmic reticulum has been shown to be the site of phospholipid synthesis (18). In the rats fed rapeseed oil, triglyceride synthesis seemed also to have occurred on the endoplasmic reticulum, for appreciable amounts were readily detected in the microsomal preparation. Also, the floating fat would be derived from the histologically observed droplets which would be composed of triglyceride. Their fatty acid composition was similar to that of the dietary rapeseed oil. The long chain fatty acids from the diet, therefore, must have been activated and then esterified before being stored as triglycerides. These data do not provide any information about possible changes in rates of triglyceride synthesis or lipolysis.

Although free fatty acids are a major source of energy for the myocardium, they may at high concentration uncouple oxidative phosphorylation (19,20). Furthermore, it was demonstrated that the CoA derivatives of fatty acids up to C_{18} chain lengths regulated the translocation of ADP into mitochondria (21). At the stage of lipidosis, however, the changes observed here in the mitochondrial components did not alter the translocation of ADP into the mitochondria or their capacity to oxidize substrates. This undoubtedly accounted for the lack of change in the total nucleotide content of the myocardium.

The changes in lipid components of the microsomal and floating fat fractions indicated esterification of fatty acids into triglycerides. The changes in mitochondrial lipid composition appeared not to be associated with changes in high energy phosphate metabolism.

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Fatty Acid Biosynthesis During Embryogenesis in the Amphibian *Bufo Arenarum* Hensel

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ABSTRACT

The fatty acid composition and biosynthesis of fatty acids were studied during early embryogenesis of the toad *Bufo arenarum* Hensel. The ova and stages up to the 6½ day embryo have similar fatty acid compositions, with ca. 70% unsaturated acids. The eggs and embryo were permeable to acetate and impermeable to palmitic, linoleic, and eicosa-8,11,14-trienoic acid. Labeled acetate was incorporated by the eggs into the saturated acids—lauric, myristic, palmitic, stearic, arachidic, and behenic—and into the unsaturated acids—myristoleic, palmitoleic, oleic, and eicosaenoic acids. During segmentation and gastrulation, de novo biosynthesis of fatty acids increased, desaturation to myristoleic, palmitoleic, and oleic acids was enhanced; and fatty acids were esterified to triglycerides, phosphatidyl choline, and phosphatidyl ethanolamine. The feeding embryo (11 days) changed the pattern of incorporation to less incorporation into triglycerides.

INTRODUCTION

Several aspects of the biochemistry of lipids during the development of eggs in invertebrates have been published (1,2). However, very few studies have been done on the structure and biosynthesis of lipids during early development in vertebrates. Terner et al. (3) studied the incorporation of 1-^{14}C acetate into different lipids of fish embryo. Jiamouyiannis and Din (4) and Pasternak (5) investigated the biosynthesis of gangliosides and phospholipids during development in amphibia. In the toad *Bufo arenarum* Hensel, Barassi and Bazan (6) examined the fatty acid distribution and ^{32}P incorporation into phospholipids from unfertilized oocytes up to the blastula and neurula stages, respectively. The aim of the present work was to investigate the enzymatic activity of fatty acid synthesis during the early embryogenesis of *Bufo arenarum* Hensel.

MATERIAL AND METHODS

Materials

1-^{14}C acetic acid sodium salt (56 mCi/mmol and 100% pure), 1-^{14}C palmitic acid (57 mCi/mmol, 99% radiochemically pure), and *cis*-1-^{14}C linoleic acid (56.2 mCi/mmol, 99% radiochemically pure) were purchased from The Radiochemical Centre (Amersham, England). All *cis* 1-^{14}C eicosa-8,11,14-trienoic acid (58.9 mCi/mmol, 99% pure) was provided by New England Nuclear Corp. (Boston, MA). Purities of labeled acids were confirmed by radiochromatography.

Oocytes and Embryos

Adult *Bufo arenarum* Hensel toads, captured near La Plata, were kept in a humidified container without feeding for 3-4 weeks prior to the experiments. To induce ovulation, adult females were injected in the dorsal lymphatic sacs with a freshly prepared suspension of one toad pituitary gland in amphibian Ringer solution. The ova were artificially fertilized with an homogenate of toad testes in 10% amphibian Ringer solution. The different stages of development were sampled after removal of the jelly coat by brief treatments with neutralized 2% thioglycolic acid. Development was allowed to proceed at 22 C and followed by means of a stereoscopic microscope using as a reference the morphological characteristics described by Del Conte and Sirlin (7).

In Vivo Incubation of Embryo with 1-^{14}C Acetate

Two samples of 150 eggs of *Bufo arenarum* were incubated in Warburg flasks for each development stage. Each flask contained 3 ml of 10% amphibian Ringer solution and 0.16 mM 1-^{14}C sodium acetate. They were incubated at 25 C and shaken 2 hr at 80 cycles per min. ^{14}C-CO_2 was absorbed in 20% KOH solution in the inner well of the vessel. The reaction was stopped by chloroform:methanol (2:1, v/v) addition and lipids extracted by the procedure of Folch et al. (8). Radioactivity in lipids was measured in a Packarg Tricarb Scintillation spectrometer with a counting efficiency

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TABLE I

Fatty Acid Composition in Different Stages of Embryo Development

Fatty acids	Stages of development[a]						
	1 hr Gray crescent[b]	12 hr Mid blastula	34 hr Late gastrula	44 hr Neural plate	64 hr Neural tube	4 days Muscle response	6½ days Tadpole
14:0	1.2 ± 0.2[c]	0.6 ± 0.4	2.1 ± 0.1	1 ± 0.4	1.4 ± 0.3	1.6 ± 0.3	1.4 ± 0.2
16:0	21.1 ± 0.5	20.3 ± 0.8	18.2 ± 1.0	20.8 ± 0.7	20.9 ± 0.8	24.2 ± 0.1	24.6 ± 0.1
16:1	10.8 ± 0.2	7.5 ± 1.5	9.1 ± 0.9	9.7 ± 0.3	11.1 ± 1.0	9.0 ± 1.5	11.1 ± 0.9
18:0	4.6 ± 0.4	4.7 ± 0.2	3.8 ± 1.0	4.5 ± 0.5	4.1 ± 0.8	4.7 ± 0.3	4.8 ± 0.1
18:1	27.4 ± 1.0	25.5 ± 2.0	28.5 ± 0.3	30.6 ± 0.3	28.2 ± 0.5	26.2 ± 1.0	26.2 ± 0.5
18:2	17.2 ± 0.5	14.4 ± 2.0	16.2 ± 0.5	17.3 ± 1.0	16.2 ± 0.7	16.4 ± 0.5	14.1 ± 2.0
18:3	5.6 ± 0.0	5.6 ± 0.0	5.4 ± 0.2	5.7 ± 0.5	4.9 ± 0.8	5.6 ± 0.0	5.1 ± 0.7
20:4ω6	4.0 ± 1.0	3.8 ± 1.2	3.6 ± 1.0	4.0 ± 0.8	4.0 ± 0.5	3.9 ± 1.0	3.9 ± 1.0
20:5ω3	1.2 ± 0.8	1.9 ± 0.2	1.3 ± 1.0	1.6 ± 0.8	1.9 ± 1.0	1.3 ± 0.7	1.4 ± 0.8
22:5ω3	1.4 ± 0.2	1.3 ± 0.3	1.4 ± 0.2	1.0 ± 0.5	1.6 ± 0.3	1.3 ± 0.3	1.3 ± 0.4
22:6ω3	1.1 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.3	1.4 ± 0.1	0.9 ± 0.2	1.1 ± 0.2

[a]Time after fertilization.

[b]Stages as Del Conte and Sirlin's table (7).

[c]Percent of total fatty acids. Results are the means of three experiments each analyzed by duplicate determination ± SEM. Traces of 14:1, 15:0, 20:2, 20:3, 20:4ω3, 22:4ω3, and 22:5ω6 were not included in the table.

of 84.6%. Expired CO_2 radioactivity was also measured by counting the KOH solution in the same apparatus. The lipids were esterified with 3 N HCl in methanol, and radioactivity distribution in the different fatty acids was measured by gas-liquid radiochromatography in a Pye apparatus with proportional counter (9). Fatty acids were identified by comparison with authentic samples.

Embryo Homogenization

Oocytes immediately after fertilization and embryos were homogenized in the cold in a Potter Elvehjem apparatus with a Teflon pestle. The homogenizing solution consisted of 5 mM $MgCl_2$, 1.5 mM reduced glutathione, 0.25 M sucrose, 62 mM phosphate buffer (pH 7), 0.1 M KCl, and 0.1 M ethylenediaminetetraacetic acid. Three ml of solution were used per mg of tissue.

The homogenate was used to measure fatty acid incorporation, desaturation, and elongation. Protein concentration was measured by the method of Lowry et al. (10). The homogenate was fractionated by differential centrifugation for the separation of a 105,000 x g pellet as already described (11).

Assay for Oxidative Desaturation and Elongation of Fatty Acids

Desaturation and elongation of 1-^{14}C palmitic, 1-^{14}C linoleic, and 1-^{14}C eicosa-8,11,14-trienoic acids were measured by the procedures described elsewhere (11,12). Two nmol of each labeled acid were incubated 30 min at 35 C in a total volume of 1.5 ml in the presence of 10 mg protein of total homogenate or 2 mg protein of the 105,000 x g pellet suspension. The concentration of the labeled acid was reduced compared to other similar experiments (11,12) to increase the sensitivity of the detection. The reaction was terminated by addition of 2 ml of 10% alcoholic KOH. The methyl esters of the fatty acids were prepared by the procedure described previously (9) and were analyzed in a Pye gas liquid radiochromatograph provided with a proportional counter. The distribution of radioactivity between substrate and products was calculated.

Incorporation of Labeled Fatty Acids in Lipid Classes

After incubation of 2 nmol of labeled fatty acids with 10 mg embryo homogenates for 30 min under the conditions already described, the lipids were extracted by the procedure of Folch et al. (8). Lipids were fractionated by thin layer chromatography on Silica Gel G. Chloroform: methanol:water (65:25:1, v/v) was used for phospholipids and hexane:ethyl ether:acetic acid (80:20:1, v/v) for neutral lipids separation.

TABLE II

1-^{14}C Acetate[a] Oxidation and Incorporation in Early Stages of Development of *Bufo arenarum*

	Eggs (cpm/150 embryos)	Gastrula (cpm/150 embryos)
Total incorporation	231,300	459,000
$C^{14}O_2$ eliminated	85,200	107,000
	(cpm/mg lipid)	(cpm/mg lipid)
Incorporation into lipids	143	254

[a]25.8 mCi of 1-^{14}C acetate were incubated with 150 embryos. Results are the mean of two experiments.

The spots were identified by comparison with authentic standards. The radioactivity of the separated compounds was measured by suspending the material in a liquid scintillation solution and counting in a Packard Tri-Carb Scintillation Spectrometer Model 3003.

Fatty Acid Composition

The lipids of different embryo stages were extracted with chloroform:methanol (2:1, v/v) (8) and converted to the methyl esters. They were analyzed by gas liquid chromatography in a Pye apparatus equipped with an ionization detector. The column was packed with 12% polyethylene glycol succinate on Chromosorb W (80-100 mesh). The fatty acids were identified by comparison with standards and by hydrogenation and rechromatography.

RESULTS AND DISCUSSION

Fatty Acid Composition of Different Stages of Embryo Development

Table I shows the total fatty acid composition of *Bufo arenarum* oocytes immediately after fertilization and during different stages of embryo development until the 6½ day larva (tadpole). During this period, important events take place from the developmental point of view, including segmentation, gastrulation, neurulation, and organogenesis. Besides, metabolic changes have been shown by different investigators to be produced during these periods (13,14). In the energetics of early development, it is generally accepted that carbohydrates represent the first energy-rich component to be used by the segmenting egg (15). However, lipids constitute an important energetic depot in amphibian egg yolk (16).

Because amphibian eggs constitute a semiclosed system that falls between cleidoic and non-cleidoic types (17), they use their own supply of organic substances required for early embryogenesis. Considering these arguments, it was expected that the fatty acid composition of the embryo would change during development due to fatty acid consumption and the biosynthesis of special lipids during membranogenesis. However, the results of Table I were unexpected in that no important changes in fatty acid composition were seen. The constancy of fatty acid composition may be due to the high content of reserve lipids present in amphibian egg yolk (16) that mask minor changes in fatty acid composition. Barassi and Bazan (6) have also found a similar constancy in total lipids and phospholipids from unfertilized eggs up to the stage of neurula formation.

The toad embryo contains ca. 70% unsaturated fatty acids (Table I). Ca. half of them belong to the monoethylenic fatty acids, palmitolic and oleic acids. Linoleic and α-linolenic acids, as well as higher homologs, were present in measurable amounts. In spite of the constancy of fatty acid composition during embryogenesis, the synthesis of unsaturated and saturated fatty acids during this period was studied to determine whether or not increased membranogenesis was related to increased synthesis of unsaturated fatty acids.

Incorporation of 1-^{14}C Acetate into Fatty Acids

Incubation of labeled acetate with eggs immediately after fertilization or with embryos with gastrula showed the permeability of embryonic membrane to acetate and the incorporation into the lipids (Table II). Some acetate was oxidized. However, the major amount of labeled acetate was incorporated into the lipids, and this incorporation increased from the ova to the gastrula stage.

When the distribution of labeled acetate in different fatty acids was measured (Table III), it was found that both the egg and the gastrula were able to synthesize the saturated fatty acids of 12, 14, 16, 18, and 20 carbons and also the monoethylenic acids—myristoleic, palmitoleic, and oleic. However, the egg synthesized preferentially the saturated fatty acids, including 22:0, whereas in the gastrula stage there was a

TABLE III

1-^{14}C Acetate Incorporation in *Bufo arenarum* Fatty Acids During Early Development[a]

Fatty acids	Egg (%)	27 hr Embryo (Gasrtula) (%)
12:0	2.1	2.2
14:0	1.9	4.1
14:1	2.4	7.0
16:0	23.1	28.0
16:1	9.2	29.8
18:0	35.4	4.7
18:1	9.3	15.8
20:0	3.9	1.8
20:1	2.0	----
22:0	10.7	----

[a]Labeling distribution in fatty acids after incubation of eggs and gastrula with 1-^{14}C acetate. Minor components in gastrula not identified are not included in the table. Results are the mean of two incubations.

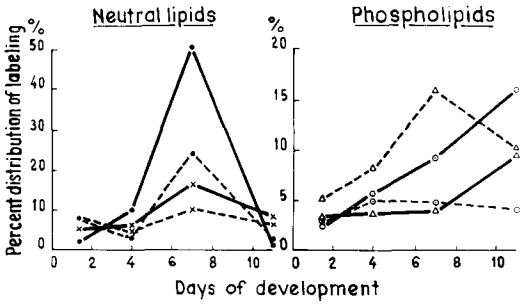

FIG. 1. Incorporation of 1-^{14}C linoleic (——) and 1-^{14}C palmitic (---) acids in triglycerides (x–x), diglycerides (●—●), phosphatidyl choline (⊙—⊙) and phosphatidyl ethanolamine (△—△) of embryos in different stages of development.

greater amount of desaturation to myristoleic, palmitoleic, and oleic acids (Table III). The increase of Δ9 desaturation with enhanced membranogenesis may be related to an increased necessity of unsaturated fatty acids for the new lipoprotein structures.

Incorporation, Desaturation, and Elongation of Labeled Palmitic, Linoleic, and Eicosa-8,11,14-trienoic Acids

Incubation of ova, gastrula, or neurula with labeled palmitic, linoleic, or eicosa-8,11,14-trienoic acids, each complexed with albumin, failed to demonstrate any incorporation of the acids into the embryo, indicating that the cells were impermeable to the fatty acids. For this reason, the homogenates of gastrula, neurula, 7 day embryo, and 11 day embryo were incubated with 1-^{14}C palmitic and 1-^{14}C linoleic acids. Eleven day embryos (tadpoles) were fed lettuce leaves ad libitum. Both palmitic and linoleic acids were incorporated into the cell lipids (Fig. 1). The distribution pattern of labeled acids in the different lipids was similar for both acids with greatest labeling in the triglycerides, less in phosphatidyl choline, and even less in phosphatidyl ethanolamine. The incorporation pattern changed with the stage of development. An increased incorporation of both palmitic and linoleic acid into triglycerides and phosphatidyl choline was found up to the 7th day. However, the feeding embryo (11 day embryo) had drastically decreased palmitic and linoleic acid labeling of triglycerides and diglycerides, whereas 1-^{14}C linoleic acid kept increasing in phosphatidyl choline. Active phospholipid and triglyceride synthesis occurred in early embryogenesis due to the necessity to build new membranes. However, feeding undoubtedly evoked great changes in the metabolic pattern of the embryo with the availability of new energy-rich compounds.

Incorporation of labeled palmitic, linoleic, and eicosa-8,11,14-trienoic acids into cell lipids demonstrates the existence of an active acyl-CoA synthetase. Some desaturation of palmitic to palmitoleic acid was found in either embryo homogenates or 105,000 x g pellet (Table IV) when 1-^{14}C palmitic acid was incubated. The conversion found for the three embryo stages studied demonstrates the existence of a Δ9 desaturase activity as early as the ovum stage. However, no Δ6 desaturation of linoleic acid to γ-linolenic acid and Δ5 desaturation of eicosa-8,11,14-trienoic acid to arachidonic acid could be measured in the ova, gastrula, or 7 day embryo.

It has been shown that the Δ9, Δ6, and Δ5 desaturations are produced in the animal by different enzymes within the endoplasmic reticulum (12,18). In spite of the activity of the Δ9 desaturase, no measurable activity of Δ6

TABLE IV

Desaturation of Labeled Palmitic Acid to Palmitoleic Acid by Cellular Fractions of Embryos in Different Stages[a]

	Eggs	27 hr Embryos	7 day Embryos
Homogenate	2.5 ± 0.5	2.2 ± 0.6	2.6 ± 0.5
Pellet of 105,000 x g	2.2 ± 0.4	2.4 ± 0.4	9.1 ± 0.4

[a]Results are expressed in percent conversion. They are the mean of three different experiments in duplicate ± SEM. Experimental conditions described in Materials and Methods.

TABLE V

Elongation of Palmitic Acid to Stearic Acid by Subcellular Fractions of Embryos in Different Stages[a]

	Eggs	27 hr Embryos	7 day Embryos
Homogenate	0.6 ± 0.2	0.9 ± 0.1	0.9 ± 0.01
Pellet of 105,000 x g	0.5 ± 0.1	0.8 ± 0.1	0.9 ± 0.1

[a]Results are expressed in percent conversion ± SEM. They are the mean of three experiments in duplicate. Experimental conditions are described in Materials and Methods.

and Δ5 desaturases was found. Whereas the Δ9 desaturase is a very early enzyme in toad embryogenesis, the Δ6 and Δ5 desaturases are transcribed and synthesized later. Different genetic control might be exerted on the Δ9 and on the Δ6 and Δ5 desaturases. However, the results obtained may also indicate a disorganization of the membranous structure during homogenization of the embryo, inasmuch as the 105,000 x g pellet did not show the typical microsomal aspect when examined by electron microscopy. If this were the case, it would mean that the Δ6 and Δ5 desaturases had been selectively destroyed, which is very improbable.

Table V shows that elongation of palmitic acid to stearic acid is also active both in ovum and embryo. However, neither the 105,000 x g fraction nor the homogenate was able to elongate linoleic acid or eicosa-8,11,14-trienoic acid. The similarity with the desaturation reaction is striking and may be explained in a similar way.

These experiments indicate that the eggs of *Bufo arenarum* Hensel possess active enzymatic systems for synthesizing fatty acids de novo, elongating palmitic to stearic acid, and desaturating saturated acids to monoethylenic acids. Very early embryogenesis is characterized by increased de novo synthesis of saturated and unsaturated fatty acids. Desaturation of myristic, palmitic, and stearic acids is particularly increased during development from ova to gastrula, a period which correlates with the period of increased membranogenesis.

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Oxidation of N-Methyl-γ-Tocopheramine to a Nitroxide[1]

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ABSTRACT

An unstable nitroxide identified by electron paramagnetic resonance spectra was obtained by oxidation of N-methyl-γ-tocopheramine with peroxide and also by ultraviolet photooxidation.

INTRODUCTION

The observation that lipid antioxidants containing secondary amino groups–dioctylamine (1), ethoxyquin (2), proline (3)–are readily oxidized to nitroxides supports the concept that this type of free radical may be the active intermediate that accounts for their antioxidant activity. The tocopheramines are analogs of the tocopherols in which an amino group replaces the hydroxyl group. Previous investigators have studied the vitamin E activities of the tocopheramines corresponding to α, β, and γ-tocopherol and of their N-methyl derivatives (4-8). α-Tocopheramine, N-methyl-β- and N-methyl-γ-tocopheramines (NMβTA and NMγTA) were approximately equal to α-tocopherol in this respect. Since NMβTA and NMγTA were unexpectedly more effective than their non-methylated parent compounds, it occurred to us that these secondary amines might be easily oxidized to nitroxides and that this property might account for their enhanced vitamin E activity. In this paper we describe some properties of the nitroxide of N-methyl-γ-tocopheramine (Fig. 1).

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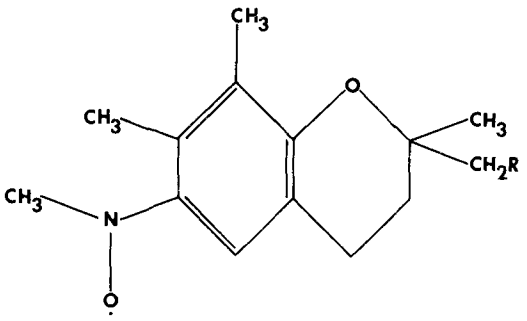

FIG. 1. Structural formula for N-methyl-γ-tocopheramine nitroxide (R=$C_{15}H_{31}$).

EXPERIMENTAL PROCEDURES

Oxidation of NMγTA

Peroxide oxidation: 0.7 ml (6 x 10^{-3} mol) 30% H_2O_2 (Mallinckrodt, St. Louis, MO) and 30 mg sodium tungstate (Mallinckrodt) were dissolved in 10 ml 95% ethanol and added dropwise with stirring to 0.25 g (6 x 10^{-4} mol) NMγTA in 10 ml 95% ethanol over a 10 min period. The solution was stirred an additional 30 min. Portions were then subjected to thin layer chromatography (TLC) and electron paramagnetic resonance (EPR) spectroscopy.

Ultraviolet (UV) oxidation: 0.1 g NMγTA were dissolved in 1 ml chloroform or methylene chloride (Mallinckrodt, nanograde) and bubbled with purified N_2 to remove excess O_2. An aliquot was placed in a quartz EPR flat cell and irradiated in the spectrometer cavity with a high pressure mercury lamp (Oriel Universal Arc Lamp, Oriel Optics Corp., Stamford, CT).

UV oxidation of NMγTA was also successful in the following solvents: ethanol (95%), heptane (Mallinckrodt, spectAR grade), and iso-octane (Eastman, Rochester, NY; practical grade, redistilled before use).

TLC plates were prepared with SilicAR TLC-7GF (Mallinckrodt). The plates were allowed to develop (benzene:EtOH [95:5]) in saturated tanks at room temperature to within 2 cm of the top of the plate, then dried under N_2.

Spots were visualized by UV light at 365 nm and 254 nm (Chromato-VUE, Model cc-20, Ultra Violet Products, San Gabriel, CA). Scrapings were dissolved in chloroform and centrifuged for EPR examination. Spots were also developed with *aqua regia* spray, in which case they appeared in ca. 10 min at 110 C or in several hours at room temperature.

EPR spectra were obtained with a Varian E-3 EPR Spectrometer, equipped with a Varian Variable Temperature Controller and multipurpose EPR cavity. Infrared (IR) spectra were obtained with a Perkin Elmer Model 137 Infracord Spectrometer with a thin film of the sample between sodium chloride discs. A Cary Model 15 was used for UV and visible spectra (solutions in 95% ethanol).

RESULTS AND DISCUSSION

The EPR spectrum obtained from photo-oxidation of NMγTA is shown in Figure 2. The exact same spectrum was seen with peroxide oxidation (not shown). The six line spectrum has a g-value of 2.0063 and can be interpreted in terms of hyperfine splittings from nitrogen with a coupling constant a_N = 12.55 G and from the N-methyl group with a coupling constant a_{CH3} = 10.75 G. A computer simulation, assuming a gaussian line shape and a line width of 3.8 G, confirmed these assignments. The g-value and the nitrogen and methyl coupling constants are in the range found for arylalkylnitroxides (9). The line width and shape of the hyperfine component suggests the presence of unresolved structure. Under conditions of higher resolution, the first and last peaks of the sextet pattern each exhibited at least 15 lines with a separation of ca. 0.5 G (10). These splittings are believed to be due to coupling from the ortho hydrogen and ortho methyl group protons and from protons of the meta methyl group. Incomplete resolution and the complexity of the spectrum, however, did not allow unambiguous assignments to be made. Similar spectra have been observed for ortho, meta, and para substituted aryl-t-butyl nitroxides by Calder and Forrester (11).

UV irradiation of NMγTA in $CHCl_3$ or CH_2Cl_2 at room temperature produced a nitroxide signal within seconds. In 95% ethanol, heptane, or iso-octane, development of the signal took minutes, but the signals were identical to those obtained from peroxide oxidation, or from the $CHCl_3$ or CH_2Cl_2 solutions. The rates of formation in the chlorinated solvents were faster, presumably because the solvents themselves produced very short-lived radicals (in comparison to the nitroxides) that abstracted the amine proton as the first step in nitroxide formation.

When TLC was performed on the irradiated mixtures or on the peroxide oxidation product, two fractions were separable: one bright yellow (R_f = 0.79), identified as unchanged NMγTA, and the other bright red (R_f = 0.02). Neither fraction had an EPR signal. When the substance responsible for the top yellow spot was irradiated again, more nitroxide free radical was produced. Irradiation of the material responsible for the red spot gave no EPR signal. When the orange oxidized solution was chromatographed on a column (SilicAR CC-7, benzene: EtOH [98:2]), ca. 50% by wt of the original material was recovered.

The relative stability of the NMγTA nitroxide was determined at several temperatures. A semilogarithmic plot of relative peak amplitude vs. time at three different temperatures (Fig. 3) shows the decay to be first order. The activation energy for radical decay obtained from an Arrhenius plot was 2.30 Kcal mol^{-1}, compared to 26.4 Kcal mol^{-1} for the more stable proline nitroxide (12).

The nonradical red fraction obtained by

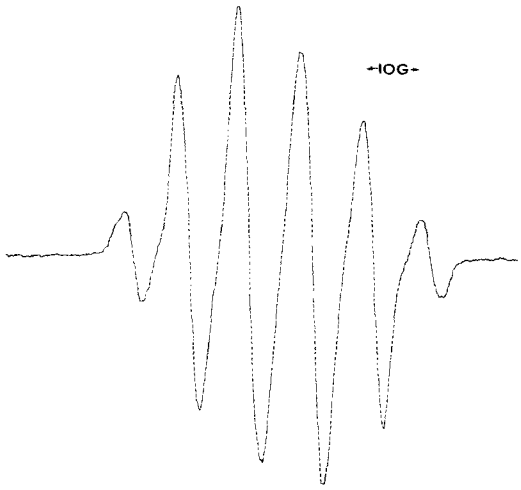

FIG. 2. Electron paramagnetic resonance spectrum of N-methyl-γ-tocopheramine nitroxide obtained by UV irradiation.

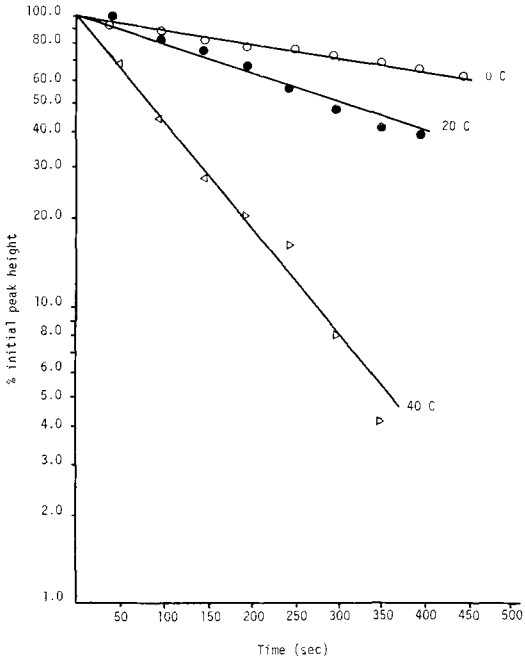

FIG. 3. Effect of temperature on rate of decomposition of N-methyl-γ-tocopheramine nitroxide.

oxidation of NMγTA was isolated and partially characterized. It was expected that it might be a nitrone (13,14), but the analytical and IR and UV spectral data (10) were not readily interpretable. Further work will be required for its identification.

Evidence for an N-hydroxy compound was not found, although such a substance has been proposed as an end product in nitroxide decomposition (13). However, in the oxidation of substituted t-butylarylamines, Calder and Forrester (15) reported that, when the nitroxide decomposed, the products were the regeneration of the parent amine and the formation of a quinone-nitrone.

A solution of NMγTA was acidified with several drops of formic acid, a strong reducing agent, and irradiated. The signal obtained had a complex spectrum without nitroxide characteristics (10) and was not investigated further.

With α-TA an EPR signal was noted almost immediately upon exposure to UV light (10,16). The signal was less stable than that obtained from the NMγTA nitroxide (10).

Assays for antioxidant activity in squalene (17) and methyl linoleate (10) indicated that NMγTA was more effective than γ-TA and less effective than α-tocopherol. Bieri and Prival (7) reported that, in methyl linolenate, NMβTA was equal to α-tocopherol and considerably more effective than β-TA.

The vitamin E activity in the TAs may be due to several reasons: of primary importance may be the ability to form nitroxide free radicals in vivo as an antioxidant in a manner similar to that proposed for the tocopherols. The fact that secondary amines oxidize quite easily to nitroxides may account for the fact that the N-methyl derivatives of β- and γ-TA are biologically more active than the corresponding free amines. α-TA is active even as a primary amine both as vitamin and as antioxidant. However, its N-methyl derivative might be expected to be more active than α-TA, but it is not. This theory also offers no explanation why N-ethyl derivatives were much less active. The dimethyl derivative has neither antioxidant nor vitamin activity. Whether free radical intermediates either of TAs or of the tocopherols themselves (16,18) are involved in their biological activities remain to be investigated.

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Some Nutrient Interrelations During Total Intravenous Alimentation in Adult Man—A Review[1]

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ABSTRACT

Selected aspects of total parenteral nutrition in man are reviewed with emphasis on carbohydrate, lipid, and hormonal interactions. In an experiment to test for the essentiality of a specific amino acid, a normal adult man received all major and minor nutrients, except lipids, by intravenous alimentation for 48 days. A chemical deficiency of essential fatty acids without clinical symptoms was promptly observed in four plasma lipid fractions and the phospholipid fraction of erythrocytes. With the glucose providing 2,600 kcal/day, blood glucose remained normal, but a hypertriglyceridemia was observed. Plasma insulin rose and cortisol fell. To meet the essential fatty acid and energy requirements of man, critical experiments are needed to define the safe utilization of lipid emulsions during prolonged intravenous nutrition.

INTRODUCTION

In 1656 the famous architect Sir Christopher Wren initiated intravenous (I.V.) alimentation by using a goose quill to introduce ale, wine, and opium from a pig's bladder into dog veins (1). Marked progress in total parenteral nutrition has been made subsequently, particularly in the last decade. (With the rapid growth in knowledge [numerous research papers and 12 books] at the time of writing, some areas cited in the text are presented in abbreviated form using citation of reviews.) Some of the major research milestones that led to this modern development are depicted in Figure 1. With respect to proteins, Rose (2) discovered threonine in 1934, identified the qualitative essential amino acid requirements of the weanling rat and then adult man, and finally ascertained the quantitative requirements of the essential amino acids for adult man. The nature of amino acid balance-imbalance, essential:nonessential amino acid ratio, calorie-nitrogen relation and other considerations, as delineated by Harper (3) and others (4-6), have been crucial for the development of I.V. alimentation. From many isolated experiments and fragments of knowledge, Munro (in 7-9) has brought coherence to help understand the overall relationships for complete, long-term I.V. nutrition.

Renner (10), Renner and Elcombe (11,12), and Goldberg (13) have shown that carbohydrates under specific conditions have an "essential role" in addition to being a fuel source. Burr and Burr recognized the essential fatty acids (EFA) during 1929-32 for the weanling rat. Later, Hansen and Holman and associates found that the human infant needed a source of EFA. These studies have been reviewed (14,15). The key investigations of Geyer (16—a good review of earlier historical developments; 4,9), Wretlind (in 4,17), and others were crucial for the modern era of I.V. lipid emulsions.

Until recently, clinicians had to rely on peripheral veins for I.V. infusions. The surgical research team of Rhoads, Dudrick, Wilmore, and Vars (18,19) opened a new era in the late 1960s with reliance on central I.V. administra-

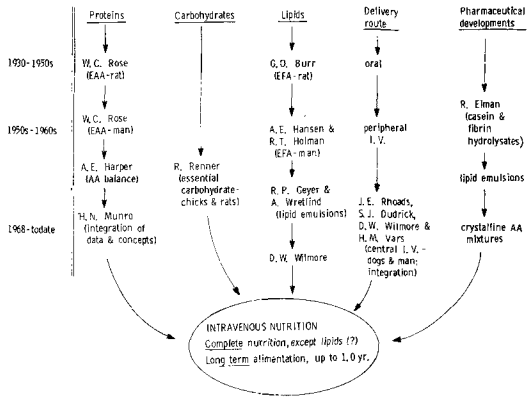

FIG. 1. Past research highlights leading to present clinical total parenteral alimentation. Other research contributions on vitamins, electrolytes, and trace elements have also been incorporated in present clinical practice. EAA = essential amino acids, EFA = essential fatty acids.

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TABLE I

Indications for Parenteral Alimentation
(i.e., "Life Without an Intestine")

Patients who:	Example
Cannot eat:	intestinal obstruction, or anomalies chronic vomiting, or diarrhea malabsorption, etc.
Should not eat:	pre- and/or post-GI operations regional enteritis ulcer complications, etc.
Cannot eat enough:	pre- and/or post-operations for malnourished patients short bowel syndrome burns and/or trauma, etc.
Will not eat enough:	adjunctive radio- or chemotherapy of cancer nonterminal coma anorexia nervosa, etc.
Have *contraindications:*	impaired renal function severe liver failure, etc.

tion of complete nutrient solutions and the successful correlation of basic experimental knowledge with the practical solution of pressing clinical problems. Their investigations produced the experimental model of litter-mate, male, beagle puppies. Puppies supplied nutrients by the superior vena cava grew slightly faster than those fed orally for 72-256 days. X-ray examinations of bone development and many biochemical measurements were found to be normal.

The pioneering investigations of Elman (20) in the late 1930s and '40s on protein hydrolyzates contributed also to the advent of modern I.V. alimentation. Subsequent research developments by pharmaceutical companies made possible the widespread clinical use of casein and fibrin hydrolyzates; later lipid emulsions were developed and then, more recently, crystalline amino acid mixtures. The earlier problems, developments, and contributions of other investigators in I.V. nutrition have been reviewed elsewhere in detail (6,8,9,16,17).

This review will concentrate on the investigations of I.V. nutrients primarily in man and indicate some of the progress, problems, and deficits in information.

TOTAL PARENTERAL NUTRITION

After the I.V. experiment on beagles (18), Dudrick et al. (19) rapidly turned their attention to the human. For the adult patient, the sterile catheter is inserted aseptically into the subclavian vein and directed into the superior vena cava of the patient or human volunteer. For the human infant, the catheter is usually delivered through a skin tunnel under the scalp to the external jugular vein and then to the superior vena cava (21). Sterile I.V. nutrient solutions are delivered by gravity or roller pump to this central vein. A cellulose membrane (Millipore filter, 0.22 μ) is usually used to remove possible microbes or solids. This delivery route leads to the rapid mixing of hyperosmolar solutions and avoids the thrombosis associated with administration by peripheral vein.

The gastrointestinal tract is bypassed in parenteral nutrition; nutrients are provided to the liver mainly via the hepatic artery rather than the usual portal vein. The term "life without an intestine" has already appeared several times in the scientific literature. To balance the possible risks, the clinical indications for parenteral alimentation must be clear cut and can be divided into four main categories of patients (Table I); similar and longer lists of clinical indications are reported in other reviews (4,7,17,22). One infant in our hospital received I.V. nutrients for 4 months during three surgical interventions to correct congenital atresia of the intestine. Patients needing gastrointestinal operations are now receiving pre- and/or post-operative alimentation. Patients with various gastrointestinal tract problems may require the removal of sections of the large and/or small intestine, leading to a condition called the "short bowel syndrome"; with limited absorptive capacity, nutrients may be supplied by vein. The requirement of severely burned patients for 5,000-8,000 kcal/day and 3-4 times

the nitrogen intake of the normal human indicates again the need for I.V. nutrition when oral feeding or tube feeding is impossible or inadequate.

One contraindication for I.V. alimentation is irreversibly impaired liver function. Patients with renal failure must be watched carefully but can benefit from an I.V. infusion of solely the essential amino acids and reduction of the nonessential amino acids (5,8,9,22).

Patients on I.V. alimentation must be followed carefully. Identification of the possible risks, such as cannula problems, infection-pyrogenic reactions, hyperosmolarity leading to dehydration and coma, electrolyte imbalance(s), and nutrient deficiency(s), facilitates clinical alertness for their prevention and enhances the utility of this method of treatment (7,17,23-26).

LIPID EMULSIONS IN TOTAL PARENTERAL NUTRITION

The osmolality of plasma (290 mosm/kg H_2O) serves as a reference point for the I.V. use of lipid emulsions. Ten-, 20-, and 30% glucose solutions are clearly hypertonic (523, 1,250, and 2,100 mosm/kg H_2O, respectively) (17) and lead to peripheral venous sclerosis. The volunteer in the experiment soon to be described received 38% glucose by central vein. On the other hand, the 10 or 20% lipid emulsions provide osmolalities of 280 and 330, respectively, and therefore higher caloric densities. If the Food and Drug Administration allowed utilization of lipid emulsions, it would be possible to reduce the hyperosmolarity and/or volume to be delivered to burn patients or other hypermetabolic patients. To restate this, one liter of 10% glucose solution provides 400 kcal, but one liter of 20% lipid emulsion solution with some carbohydrate for isotonicity will provide over 2,000 kcal (26). Administration by the peripheral vein would also be possible with lipid emulsions; clinical trials are now in progress in several laboratories.

In the 1950s and early 1960s, a cottonseed oil emulsion was used in this country but due to complications was withdrawn by FDA (7). In contrast, European clinicians have a choice of several emulsions to use clinically when justified. These emulsions are composed of 10-20% cottonseed or soybean oil, 0.75-2.0% soybean or egg yolk phosphatides, and one of several carbohydrates to maintain isotonicity. The research of Wretlind, Hallberg, and colleagues in Sweden (cf. 4,6,17) on a soybean oil-egg phospholipid emulsion has led to its large scale pharmaceutical preparation and wide use in Europe. Many other oils and emulsifying agents have been tested and eliminated to meet the stringent requirements for direct introduction into the human bloodstream (4). The emulsions in use are stable, sterile, have chylomicron size (0.20-0.50 μ), and are prepared by high pressure homogenization (2000-4000 psi).

Meng (in 7) has summarized many earlier research papers on the complications of the earlier, now withdrawn, cottonseed oil emulsion: anemia, decrease in blood platelets and extended coagulation time, altered liver function as measured by elevated bromsulfophthalein test and decreased prothrombin time, the symptoms which have been cryptically called the "overloading syndrome," and deposition of fat pigments in the reticuloendothelial system, such as the liver and spleen (27). By contrast, Wretlind's group reported in 1967 on a 4-year clinical trial with over 2,700 infusions of their emulsion (28-29). One patient received 15,000 g of emulsion over a 5 month period! Since 1967, thousands of patients in Scandinavia and Europe have received I.V. lipid emulsions (17,26), with only rare side effects.

After examining for years the parenteral administration of lipid emulsions, Geyer (in 4) has summarized his own research and that of others. The combined evidence shows that I.V. lipid emulsions leave the bloodstream rapidly, do not accumulate in tissues, provide a favorable body wt response, produce little lipid loss through excretion, show a respiratory quotient shift towards fat oxidation, and lead to an increase in blood ketone concentrations. Furthermore, labeled lipids are metabolized, and complete parenteral nutrition has been successful with favorable effects on nitrogen balance.

Subsequent to the earlier recognition of the requirement for EFA for the human infant, Collins et al. (30) reported in 1971 finding an EFA deficiency in two adult patients. One patient with the short bowel syndrome receiving lipid-free parenteral solutions for 100 days developed a skin rash, hypertriglyeridemia, serum EFA deficiency, and increased serum 5,8,11-eicosatrienoic acid. After several I.V. infusions of soybean fat emulsion and continuation of I.V. alimentation to 240 days, these symptoms were reversed. This patient developed a refractory anemia that was not due to a deficiency of iron, cyanocobalamin, nor folic acid; after lipid infusion, erythropoiesis returned to nearly normal. The second patient with severe malnutrition related to total colectomy had one complete plasma lipid analysis with similar findings. Anemia associated with EFA deficiency has been previously reported in beagle puppies (31; Table 2).

Paulsrud et al. (32) also reported an EFA deficiency in seven infants under 6 months of age induced by lipid-free I.V. feeding which was reversed by an oral dose of methyl linoleate (2% of daily calories). Sgoutas and Jones (33) and Richardson and Sgoutas (34) found an EFA deficiency in four malnourished adults after 6-8 weeks on lipid-free parenteral nutrition; oral safflower oil reversed the EFA deficiency. For perspective, the Food and Nutrition Board of the National Academy of Science-National Research Council recommends that the EFA requirement of man lies within 1-2% of the total caloric intake (35).

The group headed by Wilmore, Brooke Army Medical Center (Houston), has been very active in recent research with hypercatabolic, thermally injured, adult patients (36,37, and other papers). These investigators have recognized the EFA deficiency in acutely burned patients. Patients with burns covering 15-75% of the total body surface area responded to the I.V. administration of soybean lipid emulsion with an increase in the 18:2ω6 (linoleate), 20:4ω6 (arachidonate), and 22:6ω3 (decosohexanoate) in the red cell membrane phospholipids. As a control, another patient receiving a lipid-free I.V. solution with adequate calories failed to restore to normal his altered lipid pattern. Tube feeding of a lipid emulsion for one burn patient corrected the EFA deficiency in the erythrocytes. In contradistinction to the above reports with the stress of growth in infants, severe malnutrition cases, and acute hypercatabolic patients, an EFA deficiency in a normal, adult male will be reported shortly.

So far, comments have emphasized the role of I.V. lipids as a caloric and EFA source. Similar literature has been reviewed for I.V. glucose and such alternative caloric sources as fructose, sorbitol, xylitol, ethanol, etc. (4,6,9,17), and comparison of sources of amino acids (4,6,17). Other reported I.V. investigations to date have concentrated mainly on the intake of macro- and micro-nutrients, some directly related biochemical measurements, the patient's response, and supplementation for surgical and medical conditions. However, "life without an intestine" is in its infancy in research with respect to the overall interrelationships and the hormonal regulation of metabolic fuels. Considerable information obtained from the many elegant and detailed investigations of Cahill and Owen on human fasting and altered metabolic states has been applied to the I.V. subject (7-p. 45, 8-p. 20).

A SPECIFIC HUMAN IV EXPERIMENT

The emphasis will now shift to a specific I.V. experiment with the primary objective of testing for the essentiality or nonessentiality of histidine (38). In the 1950s, Rose (2) reported that an oral source of histidine was not needed for the maintenance of positive nitrogen equilibrium in some 50 normal adult human subjects for 3-4 weeks and in seven young men for 2.0-2.5 months. However, the writers of several recent reviews have questioned the conclusion that histidine is a nonessential amino acid for adult man.

To resolve the differences among these and other later reports, the present I.V. experiment (Nov. 1972-Jan. 1973) with crystalline amino acid solutions for I.V. administration was designed to test the effects of the complete omission of histidine. These amino acid solutions (providing 10.0 g N/day) were aseptically mixed with 70% sterile dextrose to provide 2,200-2,600 kcal/day, 12 vitamins, and electrolytes (38). Five trace elements were supplied in two 50 ml oral aliquots per day. The daily 2,000 ml of I.V. solution was delivered via the subclavian vein at a flow rate of 83 ml/hr to a normal healthy male subject.

The experimental plan consisted of two oral food periods (I and VII) and five intravenous periods: complete amino acid intake (Period II), minus lysine (Period III), complete (Period IV), minus histidine (Period V), and complete amino acid intake (Period VI). The 3-day lysine deficiency produced a marked negative nitrogen balance, whereas the 27-day histidine deficiency allowed continued mild positive nitrogen balance. Other facets of nitrogen metabolism have been described elsewhere (38; manuscript in preparation) and will be omitted here in order to concentrate on the lipid-carbohydrate-hormone interrelationships.

Hematological measurements indicated that total leukocyte counts were unchanged by alterations in the oral and intravenous amino acid intakes (39). After insertion of the catheter (Period II), the erythrocyte counts declined slowly to a subnormal level. The slope and direction of the linear regression line for three indicators of erythrocyte performance are shown in Table II. The negative slope of the erythrocyte count preceded the omission of histidine, continued throughout the intravenous administration (Periods II-VI), and then became positive upon return to oral food (Period VII). Similar results were observed for the hemoglobin and hematocrit (Table II). These plus other data are consistent with a mild hemolytic anemia (31,39; manuscript in preparation).

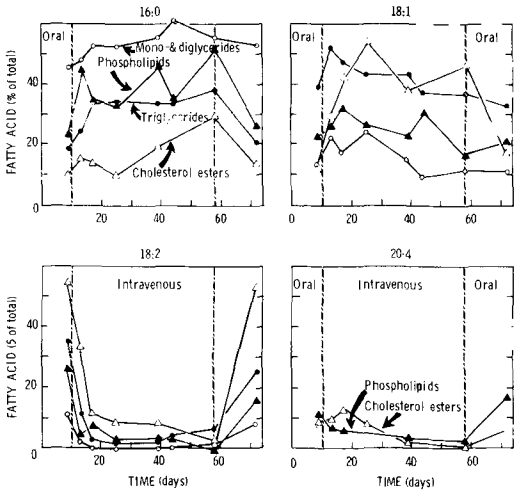

FIG. 2. Fatty acid composition of four plasma fractions. The 48 days of lipid-free I.V. alimentation, occurring between the two vertical dashed lines, was preceded and followed by a constant usual oral food intake (10.0 g N/day). Variation of the I.V. amino acid intake did not affect the lipid data here nor in subsequent figures.

BLOOD LIPIDS DURING TOTAL PARENTERAL NUTRITION

The plasma lipids were extracted with chloroform-methanol, separated into four lipid classes by silicic acid column chromatography, methylated, and analyzed by gas liquid chromatography (GLC) (39; manuscript in preparation). Palmitic acid (16:0) in all four plasma fractions increased during the intravenous period over the two control oral fractions (Fig. 2). Stearic acid (18:0) was constant (not shown) and oleic acid (18:1ω9) fluctuated. At the same time, the linoleic acid (18:2ω6) decreased rapidly in all four plasma lipid fractions by the 5-15th day. Arachidonic acid (20:4ω6) decreased in the two lipid fractions shown (cholesterol ester and phospholipids). An increase in palmitoleic acid (16:1ω9) was also observed but is not shown here. The amount of eicosatrienoic acid (20:3ω9) was negligible and was not readily measured in all the samples. Since this fatty acid usually rises in EFA deficiency (14,15,32), nutritional stress(es) such as growth, wound healing, degree and duration of deficiency, etc., may account for its appearance or absence.

The left graph of Figure 3 shows the definite decline in total plasma cholesterol during the intravenous periods, i.e., due to the absence of exogenous cholesterol intake. The total individual fatty acids in the cholesterol esters also dropped in the same time interval.

On the right graph (Fig. 3), the total plasma

TABLE II

Erythron Measurements during Oral- and Lipid-free Intravenous Intakes

Route of intake	Period in expt	Days in period	Number of determinations	Nature of amino acid intake	Slope of linear regression line		
					Erythrocyte count	Hemoglobin	Hematocrit
Oral	I	10	4	Complete	+0.33[a][1]	-0.01[a]	+0.19[a]
I.V.	II→IV	14	5	See text	- 0.20[b]	-0.05[a]	- 0.17[b]
I.V.	V	27	15	-Histidine	- 0.20[b]	-0.09[a]	- 0.26[b]
I.V.	VI	6	5	Complete	- 0.10[b]	-0.07[a]	- 0.14[b]
I.V.	II→VI	48	26	See text	- 0.17[b]	-0.07[a]	- 0.20[b]
Oral	VII	14	4	Complete	+0.45[a]	+0.06[b]	+0.52[a]

[1]Superscripts with different letters identify the slopes that are significantly different ($P<0.05$).

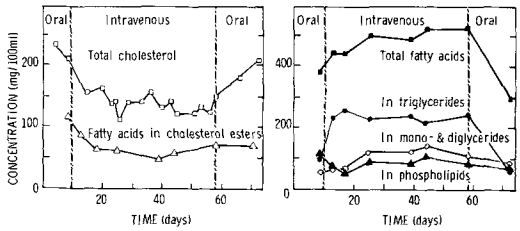

FIG. 3. Total fatty acid concentration in plasma fractions. Total cholesterol was determined separately on an autoanalyzer.

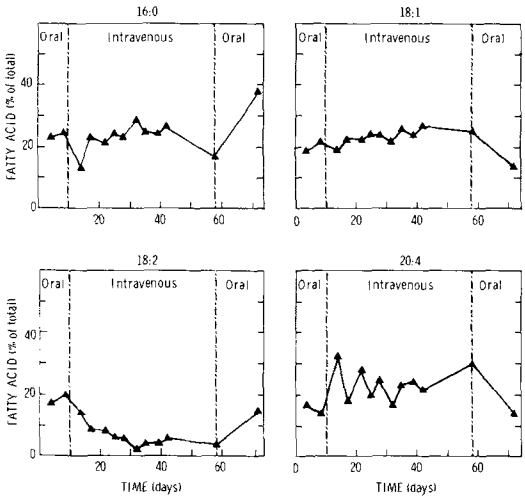

FIG. 4. Fatty acid composition of erythrocyte phospholipids.

fatty acids increased during the I.V. glucose alimentation and then declined in the final oral period. This increase was due primarily to the elevation of the plasma triglycerides. DenBesten et al. (40) compared eight men fed fat-free diets for 12 days by either nasogastric tube or I.V. alimentation. The nasogastric subjects had elevated serum triglycerides but the I.V. subjects did not. Both routes of administration lowered blood cholesterol. Serum insulin levels were higher during the I.V. feeding. While most of these findings are similar to those observed in the present experiment, one observed difference is the elevated plasma triglycerides (Fig. 3) during I.V. alimentation, a finding consistent with the hypertriglyceridemia during the oral ingestion of high carbohydrate diets (see 30 and refs. in 40). Possible clues to interpret this difference may be the length of the experiment (48 vs. their 12 days), extent of daily exercise as related to energy consumption, or other unknown factors. Hypertriglyceridemia during I.V. lipid-free alimentation was observed earlier in dogs (30). One interpretation of the hyper-

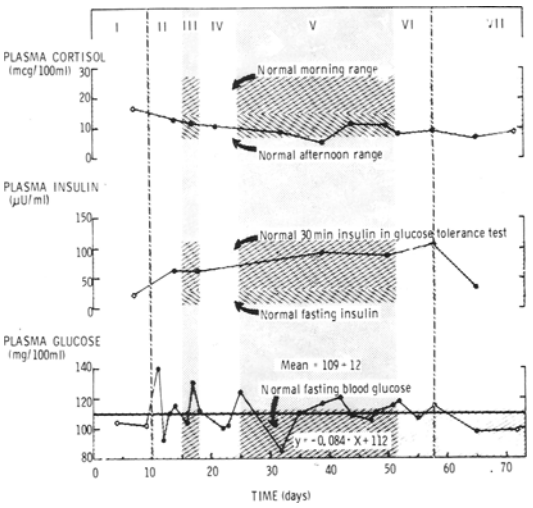

FIG. 5. Plasma cortisol, insulin, and glucose during intravenous alimentation.

triglyceridemia during a lipid-free intake may be an insulin-stimulated, hepatic lipogenesis of the very low density lipoproteins (note: 50% triglyceride concentration) and subsequent release to the plasma. These hypotheses are undergoing study but need further investigation.

The erythrocytes were washed and lysed for the isolation of the predominant lipid fraction, e.g., phospholipid. The GLC analyses showed little change in the 16:0 and 18:1ω9 fatty acids (Fig. 4). In contrast, the 18:2ω6 fatty acid declined promptly and steadily for the first 30 days of I.V. alimentation. The new data herein strengthen the need for resolving the problems involved in administration of lipid emulsions for patients (41).

HORMONAL REGULATION DURING INTRAVENOUS ALIMENTATION

The data to date suggest that the interrelationships of carbohydrate and lipids as biological fuels should be examined. Furthermore, the constant I.V. glucose flow (31.8 g/hr, 260 carbohydrate kcal/g N) is not typical with respect to the usual three oral meals per day of mixed food.

Despite the high I.V. glucose intake, blood glucose remained at a high normal concentration as compared with the post-absorptive control range shown in the bottom horizontal stripe of Figure 5. This high degree of regulation was attained in large part due to the elevated circulating insulin shown in the middle graph. These high insulin levels are of the same magnitude as those found at the 30 min glucose

peak in the human glucose tolerance test. This insulin rise is consistent with the results of Porte et al. (42), who reported that a 2-hour infusion of glucose (300 mg/min) was associated with a rise in insulin and a fall in plasma free fatty acids concentrations in man. Future studies should focus on the effect of long-term I.V. feeding on the hypersecretion of insulin and on the functional integrity of the beta cells of the Islets of Langerhans, particularly for patients with a family history of diabetes mellitus.

The top graph (Fig. 5) presents the cortisol levels collected regularly at 8:00 a.m. and indicates a downward shift during the intravenous periods to the reported afternoon range for normal man. In other words, the usual daily circadian rhythm for cortisol levels was suppressed during continuous alimentation (43). Further experiments are needed to confirm this observation.

Hormonal levels during long-term human I.V. alimentation have not been extensively investigated, although the endocrine secretions have a major role in the metabolic fuel regulatory systems (44). Exceptions which have come to our attention are the recent papers of DenBesten et al. (40) and Freeman et al. (45,46), who reported elevations in plasma free fatty acids, ketone bodies, and insulin in human volunteers receiving I.V. alimentation. The known regulatory loops (7-p. 45, 8-pp. 20 and 44) for insulin-glucose feedback, insulin-fatty acid-glucose, and insulin-amino acid-glucose have been applied to several altered metabolic states, including total parenteral nutrition. Hopefully, cortisol, glucagon, epinephrine, and growth hormone concentrations will be monitored in future I.V. investigations.

The considerable information known on the hormonal regulation of carbohydrate, lipid, and protein metabolism in laboratory animals must be correlated with that in man. Hopefully, future investigators will recognize this informational deficiency in the integrated hormonal and other controls of metabolism during long-term I.V. delivery of nutrients for humans.

To summarize, a normal human volunteer remained in positive nitrogen balance during an overall 48-day lipid-free intravenous diet; within this period, histidine was omitted 27 days. A mild anemia and an early chemical EFA deficiency were observed with this lipid-free intake. The scientific and clinical reasons for a major renewed research effort on I.V. lipid emulsions were documented. Insulin concentrations rose and cortisol fell during the continuous I.V. alimentation. Our literature review indicates an infancy in research on the integration of hormonal regulation with metabolism during human parenteral alimentation.

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NOTE: In November 1975, after submission of this manuscript, the Food and Drug Administration approved the clinical use of an intravenous soybean oil emulsion.

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Changes in Muscle Lipid Composition and Resistance Adaptation to Temperature in the Freshwater Crayfish, *Austropotamobius Pallipes*

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ABSTRACT

The phospholipid and fatty acid composition of muscle lipid extracts from crayfish acclimated to 4 C and 25 C (18 hr-light photoperiod) were analyzed. The phospholipid content and class distribution, and cholesterol content were unaffected by the acclimation treatment. Unsaturation of muscle phosphoglycerides was higher in cold acclimated crayfish. Serine/inositol phosphoglycerides from cold-acclimated animals showed somewhat higher proportions of mono- and polyunsaturated fatty acids, whereas choline and ethanolamine phosphoglycerides were less affected. This was correlated with a decreased resistance of cold-acclimated crayfish to lethal high temperature. Acclimation at 4 C under an 8 hr-light photoperiod caused an increased fatty acid unsaturation of the total phospholipid fraction compared to the 4 C, 18 hr-light photoperiod acclimated animals. The resistance of 4 C acclimated crayfish to lethal high temperature, however, was unaffected by daylength treatment. The resistance of freshwater crayfish to lethal high temperature is not simply related to the degree of saturation of the muscle phospholipids. It is suggested that a breakdown in the integrity of a bulk-lipid bilayer is not involved in the process of heat death; rather, that a membrane-bound protein factor, whose thermal sensitivity is modified by changes in its phospholipid environment during temperature adaptation but not during photoperiod adaptation, is the primary site of heat injury.

INTRODUCTION

Heilbrünn (1) first drew attention to a correlation of the resistance of various organisms to the lethal effects of high temperature with the melting points of their constituent fats. Belehrádek (2,3) later enlarged upon this and formulated the "lipoid liberation theory," which correlated the changes in lipid saturation during thermal acclimation with the resistance adaptation of various organisms. It was thought that cellular heat injury was caused by the melting of lipid constituents of the cell and that the fats formed at higher acclimation temperatures with a higher melting point would render an organism more resistant to thermal stress. Early hypotheses such as these made no distinction between depot or storage fats and membrane lipids. Indeed, they were erected without a good understanding of the structure, function, and biochemical composition of cellular membranes.

Evidence has recently been accumulating that cellular membranes as they are currently understood (4) are intimately involved in both resistance and capacity adaptation (5,6). Thus, Bowler and his colleagues (7-9) have shown that heat death in the freshwater crayfish *Austropotamobius pallipes* is accompanied by dramatic changes in haemolymph Na^+ and K^+ concentrations. This was attributed to a breakdown of the permeability barrier that separates the intra- and extracellular compartments, resulting in a decline of the ionic gradients. In addition, it has been shown (9) that several electrophysiological parameters of crayfish abdominal muscle are sensitive in vitro to exposure to temperature and time combinations that cause heat death in the intact organism. For these and other reasons, it was postulated (6) that the primary lesion of heat death occurs at the muscle membrane and that changes in the properties of this membrane during thermal acclimation may be responsible for the resistance adaptation displayed by this organism (7). Indeed, evidence has been presented (10) that the (Na^+ -K^+-) activated ATPase and the Mg^{++}-dependent ATPase of the sarcolemma are both irreversibly inactivated in vitro by exposure to temperatures that cause heat death in the whole organism. Gladwell (11) subsequently demonstrated that thermal inactivation characteristics of the Mg^{++}-dependent ATPase, but not the (Na^+ -K^+) activated ATPase, are somewhat affected by thermal acclimation of the organism.

The repeated demonstration that poikilo-

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therms possess the ability to alter the fatty acid composition of their phospholipids in response to changes in environmental temperature suggests a possible mechanism to account for the phenomena described above. Consequently, the lipid composition of the abdominal muscle from crayfish acclimated to 4 C and 25 C were examined for differences that may correlate with the altered heat death characteristics of the whole animal. In addition, the effect of different photoperiods upon the muscle lipid composition and heat death characteristics of the freshwater crayfish was studied.

EXPERIMENTAL PROCEDURES

Materials

Sphingomyelin (SPH), phosphatidyl inositol (PI), and cardiolipin were purchased from Koch-Light Laboratories (Colnbrook, Bucks, U.K.). Egg lecithin, phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), and lysolecithin were obtained from Lipid Products Ltd. (Nutfield Nurseries, South Nutfield, Nr. Redhill, Surrey, U.K.). Cholesterol and di-tert-butyl-p-cresol were purchased from Sigma Chemical Co. (St. Louis, MO). Authentic fatty acid methyl esters were purchased from Sigma Chemical Co., Supelco Inc. (Bellefonte, PA), and Applied Science Labs (State College, PA).

Water was deionized and twice-distilled, the second time from potassium permanganate in a borosilicate apparatus. Silica Gel H (type 60) for thin layer chromatography (TLC) was obtained from E. Merck (Darmstadt, West Germany) and was washed following the procedure of Parker and Peterson (12).

Treatment and Acclimation of Crayfish

Freshwater crayfish (*Austropotamobius pallipes*, Lereboullet) with a carapace length of 30-50 mm were caught locally in streams and reservoirs. They were maintained in the laboratory at 15 C for several weeks in stainless steel tanks (0.90 x 0.30 x 0.15 m) filled with clean, aerated tapwater. Animals were acclimated for at least 3 weeks prior to sacrifice at either 4±0.5 C or 25±0.1 C. (It has been shown [7] that acclimation as measured by a change in the thermal resistance was complete within 3 days at 25 C and 8 days at 8 C.) An 18 hr-light photoperiod was provided by fluorescent lamps except where indicated in the text. Crayfish were fed daily at 25 C, every 2 days at 15 C, and once every 3-4 days at 4 C, with trout pellets (Grade II pellets, Trouw Co. Ltd., Harston, Cambridge, U.K.). Crayfish undergoing ecdysis were excluded from the study.

Extraction of Muscle Lipids

The total lipid fraction was extracted from the abdominal flexor muscle of crayfish by a method modified after Folch, Lees, and Sloan-Stanley (13). Where possible, all operations were performed under an atmosphere of oxygen-free nitrogen. Muscle was thoroughly broken up in 20 vol chloroform:methanol (2:1, v/v) containing 0.005% 2,6,-ditert-butyl-p-cresol (14) using a top-drive macerator. The suspension was further homogenized using an all-glass homogenizer and allowed to stand for 30 min.

The homogenate was filtered by suction through a scintered-glass filter (medium porosity). The residue was reextracted with 10 vol chloroform:methanol (2:1, v/v) followed by chloroform:methanol (1:1, v/v) with 1.0% HCl, and chloroform:methanol (1:1, v/v) with 0.5% ammonia (15).

The pooled filtrate was dried down to ca. 80 ml in a rotary evaporator at 25 C and washed in 0.2 vol 0.79% aqueous KCl. The emulsion was allowed to settle, the organic and aqueous phases were separated by centrifugation, and the organic phase was concentrated using the solvent-replacement technique (15). Lipids in chloroform:methanol (2:1, v/v) with 0.005% di-tert-butyl-p-cresol were stored under an atmosphere of nitrogen in a sealed glass ampoule and placed in the dark at -20 C until required.

Thin Layer Chromatography

Phospholipids were fractionated using two-dimensional TLC on 200 x 200 mm chromatoplates using Silica Gel H (250 μm) slurried in 0.01 M KOH. Plates were air-dried and activated at 105 C for 1 hr before use.

Ca. 500 μg of the total lipid extract was applied using an all-glass Agla micrometer syringe (Burroughs-Wellcome Co. Ltd., London, U.K.) mounted on a Prior manipulator and fitted with a squared-tip hypodermic needle (25 gauge). Plates were developed in the first dimension with chloroform:methanol:7M ammonia (230:90:15, v/v) and dried under nitrogen for 30 min. Plates were developed in the second dimension with chloroform:acetone:methanol:glacial acetic acid:water (9:12:3:3:1.5). Neutral lipids were separated in one dimension using petroleum ether (40-60 C bp):diethyl ether:glacial acetic acid (70:30:2). Lipids were located on the chromatoplate with iodine vapor for subsequent phosphate analysis and 0.005% aqueous rhodamine 6G spray reagent (washed according to Parker and Peterson, 12) for subsequent analysis of the fatty acid composition. Phospholipids were identified

TABLE I

Comparison of the Phospholipid Composition, Total Phospholipid, and Cholesterol Content of Muscle Lipid Extracts from Cold and Warm Acclimated Crayfish

		4 C acclimated crayfish	25 C acclimated crayfish
Phospholipid composition[a]	Sphingomyelin	4.77 ± 0.22[b]	6.71 ± 0.67[b]
	Choline phospholipids	54.09 ± 1.00	54.97 ± 1.25
	Ethanolamine phospholipids	26.20 ± 1.67	22.85 ± 0.81
	Serine/inositol phospholipids	10.56 ± 0.58	10.04 ± 0.25
	Phosphatidic acid	0.50 ± 0.16	0.60 ± 0.12
	Cardiolipin	1.05 ± 0.25	1.26 ± 0.44
	Unknown	0.94 ± 0.32	1.43 ± 0.37
	Neutral lipids	0.84 ± 0.13	1.96 ± 0.63
Phospholipid:cholesterol molar ratio		2.47 ± 0.39	2.51 ± 0.29
μM Phospholipid/g wet muscle wt		7.44 ± 0.81	7.13 ± 1.54
μM Cholesterol/g wet muscle wt		3.06 ± 0.15	2.90 ± 0.55

[a]Percent of total phosphorus recovered from chromatoplate.

[b]Means ± SEM for four analyses from each acclimation group.

by comparison and co-chromatography with authentic standards and by reaction with specific spray reagents (16).

Inorganic phosphate was determined using the method of Roots (17), except that the ammonium molybdate and Fiske and Subbarow reagents were mixed prior to use. Recovery of authentic phospholipids from the chromatoplates was between 96 and 101% of an identical sample analyzed directly. Cholesterol was determined using the method of Watson (18).

Gas-Liquid Chromatography (GLC)

Phospholipid classes were fractionated as described. Each phospholipid fraction was eluted from the silica gel using an elution trap (19) with a 1 ml portion of chloroform:methanol (2:1, v/v) followed by two 0.5 ml portions. Total phospholipid fractions were prepared by removal of neutral lipids by TLC. The fatty acids of each fraction were converted to their methyl esters using 14% (w/v) boron trifluoride in methanol (20) in sealed glass ampoules (21).

Methyl esters were analyzed using a Pye Unicam 104 Gas Liquid Chromatograph fitted with flame ionization detectors, using a 1500 x 4 mm glass column filled with 10% polyethylene glycol adipate on acid-washed celite (100-120 mesh). Nitrogen carrier gas flow rate was 45 ml/min, and column temperature was 198±0.5 C.

All retention times are reported relative to methyl palmitate and were measured from the leading edge of the rising portion of the solvent front. Peaks were tentatively identified by comparison of relative retention times (RRT) with authentic standards and by the "semilog plot" and "separation factor" procedures of Ackman (22,23). These tentative identities were supported by argentation chromatography of fatty acid methyl esters on 250 μm chromatoplates impregnated with 10% (w/w) $AgNO_3$ using petroleum ether (40-60 C bp):diethyl ether:glacial acetic acid (70:30:2, v/v/v) followed by GLC analysis of the separated fractions.

Peak areas were quantitated using the method of Carroll (24) as modified by Brandt and Lands (25). The structural notation used here is that of Ackman (26). It was assumed that all olefinic bonds in unsaturated fatty acids were *cis* in structure and methylene interrupted.

RESULTS

Phospholipid Composition

Choline, ethanolamine and serine phosphoglycerides, SPH, and cholesterol were positively identified after TLC by specific spray reagents. It was not possible to separate authentic PS and PI using the solvent systems employed, and the possibility remains that PI may form part of this TLC fraction. However, GLC analysis of the trimethylchlorosilane (TMS) derivatives of the acid hydrolysis products of this fraction did not yield a peak that corresponded to the TMS derivatives of authentic meso-inositol or the acid hydrolysis products of authentic PI.

The total phosphorus and cholesterol content and the percent phospholipid composition of the lipid extract from each animal were analyzed in replicate. The values for several animals in each acclimation group were averaged, and the results are compared in Table I. Acclimation of the crayfish had little effect upon the phospholipid composition of the abdominal muscle, and only SPH comprised

TABLE II

Relative Retention Data of Fatty Acid Methyl Esters Derived from Purified Choline Phosphoglycerides[a]

Number of unsaturation bonds	Fatty acid species	Predicted RRT[b]	Fraction number I	II	III	IV	V
0	16:0	1.00	1.00				
	17:0	1.37	1.37[c]				
	18:0	1.83	1.83				
	19:0	2.46					
	20:0	3.31					
1	16:1ω9	1.14	1.14	1.14[c]			
	17:1ω9 (iso 16:0)	1.51	1.51[c]				
	18:1ω9	2.04	2.05	2.05			
	19:1ω9	2.72	2.77[c]				
	20:1ω9	3.68	3.73		3.67		
2	16:2ω6	1.36		1.42[c]			
	18:2ω6	2.44		2.46			
	20:2ω3	4.92		4.99[c]			
	20:2ω6	4.48		4.46			
	20:2ω9	4.22		4.12[c]		4.23[c]	
	22:2ω9	7.63		7.45[c]			
3	18:3ω3	3.13			3.13		
	18:3ω6	2.80			2.78[c]		
	20:3ω3	5.67			5.71		
	20:3ω6	5.02			5.01[c]		
	20:3ω9	4.78			4.82[c]		
	22:3ω6	9.15			9.00[c]		
4	16:4ω6	1.67				1.67[c]	
	18:4ω3	3.58					3.45
	18:4ω6	3.02				3.07[c]	
	20:4ω6	5.52				5.51	
	20:4ω3	6.39				6.40[c]	
	22:4ω6	10.01				10.02[c]	
5	20:5ω6	6.40					6.12[c]
	20:5ω3	7.03					7.04
	22:4ω9	9.70					9.85
	22:5ω6	11.06					11.20
	22:5ω3	12.75					12.70
6	22:6ω3	14.09					14.19

[a]Methyl esters were separated by argentation chromatography, and each fraction was analyzed by gas-liquid chromatography.

[b]Relative retention data of authentic standards, or predicted by the methods of Ackman (22,23).

[c]Minor or trace components.

significantly different proportions of the total phospholipid for the different acclimation groups (P = 0.02-0.05).

The total lipid phosphorus content and the cholesterol content proved to be somewhat variable for animals within each acclimation group (Table I), although the mean values for the two groups were not statistically different. It was concluded that the phospholipid composition, and content, and cholesterol content of crayfish abdominal muscle were not affected by thermal acclimation.

Identification of Fatty Acids

GLC retention data provides at best only a tentative identification of a chromatographic peak. In addition, the complexity of the fatty acid mixture and the possibility of peak overlap make identification by this means somewhat more difficult. In view of these uncertainties, it was considered desirable to provide further evidence for the identity of the fatty acid components of muscle phospholipids.

Thus, a purified fatty acid methyl ester sample prepared from choline phosphoglyceride was separated using argentation chromatography (Methods). Five fractions were located on the chromatoplate using the rhodamine spray reagent. Each was eluted from the silica gel and analyzed by GLC. The results are tabulated in Table II where the RRT for each peak is compared with those values obtained using the procedures of Ackman (22,23) and using authentic standards (Methods).

There was some slight contamination of each argentation fraction by minor amounts of methyl esters from adjacent fractions. For example, both Fractions I and II contained peaks that corresponded to 16:1ω9 and 18:1ω9. Nevertheless, this procedure did permit positive identification of the major (>1%) components and observation of a number of minor and trace components which would have been unnoticed in a single analysis. It was also possible to separate 20:3ω3 (RRT = 5.71, Fraction III) from 20:4ω6 (RRT = 5.51, Fraction IV), which otherwise would completely overlap. The former was present in small amounts, however, and was ignored in the quantitative analyses.

Fatty Acid Composition of Differently Acclimated Crayfish

The analysis of fatty acid composition was based upon a comparison of the total lipid extract from the pooled abdominal muscles of ten crayfish from each acclimation group. The fatty acid composition for the total phospholipid fraction and for purified choline, ethanolamine, and serine/inositol phosphoglycerides for each lipid extract are reported in Table III.

Each phospholipid class had a characteristic fatty acid composition, irrespective of its acclimation history. For example, ethanolamine phosphoglycerides possessed relatively large amounts of 22:6ω3 (6-10%). whereas the other phospholipid fractions contained 2% or less. Similarly, choline and ethanolamine phosphoglycerides had large proportions of 18:1ω9 (16-26%) and smaller proportions of 18:0 (1-7%), whereas the serine/inositol phosphoglycerides fraction had a greater proportion of 18:0 (20-28%) than 18:1ω9 (5-8%).

At the lower acclimation temperature, the total phospholipid fraction showed decreases in the proportion of the saturated fatty acids, 16:0 and 18:0, with corresponding increases in the mono- and polyunsaturated fractions. Paradoxically, the cold-acclimated crayfish muscle contained a slightly smaller proportion of 22:6ω3 than warm-acclimated crayfish muscle.

The choline phosphoglyceride fraction of cold-acclimated crayfish muscle showed minor decreases in 16:0 and 18:0 with increases in 16:1ω9 and 18:2ω6. The more polyunsaturated fatty acids were unaffected. The ethanolamine phosphoglyceride fraction exhibited reduced proportions of 16:0, 18:0, and 22:6ω3 at the lower acclimation temperature, with increased proportions of 17:1ω9 and 20:5ω3.

The serine/inositol phosphoglycerides showed the largest changes in fatty acid composition as a result of acclimation treatment, with marked increases in the proportion of the polyunsaturates 20:4ω6 and 20:5ω3 in cold-acclimated animals and a decreased proportion of 18:0. Docosahexaenoate was unaffected.

Interestingly, the acclimation treatment had qualitatively different effects upon each phospholipid fraction. Thus, the serine/inositol phosphoglycerides showed changes in the proportions of saturated and highly polyunsaturated fatty acids, while the latter fraction of the choline phosphoglycerides were relatively unaffected. In addition, the ethanolamine phosphoglycerides alone showed marked changes in the proportion of 22:6ω3.

Effect of Photoperiod on Fatty Acid Composition

Under natural conditions, the two most important seasonal changes in the physical nature of the environment are temperature and photoperiod. In all analyses described previously, crayfish were acclimated at their respective temperatures under an 18 hr-light photoperiod ("summer" daylength) in an effort to control the effect of this factor upon the acclimation response. Subsequently, it was considered desirable to know whether photoperiod had any influence upon the lipid composition of crayfish.

Three crayfish were maintained at 4±0.5 C under 8 hr-light photoperiod ("winter" daylength) conditions for 21 days, and the fatty acid composition of the total phospholipid fraction from the pooled muscle of these animals was analyzed as before (Table III). It is apparent that the total phospholipid fraction from the 4 C, 8 hr daylength acclimated crayfish was somewhat more unsaturated than the corresponding fraction from 4 C, 18 hr daylength acclimated crayfish. This was attributed mainly to a reduced proportion of 16:0 and 18:1ω9 and 18:2ω6, and increased proportions of the major polyunsaturated fractions, 20:4ω6 and 20:5ω3. Docosahexaenoate (22:6ω3) was unaffected by this treatment.

The hypothesis of a relationship between the degree of unsaturation of the muscle membranes and the thermal sensitivity of the whole organism predicts that a short-daylength acclimated crayfish should be somewhat less resistant to lethal high temperature than a corresponding long-daylength acclimated crayfish. This was tested by measuring the resistance to lethal high temperature of crayfish acclimated to 4 C and 8 hr or 18 hr-light photoperiod, since photoperiod was not a factor that was considered or controlled by earlier workers (7,9). The experimental details and results are presented in Figure 1.

TABLE III

Fatty Acid Composition of the Different Phospholipid Fractions from Crayfish Acclimated to 4 C and 25 C, 18 hr-Light Photoperiod and 4 C, 8 hr-Light Photoperiod

Observed RRT[a]	Identified fatty acid	Predicted RRT	Total lipid fraction			Choline phosphoglycerides		Ethanolamine phosphoglycerides		Serine/inositol phosphoglycerides	
			4 C,[b] 18 hr acclimated	25 C, 18 hr acclimated	4 C, 8 hr acclimated	4 C, 18 hr acclimated	25 C, 18 hr acclimated	4 C, 18 hr acclimated	25 C, 18 hr acclimated	4 C, 18 hr acclimated	25 C, 18 hr acclimated
1.00	16:0	1.00	12.91[c]	14.63	10.53	20.33	22.75	4.36	8.40	5.69	5.47
1.16	16:1ω9	1.14	4.81	4.42	4.10	7.82	6.94	3.00	2.45	0.78	1.35
1.35	17:0	1.37	0.67	0.93	0.90	0.67	0.91	d	d	2.15	2.61
1.55	17:1ω9	1.51	1.66	1.16	2.52	1.40	1.21	6.29	3.83		
1.72	17:2ω4	1.73	d	0.96				2.39	1.96		
1.83	18:0	1.82	4.84	7.05	5.08	2.37	4.32	1.47	2.57	20.87	28.79
2.06	18:1ω9	2.04	19.20	19.29	18.17	26.07	26.14	16.47	16.08	7.58	5.47
2.46	18:2ω6	2.44	4.80	3.05	3.25	6.62	3.66	3.87	3.05	0.62	d
2.80	18:3ω6	2.80	d	d	d	d	d	d	d	0.55	0.50
3.09	18:3ω3	3.13	1.28	1.13	0.90	1.74	1.29	0.89	0.82	d	d
3.43	18:4ω3	3.58	d	d	d	d	d	d	d	d	0.61
3.75	20:1ω9	3.68	1.63	1.27	1.39	1.63	1.33	1.37	1.26	1.74	1.34
4.50	20:2ω6	4.46	1.37	1.48	1.68	1.30	1.53	0.93	1.24	1.41	0.95
5.08	20:3ω6	5.02	d	d	d	d	d	d	d	0.80	0.82
5.54	20:4ω6	5.52	9.82	8.90	13.28	6.37	6.03	10.77	10.01	18.34	14.16
7.04	20:5ω3	7.04	29.00	27.18	30.56	19.60	19.49	36.72	33.53	34.90	31.30
9.83	22:4ω9	9.70	d	0.60	0.55	d	d	0.71	d		
11.27	22:5ω6	11.D6	d	d	d	d	d	0.69	0.72	d	0.64
12.89	22:5ω3	12.75	1.24	1.05	1.27	0.54	0.71	1.63	1.63	0.88	1.18
14.18	22:6ω3	14.19	3.83	4.77	3.79	2.02	1.89	6.68	9.53	1.24	1.77
Total saturated			18.42	22.61	16.51	23.37	27.98	6.01	11.35	28.71	36.87
Total monounsaturated			27.30	26.14	26.18	36.92	35.62	27.13	23.62	10.10	8.16
Total polyunsaturated			54.30	51.27	57.31	39.80	36.37	66.86	65.00	61.22	54.96

[a]RRT = relative retention time.

[b]Conditions for thermal acclimation (temperature, photoperiod).

[c]Percent of total peak area.

[d]<0.5%. Other components present in trace quantities were 16:4ω6, 18:2ω9, 20:2ω9, 20:5ω6, 22:2ω9, 22:2ω6, 22:3ω6, and 22:5ω9.

In agreement with the results of Bowler (7), crayfish acclimated to 25 C (long daylength) were markedly more resistant to lethal high temperature stress than crayfish acclimated to 4 C (long daylength). However, although a few 4 C, short daylength animals died more rapidly at 32 C than did 4 C, long daylength individuals, there was no marked difference between the overall heat death curves of the differently treated animals. The time for 50% mortality (LD_{50}) was 28 min for 4 C, short daylength crayfish and 28½ min for 4 C, long daylength crayfish.

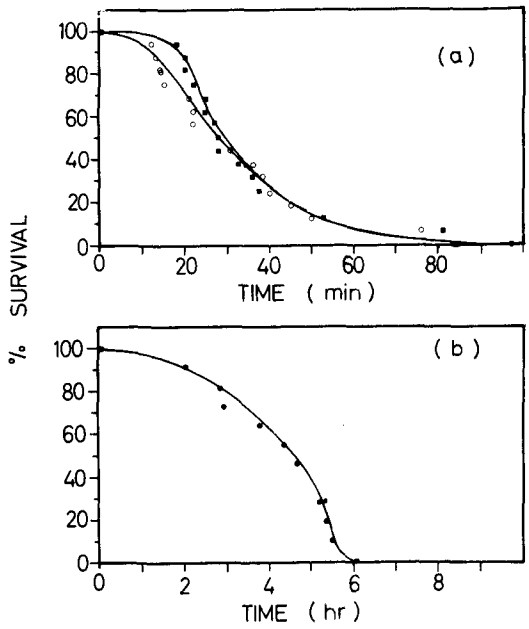

FIG. 1. Effect of thermal acclimation with 8 hr- and 18 hr-light photperiod conditions on the thermal resistance of the freshwater crayfish. Freshly trapped crayfish were acclimated under their respective conditions for 21 days. Thermal resistance was measured using the technique of Bowler (7) in clean aerated tapwater at 32 C. The results for both 4 C, 8 hr- and 18 hr-light photoperiod acclimated crayfish are shown in (a), and the results for 25 C, 18 hr-light photoperiod acclimated crayfish in (b). ○ - 4 C, 8 hr photoperiod acclimated crayfish; ■ - 4 C, 18 hr photoperiod acclimated crayfish; ● - 25 C, 18 hr photoperiod acclimated crayfish.

DISCUSSION

Thermal acclimation clearly has no major effect upon the phospholipid class distribution of crayfish muscle other than a slight elevation of the SPH content at the higher acclimation temperature. Similar results have been reported by Roots (17) and Driedzic et al. (27) on goldfish brain, Kemp and Smith (28) on goldfish intestinal membranes, Anderson (29) on subcellular fractions from various goldfish tissues, and Cullen et al. (30) on *Pseudomonas*. By contrast, Caldwell and Vernberg (31) have found an elevated PE and cardiolipin content and a reduced SPH content at lower acclimation temperatures in goldfish gill mitochondria. A similar shift in PE content of the mesophilic yeast *Candida* has been noted by Kates and Baxter (32), and a statistically insignificant increase in PE content was reported in cold-adapted goldfish brain by Roots (17).

Although the phospholipid and cholesterol content of crayfish muscle varied among individuals, it was not possible to demonstrate any consistent differences between crayfish acclimated to 4 C and those acclimated to 25 C. Similar results have been reported by Caldwell and Vernberg (31), Kemp and Smith (28), Driedzic and Roots (33), and Cullen et al. (30). In goldfish, Hoar and Cottle (34) noted that the cholesterol to phospholipid ratio varied inversely with acclimation temperature. Knipprath and Mead (35) similarly showed an increased content of neutral lipids at lower acclimation temperatures in goldfish muscle. The total lipid content of goldfish brain was found to be 18-20% less at the lower acclimation temperature (17,36). In general, it may be concluded that the acclimation response does not involve a change in the overall phospholipid content or composition of the tissue concerned but may result in an increased neutral lipid fraction at lower acclimation temperatures. This presumably reflects differences in the level of storage lipids and rates of lipogenesis (37) at different acclimation temperatures.

The differences observed in the overall fatty acid distribution of the total phospholipid fraction from the abdominal muscle of crayfish acclimated to 4 C and to 25 C were minor in extent. They consisted mainly of a smaller proportion of the saturated fatty acids in the former group, but slightly higher proportions of the mono-, di-, tri-, tetra-, and pentaenoic fatty acids. The differences in the proportions of the polyunsaturated fatty acids were clearly not mediated by changes in the choline phosphoglyceride fraction. However, both PE and serine/inositol phosphoglycerides showed increases in the proportion of the tetra- and pentaenoic fatty acids in the extract from 4 C acclimated crayfish. This was caused largely by differences in the proportions of 20:4ω6 and 20:5ω3 in serine/inositol phosphoglycerides and in 20:5ω3 in the ethanolamine phosphoglyceride fatty acids.

An interesting but paradoxical feature of

these results is the higher proportion of 22:6ω3 in the phospholipids isolated from 25 C acclimated crayfish. This was caused almost exclusively by differences in the PE fraction. This phenomenon is also apparent in the results of Knipprath and Mead (35), where an increase in 22:6 content at higher acclimation temperatures was caused almost entirely by a "cephalin" fraction. Selivonchick and Roots (personal communication) have noted marked increases in the proportion of 22:6 in PE from a number of subcellular fractions of the brain from warm-acclimated goldfish compared to cold-acclimated goldfish. By contrast, choline phosphoglycerides from the same preparations showed no appreciable change. In addition, the occurrence of significant quantities of this fatty acid in homoeotherm tissues (38,39) suggests that docosahexaenoic acid may prove an exception to the general rule that the permeability of membranes and the unsaturated fatty acid content of membranes are positively related. This is in agreement with the aberrant behavior of lecithins containing docosahexaenoic and palmitic acids, in respect of monolayer packing characteristics and liposome permeability (40).

It is concluded, therefore, that acclimation to lower temperatures at constant daylength causes an increase in the proportion of mono- and polyunsaturated fatty acids in muscle phospholipids. These changes occur mainly in the acidic phospholipids. This is in broad agreement with the results of a number of other studies (for review, 5). Kemp and Smith (28), however, noted that the fatty acids of serine and inositol phosphoglycerides were less susceptible to acclimation-dependent changes than the choline and ethanolamine phosphoglycerides. This suggests that, because of the different specialization of their component membranes, different tissues might adapt their membrane composition to temperature changes in different ways.

The adaptive properties of these described responses have usually been given in terms of capacity adaptation (41), i.e., the maintenance of the appropriate degree of 'fluidity' of the liquid-crystalline membrane that is necessary for its correct and efficient function at the environmental temperature (31,36). However, this compensatory effect has not been experimentally demonstrated for any higher poikilothermic animal, although certain prokaryotes (42-44) and protozoans (30,45) show adaptation of membrane composition and membrane properties when grown at different temperatures. These facts point to a control mechanism of membrane composition that is sensitive to the environmental temperature and/or membrane fluidity.

However, changes in phospholipid unsaturation may also prove important in those cases of resistance adaptation where membranes have been implicated. Thus, membranes from cold-acclimated organisms may show a more rapid breakdown in physical integrity than equivalent membranes from warm-acclimated organisms because of the higher proportion of unsaturated lipids. House et al. (46) made such a correlation in *Pseudosarcophaga affinis* larvae, and Esser and Souza (43) have recently suggested that the maximal growth temperature of the thermophile *Bacillus stearothermophilus* is determined by the physical properties of the membrane, which is grossly influenced by its lipid composition. In addition, Souza et al. (47) have demonstrated that a temperature-sensitive mutant of *Bacillus* has a decreased ability to modify the fatty acid composition of its membrane lipids in response to growth at different temperatures.

On the other hand, the absence of any change in resistance of freshwater crayfish to lethal high temperatures after a period of photoperiod acclimation, in spite of a marked effect upon the fatty acid composition of muscle phospholipids, indicates that the two phenomena are not invariably related, or that the relationship is not a simple one. Similar conclusions were reached by Fraenkel and Hopf (48), who found that two closely allied species of blowfly, when grown at the same temperature and possessing identical unsaturation of body lipids, had different resistances to lethal high temperatures. Ushakov (49,50) quotes work in which the lipid composition of muscle from *Rana temporaria* and *Calliphora erythrocephala* (as measured by the iodine value) was altered without affecting the thermostability of the muscle fibers. Furthermore, a decrease in the thermostability of frog muscle was not associated with a change in the nature of the lipid constituents.

The lack of correlation between changes in the resistance of crayfish to lethal high temperature and changes in muscle lipid composition during photoperiod acclimation may indicate that a breakdown in the physical integrity of the bulk-lipid bilayer of the membrane (4) is not involved in those phenomena that are associated with the process of heat death, since the lipid composition of the bulk-lipid membrane is presumably a function of the overall phospholipid composition of the tissue. It may be that the primary event leading to the breakdown of muscle permeability barriers may occur at some membrane-bound protein. Indeed, Bowler et al. (6) have suggested that

"cellular heat injury (and therefore heat death) . . .may well be related not solely to the state of either phospholipids or proteins, but rather to the stability of lipoprotein complexes." Intuitively it is possible to see how the thermal stability of a protein molecule may be influenced by the physical state of its immediate microenvironment. The presence of a distinct, physically differentiated lipid halo around certain membrane-bound enzymes has been demonstrated in a number of well-characterized systems (51,52), although it is not known whether the lipids that contribute to halo formation are chemically different from the bulk lipid or whether all membrane lipids participate in halo formation. It is possible that modification of this phospholipid halo during temperature acclimation may occur, thereby changing the thermal lability of that membrane-bound enzyme, as has been observed with the Mg^{++} ATPase of the crayfish sarcolemma (11). It must be postulated, however, that changes in lipid composition of the membrane as a result of photoperiod acclimation do not contribute to the specific environment of the temperature-sensitive protein factor and that thermal acclimation and photoperiod acclimation are quite distinct processes, involving qualitatively different effects upon the lipid composition.

The fact that the analysis of short-daylength, 4 C acclimated crayfish was performed on the muscle from only three animals indicates that the results are not as representative of the acclimation group as in earlier analyses of the lipid extracts from ten crayfish. This possibility could only be tested by repeating the analysis to ensure that the biological variation in fatty acid composition between animals is not as great as the differences between acclimation groups. In either case, it is apparent that changes in the lipid composition of membranes, organs, or whole animals which correspond to acclimation-dependent changes in the upper lethal limits of a physiological function or of an organism do not necessarily constitute a causal relationship. Other approaches are required to resolve this issue.

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Effect of Vitamin E Deficiency on the Lipid Class and Fatty Acid Composition of Rat Brain Gray and White Matter[1]

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ABSTRACT

Three-week old male Sprague-Dawley rats were placed on a control or vitamin E-deficient diet for 9 months. The total lipid and cholesterol contents of brain gray and white matter areas in the vitamin E-deficient group did not differ from controls. The concentration of cerebrosides was lower in white matter but higher in gray matter of deficient animals. However, sulfatide was significantly ($P<0.001$) higher in white and gray matter of deficient animals compared with controls. Lysolecithin was not found in vitamin E-deficient gray matter but was present in control gray matter lipids. No marked differences were found in the concentrations or relative amounts of sphingomyelin, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, or phosphatidyl inositol in the phospholipids of gray or white matter of vitamin E-deficient rats as compared to controls. In addition, no remarkable differences were found in the fatty acid composition of total lipid extracts of gray or white matter from vitamin E-deficient rats when compared with controls.

INTRODUCTION

A variety of studies indicate that vitamin E (tocopherol) may play an important role in maintaining the functional and structural integrity of the nervous system. The development of motor paralysis in the suckling young of rats maintained on a vitamin E-deficient diet was first reported by Evans and Burr in 1928 (1). Ringsted (2) later found that adult rats developed paresis after maintenance on a vitamin E-deficient diet for periods of 5-22 months. Pentschew and Schwarz (3) and Carpenter (4) have observed swollen axons in the sensory relay nuclei of the medulla and in the tracts afferent to these nuclei and in dorsal gray matter of the spinal cord from rats maintained on a vitamin E-deficient diet. Histological changes resulting from vitamin E deficiency in various laboratory animals such as rats, guinea pigs, rabbits, and monkeys have been reported by Einarson and Telford (5). In the adult rat, they found evidence for demyelination in the posterior fasciculi, and marked accumulation of lipofuscin pigment was observed in the neurons of rats fed vitamin E-deficient diets. These studies (5) suggested the possibility that vitamin E deficiency could result in brain lipid changes. Therefore, the alterations of brain lipid classes and fatty acids associated with vitamin E deficiency in rats during the period from post-weaning to adulthood were studied and the results are reported here.

EXPERIMENTAL PROCEDURES

Three-week old male Sprague-Dawley rats were fed either the control or vitamin E-deficient diet based on the formulations of Cawthorne et al. (6). The original diet was slightly modified to contain 0.2% DL-methionine (replacing sucrose), since the amount of casein in the diet of Cawthorne et al. (6) may not be adequate with respect to this amino acid for a growing rat (7). After a period of 9 months on the modified diet, groups of control and deficient rats were sacrificied by decapitation after overnight fasting. Serum total tocopherol determined fluorometrically (8) was found to be very low in the deficient animals (<0.05 mg per 100 ml compared with a mean of 0.58 mg per 100 ml for controls). The testicles from the deficient animals showed considerable histological degeneration as well as wt loss (mean wet testicular wt for controls was 1.6 g compared with 0.82 g for deficient animals). No rats on the vitamin E-deficient diet had developed signs of motor paralysis by the time of sacrifice, unlike the studies of Ringsted (2).

Samples consisting of predominantly gray

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TABLE I

Total Lipid Extracted from Normal and Vitamin E-Deficient Rat Brain Gray and White Matter

Sample	G total lipid/g wet tissue[a]	
	Control	Vitamin E-deficient
Gray matter	0.0812 ± 0.0010[b]	0.0879 ± 0.0015
White matter	0.1984 ± 0.0072	0.2012 ± 0.0040

[a]Mean of four determinations.

[b]± standard deviation.

matter were dissected out manually from the cerebral cortex and white matter from the region of the pons and medulla. The dissected samples were frozen immediately in liquid nitrogen and stored under nitrogen in tightly sealed containers. Wet tissues were weighed and the lipids extracted under nitrogen and in the presence of added antioxidant (butylated hydroxytoluene) using the sequence of solvents described by Rouser et al. (9). The total lipid extracts were made up to fixed volumes. An aliquot was removed with a Hamilton micro syringe and the solvent evaporated. The lipid content was obtained by weighing the residue to constant wt on a Cahn micro balance (9). Phospholipids were quantitated after two-dimensional thin layer chromatographic (TLC) separation (10). Cholesterol (11) and the sphingosine moiety in glycolipids (12) were measured by standard procedures. Methyl esters of fatty acids from total lipid extracts were prepared by methanolysis for 4 hr at 100 C using 14% boron trifluoride in anhydrous methanol as described by Morrison and Smith (13). Spectrograde hexane was used for extraction of methyl esters. Completeness of methanolysis and the lack of side reactions were confirmed by TLC on Silica Gel H (Applied Science Labs, State College, PA), Adsorbosil M-2 (Applied Science Labs), or Supelcosil 41A (Supelco, Bellefonte, PA) 9:1 (w/w) or Supelcosil 42A using hexane:ether:acetic acid, 8:20:1 (v/v/v). Gas chromatography was carried out on a Hewlett-Packard Model 5750 dual-column, dual flame ionization detector instrument. Separations were accomplished isothermally on 1/8 in. stainless steel columns, 6 ft in length, packed with 10% diethylene glycol succinate on 80-100 mesh Supelcoport (170 C) or 10% SE-30 on 80-100 mesh Diatoport S (220 C). Methyl esters were identified by comparison of their retention times with those of a large number of pure standards obtained from Supelco or from Applied Science Labs. Unsaturated fatty acids were also characterized by rechromatography of a portion of the sample after micro-hydrogenation. The range of detector linearity and relative mass sensitivity of the detector system were established using appropriate standards. Representative samples were rechromatographed after saponification in 1 N KOH in

TABLE II

Gray and White Matter Lipid Class Composition in Brain from Normal and Vitamin E-Deficient Rats

Lipid class[a]	Gray matter (mg lipid/100 mg total lipid)		White matter (mg lipid/100 mg total lipid)	
	Control	Vitamin E-deficient	Control	Vitamin E-deficient
SM[b]	2.98 ± 0.49[d]	2.64 ± 0.56	2.77 ± 0.46	3.09 ± 0.38
PC	23.55 ± 2.88	22.23 ± 3.11	11.58 ± 1.69	11.19 ± 1.71
PE[c]	22.28 ± 2.55	21.67 ± 2.89	16.98 ± 1.82	17.23 ± 2.32
PI	2.43 ± 0.99	2.77 ± 0.73	1.62 ± 0.78	1.81 ± 0.36
PS	7.57 ± 0.96	7.64 ± 1.07	5.99 ± 0.94	6.07 ± 0.94
LPC	0.82 ± 0.31	ND	ND	ND
PA	ND[e]	ND	1.23 ± 0.20	1.19 ± 0.80
Cholesterol	14.33 ± 1.29	15.36 ± 1.69	19.29 ± 2.31	17.07 ± 1.19
Sulfatides	1.05 ± 0.05	1.34 ± 0.08[f]	6.78 ± 0.34	8.86 ± 0.62[f]
Cerebrosides	3.94 ± 0.36	6.21 ± 0.62[f]	20.27 ± 1.82	16.69 ± 1.71[g]

[a]Each value is the mean of eight determinations.

[b]SM = sphingomyelin, PC = phosphatidyl choline, PE = phosphatidyl ethanolamine, PI = phosphatidyl inositol, PS = phosphatidyl serine, LPC = lysophosphatidyl choline, PA = phosphatidic acid.

[c]Includes diacyl-, alk-1-enyl-ether, and 1-alkylether forms.

[d]± Standard deviation.

[e]Not detected.

[f]$P<0.001$.

[g]$P<0.01$.

TABLE III

Relative Phospholipid Class Composition in Gray and White Matter from Normal and Vitamin E-Deficient Rat Brain

Phospholipid class[a]	Gray matter (% of total phospholipid)		White matter (% of total phospholipid)	
	Control	Vitamin E-deficient	Control	Vitamin E-deficient
SM[b]	4.62 ± 0.22[d]	4.38 ± 0.48	6.89 ± 0.71	7.93 ± 0.46
PC	36.66 ± 1.59	36.89 ± 1.27	28.84 ± 1.39	28.65 ± 1.73
PE[c]	34.79 ± 1.81	35.88 ± 1.92	42.46 ± 1.96	44.37 ± 2.06
PI	3.67 ± 1.01	4.54 ± 0.88	3.94 ± 1.71	4.61 ± 1.13
PS	11.76 ± 0.36	12.68 ± 0.57	14.89 ± 0.49	15.56 ± 0.76
LPC	1.17 ± 0.28	ND	ND	ND
PA	ND[e]	ND	3.04 ± 0.34	2.98 ± 1.35

[a]Each value is the mean of eight determinations.

[b]SM = sphingomyelin, PC = phosphatidyl choline, PE = phosphatidyl ethanolamine, PI = phosphatidyl inositol, PS = phosphatidyl serine, LPC = lysophosphatidyl choline, PA = phosphatidic acid.

[c]Includes diacyl-, alk-1-ether, and 1-akyl ether forms.

[d]± Standard deviation.

[e]Not detected.

TABLE IV

% by Wt of Fatty Acids in Lipid Extracts of Gray and White Matter from Normal and Vitamin E-Deficient Rat Brain

Fatty acid[a]	Gray matter		White matter	
	Control	Vitamin E-deficient	Control	Vitamin E-deficient
14:0[b]	3.76 ± 2.62[c]	2.92 ± 1.51	4.25 ± 4.76	7.98 ± 6.69
15:1	1.17 ± 0.04	1.12 ± 0.16	1.65 ± 0.12	1.62 ± 0.06
16:0	20.43 ± 4.19	20.02 ± 3.34	12.83 ± 3.82	10.88 ± 1.33
16:1	0.68 ± 0.18	0.66 ± 0.19	0.70 ± 0.32	---
17:0	1.45 ± 1.94	0.83 ± 0.84	0.56 ± 0.39	2.97 ± 3.81
17:1	2.55 ± 1.29	1.86 ± 0.31	2.95 ± 1.67	1.91 ± 0.54
18:0	19.04 ± 1.78	19.46 ± 0.72	16.68 ± 1.26	15.84 ± 1.36
18:1	16.52 ± 1.88	16.47 ± 0.28	25.16 ± 2.31	23.20 ± 2.29
18:2	2.11 ± 2.89	0.45 ± 0.11	---	---
20:0	---	---	1.37 ± 0.93	1.11 ± 0.56
18:3	---	0.39 ± 0.21	---	---
20:1	2.90 ± 0.06	2.53 ± 0.50	5.07 ± 1.37	6.09 ± 2.23
20:4	9.98 ± 0.22	10.82 ± 0.70	---	---
22:0	---	---	6.10 ± 1.46	6.15 ± 0.11
22:4	2.69 ± 1.31	3.36 ± 1.57	---	---
24:0	---	---	6.65 ± 1.70	5.28 ± 3.71
22:5	13.19 ± 6.13	15.45 ± 3.01	---	---
24:1	---	---	12.90 ± 0.83	14.25 ± 1.90
22:6	5.62 ± 1.55	6.12 ± 2.78	---	---

[a]Each value is the mean of four determinations. Fatty acids that are <1% of total in gray or white matter are excluded.

[b]Number before colon represents number of C-atoms. Number after colon represents number of double bonds.

[c]± Standard deviation.

methanol to correct for nonmethyl ester peaks. Blank samples of corresponding volumes of solvents were carried through the entire procedure to confirm the absence of artifacts. Areas under peaks were determined on a Columbia Scientific Industries (model CSI-208) integrator.

RESULTS

Table I shows that the total amount of lipid extracted from brain gray and white matter of control and vitamin E-deficient rats does not differ. The lipid class compositions of gray and white matter from both groups of rats were

determined, and the data is presented in Table II. To examine the phospholipid distribution more closely, each phospholipid class was expressed as a relative percentage of the total phospholipid, and the data thus calculated is presented in Table III. The fatty acid compositions of lipid extracts from gray and white matter of the brain from control and vitamin E-deficient rats are given in Table IV and are comparable with values reported for normal rat brain (14).

DISCUSSION

No significant differences were observed in the total lipids extracted per unit wt of either the gray or white matter in this study (Table I). However, a slight but not significant ($P>0.8$) increase in the total lipids extracted from the gray matter of vitamin E-deficient rats was noted (Table I). In contrast, Carpenter (15) has found a noticeable decrease in total lipid content per g wet wt of vitamin E-deficient rat testes. In addition, Albarracin and coworkers (16) have reported that rabbits on vitamin E-deficient diets have higher amounts of total lipid on a tissue wet wt basis compared with controls. Thus, the rat brain appears to be refractory to gross changes in total lipids induced by vitamin E deficiency during the age period studied.

Although a decrease in cerebroside concentration of white matter in vitamin E-deficient rats was observed (Table II), there was a corresponding increase in the sulfatide content of white matter. In addition, there was also a small but significant ($P<0.001$) increase in both cerebrosides and sulfatides in gray matter. It is interesting to note that decreased aryl sulfatase A activity has been reported in mouse brain homogenates associated with vitamin E deficiency (17). Increases in cerebrosides and sulfatides have also been observed in the muscles of rabbits maintained on a vitamin E-deficient diet (16). The exact mechanism of the changes is unknown at present. Einarson and Telford (5) have reported the existence of demyelination in adult rats as a result of vitamin E deficiency. Cholesterol esters typical of nervous tissue undergoing demyelination were not found in the lipid extracts, and free and total cholesterol levels were not altered in the white matter of deficient rats. Thus, the biochemical alterations observed suggest a complex pathological process.

The available information on the role of viatmin E in membrane function was summarized by Molenaar et al. (18). These authors (18) concluded that the main effects of vitamin E deficiency on cellular membranes are expressed in the lipid moiety rather than in the protein portion of the membrane. Table III shows that the relative composition of the major phospholipid components of rat brain lipid extracts is not altered significantly as a result of a vitamin E-deficient diet in either gray or white matter. However, deficient gray matter does not contain any lysophosphatidyl choline (LPC), which is present in small amounts in control gray matter lipids. This might suggest an increased acylation of LPC or a decreased LPC production in gray matter of vitamin E-deficient rats.

The intimate biological interrelationships between polyunsaturated fatty acids and vitamin E are well known (19). Therefore, the changes in fatty acid profiles of total brain lipids due to vitamin E deficiency in gray and white matter were studied. Data in Table IV show that there is no significant change in the fatty acid composition of total lipids in gray or white matter associated with vitamin E deficiency. Since the major lipids in gray matter are essentially unchanged by vitamin E deficiency, it is not surprising that the fatty acid composition of total lipid extract also remained unaltered. Although cerebrosides and sulfatides are important components of white matter lipids, the decrease in cerebrosides is countered by an increase in sulfatides, and changes in their constituent saturated fatty acids of chain length longer than C_{22} are not reflected by changes in fatty acid compositions of the total lipid extracts. The high standard deviations reflect variability between batches of animals rather than between multiple determinations within the same experiment. It is noteworthy that Witting (20) found an increase in arachidonic acid (20:4) in the total phospholipid fraction of vitamin E-deficient chick brain lipid extracts and this increase was specifically restricted to phosphatidyl serine.

Data from the present studies show that the major membrane phospholipids and the fatty acid composition of total lipids seem to enjoy a degree of protection against major alteration due to a long-term feeding of vitamin E-deficient diet to the Sprague-Dawley rat from post-weaning to 9 months of age. Subtle changes are seen in the glycosphingolipids of both gray and white matter. Essentially the same results have been obtained even when the animals were kept on the diets for as long as 12 months. It has been observed that the most active brain growth in the rat occurs at ca. 10 days after birth (21). Therefore, it is conceivable that greater changes in brain lipid composition might occur if suckling rats are raised

by vitamin E-deficient mothers and then fed the vitamin E-deficient diet. Experiments using such animals are at present in progress.

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Changes in Phospholipids and Acyl Group Composition of Rat Mammary Gland During Pregnant, Lactating, and Post-Weaning Periods

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ABSTRACT

The distribution of phospholipids as well as their fatty acid compositions of rat mammary tissues were examined during pregnant, lactating, and post-weaning periods. There was no apparent change in phospholipids and their acyl groups during the early and late pregnant periods. However, tissue phospholipid composition was different during pregnant, early, and late lactating periods. After parturition, there was a marked increase in the proportion of diacyl-glycerophosphorylcholine in the phospholipids of mammary tissue, but this proportion decreased gradually during lactation. The decrease in diacyl-glycerophosphorylcholine during lactation was marked by a corresponding increase in diacyl-glycerophosphorylethanolamine. Although the shorter chain fatty acids of triglycerides were increased during lactation, only a small proportion of these fatty acids was found in the phosphoglycerides. Marked changes in acyl group composition of individual phospholipids are found during these different physiological stages. In general, there was a rapid decrease in 20:4 and an increase in 18:2 in the major phosphoglycerides during parturition. The proportion of 20:4 in the phosphoglycerides remained low throughout the entire lactating period, while that of 18:2 continued to increase 2-3 fold. Most of the changes in acyl group of the phosphoglycerides during lactation returned to normal ca. 10 days after weaning. A possible relationship of the variation of phospholipid and acyl group compositions in mammary tissue to changes in hormonal pattern during different physiological stages is discussed.

INTRODUCTION

Lipids in the mammary tissues are characterized by a high content of triglycerides which are secreted as fat droplets in milk during lactation (1-6). It is generally known that the triglycerides from milk fat contain a large proportion of fatty acids with shorter chain length ranging from C_8-C_{14} (7-9). The synthesis of shorter chain length fatty acids by the mammary gland during lactation seems to vary among different animal species.

In the past, hormonal regulation of fatty acid synthesis in the mammary tissue during lactation has been the subject of some investigations (10-15). However, relatively little information has been obtained on the fatty acids of phosphoglycerides in mammary tissue (16). Since phosphoglycerides are important constituents of cellular and subcellular organelles, possible association of the shorter chain fatty acids with tissue phosphoglycerides during lactation and the hormonal regulation of fatty acids of phosphoglycerides during various physiological stages of the gland remain to be investigated. The aim of this investigation is to study these aspects by examining changes of phospholipids and their acyl group compositions of rat mammary tissue during pregnancy, lactation, and post-weaning stages where major variations of hormonal milieu may have occurred.

MATERIALS AND METHODS

Normal breast tissue were dissected carefully from the Sprague-Dawley rats (Hotlzman Co., Madison, WI) at various times during pregnant, lactating, and early post-weaning periods. Tissues were weighed and finely minced with scissors before suspending in 4 vol of Tris-EDTA buffer (10 mM Tris and 1.5 mM EDTA, pH 7.5). The tissue suspension was homogenized with a Polytron PT-10 tissue disintegrator (Brinkmann Instruments, Westbury, NY) for two 15-sec periods at setting 13.5 with a 30 sec cooling interval. The resulting homogenate was centrifuged for 15 min at 3500 x g to remove cellular debris and red blood cells. The supernatant solution was subjected to the following procedure for lipid extraction and analysis.

Analysis of Membrane Lipids

Lipids from mammary tissues were extracted with 5 vol of chloroform:methanol (2:1, v/v).

After phase separation, solvent in the lower phase was taken to dryness by evaporating in a Büchler rotary evaporator. The total lipid extract was redissolved in 10 ml of chloroform and stored at 4 C until further analysis.

Since the mammary tissues are rich in triglycerides, the neutral glycerides and other nonpolar lipids were separated from the polar lipids by Unisil silicic acid column chromatography (Clarkson Chemical Co., Williamsport, PA). The nonpolar lipids were eluted by passing 50 ml of chloroform through the column, and the phospholipids and other polar lipids were eluted with 100 ml of methanol (17). Individual neutral glycerides and phosphoglycerides were further separated by thin layer chromatography (TLC). The thin layer plates were prepared with Silica Gel G suspended in 0.01M Na_2CO_3 (Brinkmann Instruments). The triglycerides were separated from other nonpolar lipid components by one-dimensional TLC using hexane:diethyl ether:15N ammonium hydroxide (70:30:0.1, v/v) as solvent for development. The phospholipids were subjected to separation-reaction-separation by TLC as described previously (18,19). The solvent system used for the first dimension was chloroform:methanol:15N NH_4OH (130:55:10, v/v) and that for the second dimension was chloroform:methanol:acetone:0.1M ammonium acetate:acetic acid (140:50:55:10:2.5, v/v). After solvent development, lipid spots were either visualized by exposing the thin layer plates to iodine vapor, as in the case to be used for phosphorus assay, or by spraying the thin layer plates with 2′,7′-dichlorofluorescein in ethanol, as in the case for fatty acid analysis. Individual lipid spots were scraped into test tubes, and the amount of lipid phosphorus was determined according to the method described by Gottfried (20). For analysis of the acyl group composition of individual phospholipids and triglycerides, the lipids were subjected to alkaline methanolysis (21). Methyl esters of fatty acid were then analyzed by a Hewlett-Packard Research gas-liquid chromatograph (GLC) equipped with dual flame ionization detectors and dual 6 ft columns packed with 10% EGSS-X on Gas Chrom P (Applied Science Laboratories, State College, PA). The initial temperature for the analysis was set at 170 C, which was later programmed to 195 C at 2 C/min. Peak areas were integrated by a Hewlett-Packard digital integrator. Repeated analysis of the same sample showed good reproducibility to within 5% variation of the peak area.

RESULTS

Lipids from breast tissues are rich in triglycerides, especially during the pregnant and lactating periods. To insure better analysis of phospholipids, we find it desirable to separate the nonpolar lipids from the polar lipids by column chromatographic elution prior to phospholipid analysis. As shown in Table I, the phospholipids in mammary tissues are rich in sphingomyelin, diacyl-glycerophosphorylcholine (GPC), and diacyl-glycerophosphorylethanolamine (GPE) with smaller proportions of alkenylacyl-GPE, diacyl-glycerophosphorylinositol (GPI), and diacyl-glycerophosphorylserine (GPS). Alkenylacyl-GPC and some lyso compounds were also present in smaller amounts. Analysis of the phospholipid composition from five tissue samples obtained between 11 and 20 days of the pregnant period did not reveal any apparent change in composition. Therefore, the results were pooled and mean values from these samples were represented in Table I. Immediately after parturition, there was an obvious increase in the proportion of diacyl-GPC which started to decline during the lactating period. The decrease in proportion of diacyl-GPC during lactation was then marked by a corresponding increase in diacyl-GPE. Towards the latter part of lactation, the proportion of diacyl-GPI also seemed to increase somewhat. After weaning, there was an increase in the proportion of sphingomyelin and a decrease in diacyl-GPI and diacyl-GPS in the mammary tissue. The proportion of diacyl-GPE also rapidly returned to the level shown in the pregnant period.

The fatty acid profile of diacyl-GPC of mammary gland rat contains mainly 16:0, 18:0, 18:1, 18:2, and 20:4 (Table II). The fatty acids of diacyl-GPE have a similar profile as diacyl-GPC but show higher proportions of the polyunsaturated fatty acids (Table III). The fatty acid profile of triglycerides is different from those in the phospholipids, and the major fatty acids are 16:0, 18:1, and 18:2 (Table IV). Since the proportion of 18:0 in trilglycerides was quite small (<5% of the total) as compared to that of the 18:1, these two components were not always well separated during GLC analysis and were thus grouped together. Fatty acids with shorter chain length (C_{10}-C_{14}) were present in triglycerides, and their proportions increased considerably during lactation (Table IV). In spite of this, the shorter chain fatty acids were not incorporated into the phosphoglycerides since only a small proportion of 14:0 was observed in diacyl-GPC during lactation.

There was no appreciable change in fatty

TABLE I

Phospholipid Composition of Rat Mammary Tissues during Pregnant, Lactating, and Post-Weaning Periods

Phospholipids[a]	Pregnant[b]	Lactation (days)							Post-lactation (days)					
	11-20 days (n=5)	1	2	3	7	14	15	19	1	2	2 early	5	10	
					mol %						mol %			
SPH	13.5 ± 2.25	14.8	13.0	12.9	10.2	10.6	7.2	11.4	26.2	19.7	23.3	17.8	19.6	14.5
GPI	9.9 ± 1.15	7.1	9.3	7.4	10.0	9.0	11.1	12.1	2.6[c]	6.6	3.3	9.7[c]	6.6[c]	6.4
GPS	9.2 ± 1.71	6.2	7.1	6.9	6.6	6.0	5.2	6.6		3.9	2.8			9.1
aGPC	2.9 ± 0.51	3.8	3.3	3.5	2.7	2.7	3.4	3.4	---	---	1.8	---	---	---
GPC	31.6 ± 0.52	41.4	39.1	40.1	35.4	37.4	31.5	30.0	37.9	46.2	53.1	50.1	49.6	36.5
aGPE	13.4 ± 0.71	6.5	5.2	5.2	6.0	5.7	8.4	6.6	8.1	6.0	2.7	3.3	10.2	16.5
GPE	13.9 ± 0.94	12.6	15.6	16.9	20.0	22.1	23.9	22.0	11.0	7.0	6.0	6.5	6.6	13.2
PA	1.1 ± 0.84	1.0	1.3	0.5	2.1	1.4	0.8	0.7	---	---	---	---	---	---
LPC	4.3 ± 2.69	6.7	6.1	6.5	6.8	5.2	8.4	7.6	13.6	9.0	7.4	9.9	7.4	4.0

[a]SPH = sphingomyelin, GPI = diacyl-glycerophosphorylinositol, GPS = diacyl-glycerophosphorylserine, GPC = diacyl-glycerophosphorylcholine, aGPC = alkenylacyl-GPC, GPE = diacyl-glycerophosphorylethanolamine, aGPE = alkenylacyl-GPE, PA = phosphatidic acids, LPC = lyso-GPC.

[b]Results were mean ± SE from five samples obtained during the pregnant period.

[c]GPI and GPS were not well separated and were taken as one spot.

TABLE II

Fatty Acid Composition of Diacyl-glycerophosphorylcholine from Rat Mammary Gland during Pregnant, Lactating, and Post-Weaning Periods

Acyl groups	Pregnant[a]	Lactation (days)							Post-lactation (days)			
	9-20 days (n=5)	1	2	5	7	14	15	19	1	2	5	10
					% by wt					% by wt		
14:0	---	0.5	1.7	3.2	1.9	---	2.7	2.6	---	---	---	---
16:0	40.9 ± 2.52	39.3	38.8	38.4	30.5	29.2	26.4	26.9	30.8	40.3	40.0	47.7
18:0	11.7 ± 1.71	21.3	22.7	21.8	20.1	21.9	26.4	19.7	16.0	14.8	16.3	12.3
18:1	19.0 ± 1.06	15.0	15.6	15.8	15.2	15.4	14.0	19.1	19.5	21.3	23.6	16.3
18:2	9.1 ± 1.39	12.0	16.8	16.9	22.8	24.0	24.5	26.8	25.5	17.9	11.4	10.0
20:4	18.2 ± 1.38	10.6	4.4	3.9	9.6	9.7	7.1	5.5	7.6	5.8	6.7	13.8

[a]Results were mean ± SE from five samples obtained during the pregnant period.

TABLE III

Fatty Acid Composition of Diacyl-glycerophosphorylethanolamine from Rat Mammary Gland during Pregnant, Lactating, and Post-Weaning Periods

Acyl groups	Pregnant[a] 9-20 days (n=5)	Lactation (days) 1	2	5	7	14	15	19	Post-lactation (days) 1	2	5	10
		% by wt							% by wt			
16:0	6.9 ± 1.34	11.6	6.8	6.5	6.7	4.8	4.7	4.5	6.0	6.4	13.8	10.7
18:0	28.4 ± 2.10	31.2	31.2	34.7	31.1	36.0	39.8	37.9	33.8	36.1	32.0	29.8
18:1	22.7 ± 1.13	23.7	27.4	26.1	28.6	18.9	26.2	26.0	21.2	21.3	22.4	19.0
18:2	6.8 ± 0.96	11.4	16.6	14.5	19.4	20.6	17.0	20.8	22.6	11.8	11.2	6.9
20:4	29.9 ± 1.73	19.0	16.6	18.2	14.2	19.6	12.4	10.8	17.1	22.1	20.6	25.6
22:4	2.7 ± 0.59	1.1	0.6									
22:U[b]	---	1.3	0.4									
22:6	1.6 ± 0.83	0.7	0.5									

[a]Results were mean ± SE from five samples obtained during the pregnant period.

[b]Fatty acid chain length and unsaturation not identified.

TABLE IV

Fatty Acid Composition of Triglycerides from Rat Mammary Gland during Pregnant, Lactating, and Post-Weaning Periods

Acyl groups	Pregnant[a] 9-20 days (n=5)	Lactation (days) 1	2	3	5	7	14	18	19	Post-lactation (days) 1	2	5	3 mo
		% by wt								% by wt			
12:0	0.4 ± 0.05	0.6	2.8	1.3	2.5	2.3	6.2	7.5	6.5	2.4	0.4	2.0	1.5
14:0	1.9 ± 0.57	2.2	3.8	1.6	3.2	3.1	3.6	3.5	5.2	1.7	1.4	1.6	---
16:0	26.7 ± 2.20	34.2	29.5	30.3	27.8	29.1	28.6	26.8	30.6	23.5	26.5	24.4	26.1
16:1	8.8 ± 1.67												
18:1[b]	43.5 ± 3.19	43.4	44.3	44.7	44.0	42.2	41.7	43.0	41.9	45.4	48.3	49.2	45.8
18:2	17.3 ± 2.84	19.7	19.6	22.3	22.5	23.3	21.0	19.4	15.8	27.1	23.4	22.8	26.2
20:4	2.6 ± 1.27	---	---	---	---	---	---	---	---	---	---	---	---

[a]Results were mean ± SE from five samples obtained during the pregnant period.

[b]A small percentage of 18:0 is included in this peak.

acid profiles of phosphoglycerides from the pregnant rats during the latter part of gestation. However, marked differences in acyl group profile were observed in both phosphoglycerides after parturition. In diacyl-GPC, there was a 2-fold increase in the proportion of 18:0 and a sharp decrease in 20:4 following parturition (Table II). During lactation, a gradual decrease in the proportion of 16:0 (from 39.3% to 26.9%) with a corresponding increase in 18:2 (from 12.0% to 26.8%) was also observed in diacyl-GPC. Acyl group changes found postpartum returned rapidly to normal after weaning. For example, the proportion of 18:0 declined to that found during pregnancy at ca. 10 days after weaning.

The proportion of 20:4 in diacyl-GPE was high during the entire pregnant period but decreased considerably following parturition. During lactation, the proportion of 20:4 in diacyl-GPE remained low, whereas that of 18:2 increased from 11.4% to 20.8%. These changes also gradually returned to the level of pregnant state 10 days after weaning.

DISCUSSION

One of the special functions of the mammary gland is its ability to synthesize the shorter chain fatty acids, C_8-C_{14}, during lactation (1,12). These fatty acids are esterified mainly to triglycerides and are excreted in the form of fat droplets in the milk (2-6). The present study also demonstrates the selective association of these acyl groups to the triglycerides and not phosphoglycerides of rat mammary tissue. In spite of the obvious increase in the shorter chain fatty acids in triglycerides during lactation, only trace amounts of 14:0 were detected in diacyl-GPC. On the contrary, marked changes in acyl group composition were found in phospholipids of the mammary tissues from pregnancy to lactation. The composition of phospholipids also changes during these different physiological stages. After parturition, there was a gradual increase in the proportions of diacyl-GPE and diacyl-GPI which reached maximal levels at mid-lactation. Since phospholipids are known constituents of biomembranes, the present results of an alteration in phospholipid composition and their acyl group patterns suggest a possible structural diversity of membranes during these physiological states. Alternately, these changes may be a reflection of a change in the population of specific cell types from pregnancy to lactation.

It is noteworthy to mention, in particular, the changes of 18:2 and 20:4 in phospholipids after parturition. Arachidonic acid, which is present in significant quantity in the phosphoglycerides during pregnancy, was greatly depleted following parturition. On the contrary, the proportion of 18:2 was high and remained fairly constant in triglycerides during lactation, while an obvious increase in the proportion of this acyl group in most phospholipids was observed. If the conversion of 18:2 to 20:4 is inhibited during lactation, we might expect an accumulation of 18:2 with a concomitant decrease of 20:4. This situation would be consistent with our observed result.

Hormones, such as prolactin and estrogen, influence lipid metabolism in the uterus, the mammary glands, and mammary carcinoma (10,11,22,23). These two hormones are known to undergo drastic changes during gestation and lactation. During early pregnancy, plasma prolactin level is elevated to ca. 3-fold that of diestrus, declines to a low level in mid-pregnancy, increases thereafter to reach a high maximum before mid and late lactation, and subsequently decreases (24-27). The present investigation indicates that the increase of phospholipid, namely diacyl-GPE and diacyl-GPI, during lactation follows patterns of variation similar to those indicated in prolactin. The existence of a causal relationship, however, remains to be determined.

Serum estrogen, which increases during gestation, also has a late surge at termination of pregnancy, and rapidly declines to very low levels following parturition (28). It has been suggested that estrogen plays an important role in the conversion of 20:4 to prostaglandin in the uterus (29-30) and estrogen-dependent mammary tumors contain several folds higher levels of prostaglandins than normal breast tissues (31). Furthermore, the late surge of estrogen coincides with the abrupt increase of prostaglandin levels just prior to delivery (28). Consequently, a rapid utilization of 20:4 would be obligatory for a rapid turnover of prostaglandin, as observed in late pregnancy. When estrogen becomes low following parturition, the level of 20:4 in phosphoglycerides remains low during the lactation period. The gradual increase in 18:2 in phosphoglycerides during lactation seemed to follow a different metabolic pattern as compared to 20:4. Consequently, it is possible that different types of fatty acids in the phosphoglycerides may be regulated under different types of hormonal control. This speculation is currently being investigated.

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Effect of Dietary Fats on the Incidence of 7,12-Dimethylbenz(a)-anthracene-Induced Tumors in Rats

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ABSTRACT

Female rats have been fed high fat diets containing either polyunsaturated or saturated fat. After being fed either of the diets for 4 weeks, some of the animals received an intragastric dose of 7,12-dimethylbenz(a)anthracene (DMBA). At this point, the diets of half of the animals were interchanged so that animals previously fed the polyunsaturated fat diet were fed the saturated fat diet and vice versa. The cumulative incidence of tumor-bearing rats among DMBA-dosed rats was greater when the polyunsaturated fat diet was fed. The mean induction time of tumors decreased and the proportion of tumor-bearing rats which developed malignant tumors increased when the polyunsaturated fat diet was fed. This promotional effect of the polyunsaturated fat diet was exerted only when the diet was fed after DMBA administration.

INTRODUCTION

Several groups of workers have shown that the susceptibility of rats and mice to develop mammary and skin tumors is enhanced by an increased proportion of fat in the diet (1-3). The incidence of both spontaneous tumors (4-6) and tumors induced by various carcinogenic agents (7-9) has been shown to be influenced in this way. The effect of dietary fat is due to a specific action of the fat rather than to an increased calorific intake (1,9).

Studies by Carroll and his colleagues on mammary carcinogenesis induced by the polycyclic hydrocarbon 7,12-dimethylbenz(a)anthracene (DMBA) have suggested that the nature as well as the amount of dietary fat may influence the incidence of cancer. Thus, Gammal et al. (8) found that the development of DMBA-induced tumors was enhanced in rats fed a diet containing 20% corn oil compared with rats fed diets containing either 0.5% corn oil or 20% coconut oil. Both the number of rats developing palpable tumors and the mean number of tumors per rat were greater among rats fed the high corn oil diet. Carroll and Khor (10) assessed the effect of a number of dietary fats and oils on the incidence of mammary tumors among DMBA-dosed rats. They found that there tended to be more tumors per rat when polyunsaturated fats were fed and this was reflected in a trend towards higher tumor yields with increased unsaturation of the diets.

In this study, we have examined the incidence and type of DMBA-induced tumors among rats fed diets containing either polyunsaturated or saturated fat.

MATERIALS AND METHODS

Weanling female Wistar-derived rats, bred in the Animal Breeding Establishment of the Australian National University, were maintained on a normal low fat (4.5% fat) laboratory rat pellet diet. At a mean age of 32 days, the rats were transferred to high fat (18.6% fat) diets containing either polyunsaturated or saturated fat. The principal fatty acids in the polyunsaturated fat diet were linoleic (59%) and oleic acids (27%) and, in the saturated fat diet, oleic (37%), stearic (19%), and palmitic acids (28%). Full details of the diets used have been reported elsewhere (G.J. Hopkins and C.E. West, unpublished data).

At 60 days of age, each rat received via stomach tube 10 mg DMBA (Fluka, Buchs S.G., Switzerland) dissolved in 0.5 ml sesame oil (Belleview Health Supplies, Chatswood, NSW, 2067, Australia). Control animals received 0.5 ml sesame oil. Animals were fed the low fat diet for 2 days before and 2 days after DMBA administration. This temporary change in diet was designed to minimize any possible effect of dietary fat on the absorption of the DMBA from the alimentary canal. Following administration of the carcinogen, the diets of half of the animals were interchanged, so that rats previously fed the polyunsaturated fat diet were fed the saturated fat diet and vice versa. The remaining rats were fed the same diet as before the administration of the carcinogen. Rats were weighed and palpated for the presence of tumors every 2 weeks. Rats were autopsied after they had died of natural means,

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or were killed after tumors had persisted for several months and death was considered to be imminent. Portions of tumors and other organs were fixed in 10% formol-saline, sectioned, and stained with haematoxylin and eosin. The experiment was continued until all experimental animals had died or had been killed. None of the control animals developed tumors.

Differences in the occurrence of tumor-bearing rats among animals on the different dietary regimes were analyzed using the method of Peto (11), which has been designed specifically for statistical analysis of tumor incidences in experimental animals. Using this method, the lifetime of the animals was divided into periods, and groups of animals were compared within each period. The number of animals alive and without tumors at the beginning of each period, and the number of animals developing tumors during each period, were listed. Assuming the dietary regimes to be equal in their effect on carcinogenesis, a table of the number of tumor-bearing rats observed and expected during each period was abstracted. The overall observed and expected numbers of tumor-bearing rats were then compared by chi-square analysis.

RESULTS

There were no meaningful differences in growth rate, as assessed by body wts, between control and DMBA-dosed rats fed the high fat diets containing polyunsaturated or saturated fat (Fig. 1). When rats were fed a different diet after DMBA administration, the ensuing growth rate was not altered by the change of diet.

The number of rats which developed tumors and the total number of rats in each experimental group are shown in Table I.

The final cumulative tumor incidence among rats fed the saturated fat diet before and the polyunsaturated fat diet after DMBA administration was 2.8-fold that among rats fed the saturated fat diet throughout the experiment (Fig. 2). Conversely, the final cumulative tumor incidence among rats fed the polyunsaturated fat diet throughout the experiment was 3.4-fold that among rats switched to the saturated fat diet after DMBA administration (Fig. 3). The cumulative tumor incidence among rats fed the polyunsaturated fat diet throughout the experiment was consistently higher than that among rats fed the saturated fat diet throughout the experiment (Fig. 4). However, this difference was not statistically significant.

These results may also be examined by comparing cumulative tumor incidences among all rats fed either diet after DMBA administration, regardless of the diet fed previously (Fig. 5). The final cumulative tumor incidence among rats fed the polyunsaturated fat diet after DMBA administration, regardless of the diet fed previously, was 3.2-fold that among rats fed the saturated fat diet after DMBA administration. This comparison is only valid if a similar comparison of cumulative tumor incidences is made between rats fed the different diets before DMBA administration, regardless of the diet fed after administration. In this case, the cumulative tumor incidences were not significantly different.

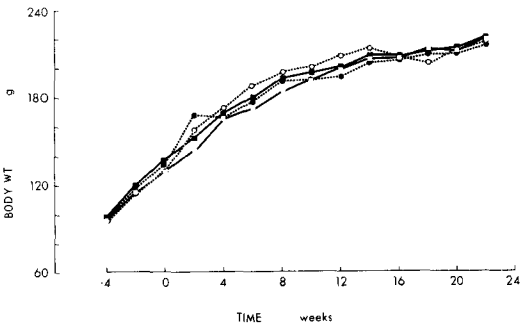

FIG. 1. Mean body wts of DMBA-dosed rats fed diets containing polyunsaturated (■——■) or saturated (□——□) fat, and control rats fed diets containing polyunsaturated (●- - -●) or saturated (○- - -○) fat. The rats which received DMBA were dosed at week zero. DMBA = 7,12-dimethylbenz(a)anthracene.

The mean times elapsed before detection of tumors (mean induction times) were less when rats were fed the polyunsaturated fat diet after DMBA administration (Table II). However, this difference was only statistically significant ($P<0.05$) between rats fed the saturated fat diet before and polyunsaturated fat diet after DMBA administration, and rats fed the saturated fat diet throughout the experiment.

A comparison of the mean induction times of tumors among all rats fed either diet after DMBA administration, regardless of the diet fed previously, revealed that the mean induction time was significantly ($P<0.05$) shortened when rats were fed the polyunsaturated fat diet after DMBA administration. The mean induction times of tumors were the same among rats fed either diet before DMBA administration, regardless of the diet fed after DMBA administration.

The majority of tumors were either benign fibroepithelial tumors or malignant epithelial tumors of the mammary gland. One animal developed a squamous cell carcinoma and another developed a squamous cell papilloma. Photomicrographs of the most common types

TABLE I

Number of Tumor-bearing Rats Discovered among Animals Fed Diets Containing Polyunsaturated (P) or Saturated (S) Fat

Diet[a]	Time after DMBA administration (wk)											
	0-4	4-8	8-12	12-16	16-20	20-24	24-28	28-32	32-36	36-40	40-44	44-48	48-52	52-56
PP	0/17[b,c]	0/17	3/17	0/14	0/14	0/14	1/14	2/13	0/11	2/11	2/9	2/7	1/2	0/1
SS	0/17	0/17	0/17	1/14	0/13	0/13	0/13	1/13	0/12	1/12	0/11	2/11	4/9	1/5
PS	0/15	0/15	0/15	0/15	1/15	0/14	1/14	0/13	1/13	0/12	0/12	1/12	1/11	1/10
SP	0/13	0/13	1/13	3/11	0/8	0/7	2/7	1/5	1/5	1/3	0/2	0/2	1/2	1/1

[a]The first and second letters refer to the diets fed before and after 7,12-dimethylbenz(a)anthracene (DMBA) administration, respectively.

[b]x/y: x = number of animals developing palpable tumor during each 4 week period, y = number of animals alive and without a tumor at the beginning of each 4 week period.

[c]Some animals died of the toxic effects of DMBA within 1 week of its administration. These animals have not been included in this table.

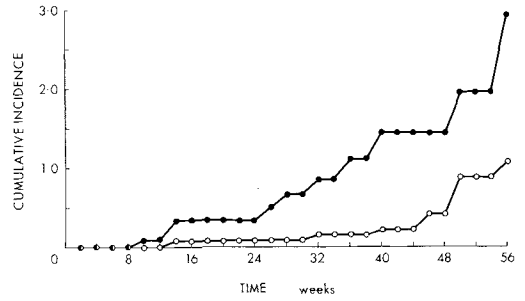

FIG. 2. Cumulative incidences of tumor-bearing rats among animals fed the saturated fat diet throughout the experiment (○——○) or fed the saturated fat diet before and the polyunsaturated fat diet after DMBA administration (●——●). The difference between the cumulative incidence lines was statistically significant (chi-square analysis: $P<0.01$). DMBA = 7,12-dimethylbenz(a)anthracene.

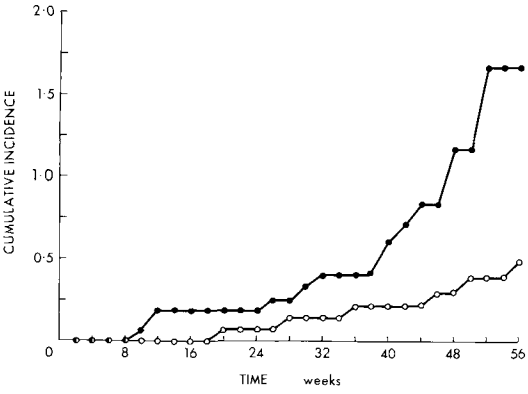

FIG. 3. Cumulative incidence of tumor-bearing rats among animals fed the polyunsaturated fat diet throughout the experiment (●——●) or fed the polyunsaturated fat diet before and the saturated fat diet after DMBA administration (○——○). The difference between the cumulative incidence lines was statistically significant (chi-square analysis: $P<0.025$). DMBA = 7,12-dimethylbenz(a)anthracene.

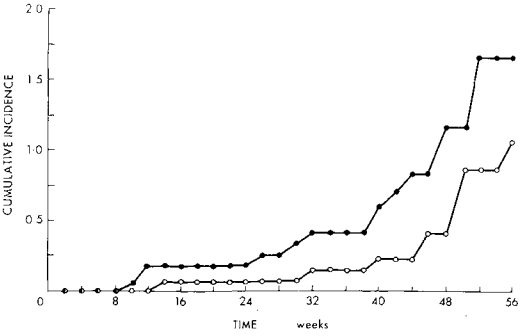

FIG. 4. Cumulative incidence of tumor-bearing rats among animals fed the polyunsaturated (●——●) or the saturated (○——○) fat diet throughout the experiment. The difference between the cumulative incidence lines was not statistically significant.

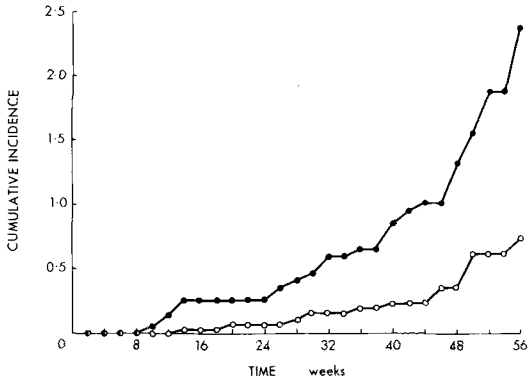

FIG. 5. Cumulative incidence of tumor-bearing rats fed the polyunsaturated (●——●) or saturated (○——○) fat diet after DMBA administration, regardless of the diet fed previously. The difference between the cumulative incidence lines was statistically significant (chi-square analysis: $P<0.001$). DMBA = 7,12-dimethylbenz(a)anthracene.

TABLE II

Mean Induction Times of DMBA-induced Tumors in Rats Fed Diets Containing Polyunsaturated (P) or Saturated (S) Fat[a]

Diet[b]	Induction time (days)
SS	294 ± 27.5 (10)[c]
SP	197 ± 32.5 (11)
PP	228 ± 28.3 (13)
PS	272 ± 37.1 (6)
SS	294 ± 27.5 (10)
PP	228 ± 28.3 (13)
PP + SP	213 ± 21.1 (24)[c]
SS + PS	285 ± 21.5 (16)
PP + PS	242 ± 22.6 (19)
SS + SP	243 ± 23.6 (21)

[a]Results are mean ± standard error (n). Comparisons are by Student's t-test. DMBA = 7,12-dimethylbenz(a)anthracene.

[b]The first and second letters refer to the diets fed before and after DMBA administration, respectively.

[c]$P<0.05$.

of tumors which were found are shown in Figure 6. The proportion of tumor-bearing rats which developed malignant tumors and were fed the polyunsaturated fat diet throughout the experiment was 64%. The proportion of malignant tumors was only 40% when rats were switched to the saturated fat diet after DMBA administration. The proportion of malignant tumors was 40% among rats fed the saturated fat diet throughout the experiment, but rose to 80% when rats were switched to the polyunsaturated fat diet after DMBA administration. Four rats which bore palpable tumors (at least 2 cm in diameter) died unexpectedly and were eaten by the other rats before autopsy could be performed. Two of these rats were fed the polyunsaturated fat diet throughout the experiment; one was switched to the polyunsaturated fat diet after DMBA administration and the other switched to the saturated fat diet after DMBA administration.

DISCUSSION

The results presented in this paper show that the feeding of a diet containing polyunsaturated fat led to an increase in the incidence of tumor-bearing rats as well as an increase in the proportion of tumor-bearing rats which

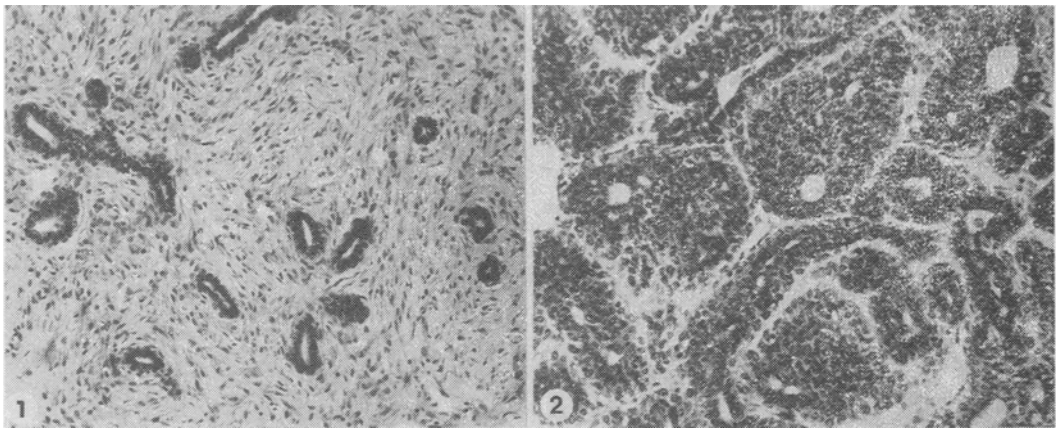

FIG. 6. Photomicrographs of the most common types of tumor found in rats dosed with 7,12-dimethylbenz(a)anthracene. (1) Example of benign fibroepithelial mammary tumor-pericanalicular fibroadenoma. Haematoxylin and eosin; magnification X200. (2) Example of malignant epithelial mammary tumor-adenocarcinoma. Haematoxylin and eosin; magnification X250.

developed malignant tumors. The mean tumor induction time among rats fed the polyunsaturated fat diet was also less than among rats fed the saturated fat diet. This promotional effect of the polyunsaturated fat diet upon carcinogenesis was only exerted when the diet was fed after DMBA administration.

These results support those of Gammal et al. (8), who showed that the incidence of palpable tumors was greater among DMBA-dosed rats fed a semisynthetic high corn oil diet rather than an isocaloric diet containing coconut oil. These authors did not find statistically significant differences between tumor incidences based on the total number of tumors discovered at autopsy 4 months after DMBA administration. As tumors may occasionally regress, we have allowed tumor-bearing rats to die naturally or to survive until their general appearance has indicated that death was imminent. We regard the death or imminent death of an animal to be a more meaningful measure of the incidence of cancer in groups of experimental animals.

The carcinogenic process can be divided into two distinct stages (12), commonly referred to as the initiation and the promotional stages. The first stage is thought to involve the rapid and irreversible conversion of normal cells into neoplastic cells by interaction with a carcinogenic agent (13). The second stage involves the cellular proliferation of neoplastic cells to form a discrete tumor (14). Since we have observed an enhancement of tumorigenesis only when the polyunsaturated fat diet was fed after DMBA administration, it is likely that this effect involved the survival and proliferation of tumor cells, rather than the intial event of neoplastic transformation. This premise is consistent with the finding that the higher increase of palpable tumors observed by Gammal et al. (8) in rats fed a high corn oil diet was not a result of a higher concentration of DMBA in the tissues or prolonged exposure of the tissues to the carcinogen (15).

In addition, Carroll and Khor (3) have found that the tumor yield in rats dosed with DMBA was enhanced by a high corn oil diet if rats were transferred from a low corn oil diet to a high corn oil diet 1 or 2 weeks after giving the DMBA. If the transfer was delayed for 4 weeks, little or no enhancement was seen.

The proportion of polyunsaturated fatty acids in hepatocyte plasma membranes of mice fed the polyunsaturated fat diet has been shown to increase, reaching a maximum after 4 weeks and returning to a lower level by 24 weeks (Hopkins and West, unpublished work). If similar changes occur in the plasma membranes of tissues of the rat, the plasma membranes of rats switched to the polyunsaturated fat diet at the time of DMBA administration would contain a maximum proportion of polyunsaturated fatty acids 4 weeks after DMBA administration. From the results of the experiments reported here and those of Carroll's group, it appears that this is a crucial period for the development of tumors from neoplastic cells. This could be related in some way to changes in the permeability (16) and the activity of lipid-associated membrane-bound enzymes (17).

It is now widely accepted that cell-mediated immune reactions can be generated in response to tumor-associated antigens (18,19). Assuming these reactions to be of physiological consequence to the potential tumor-bearing host, it is possible that the enhancement of tumorigenesis by dietary polyunsaturated fat that we have observed may have an underlying immunological basis. This hypothesis is supported by reports that polyunsaturated fatty acids inhibit the *in vitro* antigen-induced response of lymphocytes (20-22). Polyunsaturated fatty acids have also been shown to be effective in prolonging the survival of skin grafts in rodents (23-25) and to be beneficial as an adjunct to immunosuppressive therapy following human renal transplantation (26). The reports of an improved clinical course of multiple sclerosis patients fed linoleate (27), a higher incidence of cancer among atherosclerotic patients fed a diet containing polyunsaturated fat (28), and an increased susceptibility to allergic encephalomyelitis of rats bred and raised on a diet deficient in polyunsaturated fatty acids (29) may also be explicable in terms of a suppression of immune function by polyunsaturated fatty acids.

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Lipid Metabolism of the Yellow Clam, *Mesodesma mactroides:* I. Composition of the Lipids

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ABSTRACT

The lipid composition of the yellow clam, *Mesodesma mactroides,* that lives in the northern beaches of the Buenos Aires province of Argentina was studied. The main nonpolar lipids are triglycerides and alkoxyglycerides. Phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl serine are the main phospholipids. The predominant fatty acids are 16:0, 16:1ω7, 18:0, 18:1ω9, 20:5ω3, and 22:6ω3. They are mainly provided by the clam's food and stored in the hepatopancreas. The content of polyunsaturated acids increases in summer together with an increase in nonpolar lipids and is correlative with an increase in phytoplankton in the sea water. Sexual maturity modifies the lipid composition of gametes.

INTRODUCTION

The yellow clam *(Mesodesma mactroides* Desh. 1854) lives under the sand of the intertidal zone of the beaches of the Atlantic coast from the state of Sao Paulo (Brazil) to the south of the province of Buenos Aires (Argentina). A previous work reported the seasonal variations and proximal biochemical composition of the soft tissues of this mollusc (1). The aim of the present work was to determine the lipid composition of the yellow clam and its seasonal changes caused by different ecological and physiological factors. The following factors were considered: availability and composition of food, temperature, stage of sexual development, and self-regulation of fatty acid synthesis.

Accordingly, the fatty acid composition of the total lipids of clam was determined in animals collected in January, April, and September. January (summer) is a period of intensive feeding with a predominance of phytoplankton, the most important source of lipids (1). Therefore, the total lipids of the clam reach their maximum in this month. April (autumn) is a period of almost complete rest or inactivity, considering the mentioned factors. Finally, September (spring) is the period of most intense sexual activity.

MATERIAL AND METHODS

Animals

The clams were harvested after tide retreat from the sandy beaches of Mar Azul (37° 19' S; 57° 00' W, province of Buenos Aires, Argentina). The population density there (629 clams/m^2) is the highest found up to now (1). Thirty clams were chosen for each sampling regardless of sex, but with intact valves and 60-70 mm in length. They were then kept overnight in aereated sea water filtered through cotton wool. They were hung on a net to wash out sand and impurities. Then the abductor muscles were cut off and the bodies were separated and washed with distilled water. They were dried with filter paper and weighed. The compositions of the following parts of the body were determined in particular: muscular foot, mantle and gills (together), hepatopancreas and gonads.

Extraction and Fractionation of Lipids

The total lipids were extracted according to Folch et al (2) using a Virtis 23 homogenizer. The polar and nonpolar lipids were fractionated by absorption on silicic acid (3) to facilitate the ulterior separation, identification, and quantification of the components of each fraction by thin layer chromatography (TLC). With this purpose, 20 x 20 cm plates covered with 250μ of Silica Gel G (E. Merck, Darmstadt, West Germany) were used to identify the components and plates covered with 500μ were used for quantitative purposes. The following solvent system was used to analyze the nonpolar lipids: petroleum ether (60-90 C):ethyl ether:acetic acid (90:10:1, v/v); and to separate the sterols: petroleum ether (60-90 C):ethyl ether:acetic acid:methanol (90:50:2:5, v/v). Identification of the components was done by comparison of the R_f with standards run on the same plate and by means of specific reaction (4,5). The amount of the nonpolar components was measured by extraction of the lipids from the silica gel with chloroform, evaporation of the solvent, and weighing. The relative percentage

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TABLE I

Composition of Nonpolar Lipids of *Mesodesma mactroides* (January)

Lipid classes	R_f[a]	Reactions: Iodine	Reactions: Dinitrophenyl-hydrazine	Reactions: $C_{13}Fe$	Distribution (%)
Sterol esters	0.87	+	-	+	13.8
Alkoxyglycerides	0.70	+	+	-	23.8
Triglycerides	0.51	+	-	-	25.0
Free fatty acids	0.30	+	-	-	7.7
Sterols	0.12	+	-	+	10.4
Diglycerides					0.8
Monoglycerides, pigments, and phospholipids	Origin	+	-	-	12.5

[a]Thin layer chromatography in petroleum ether (60-90 C):ethyl ether:acetic acid (90:10:1, v/v).

TABLE II

Phospholipid Composition of Polar Lipids of *Mesodesma mactroides* (January)

Phospholipid classes	R_f[a]	Reactions: Dragendorff	Reactions: Ninhidrine	Reactions: -naftol	Distribution (%)
Phosphorylated glycolipid	0.85	-	-	+	7.0
Cardiolipin	0.71	-	-	-	5.6
Phosphatidyl ethanolamine	0.59	-	+	-	11.7
Phosphatidyl serine zone	0.55	-	+	-	11.2
Phosphatidyl choline	0.36	+	-	-	51.5
Sphingomyelin, sphingoethanolamine	0.28	+	+	-	5.5
Lysophosphadidyl ethanolamine	0.23	-	+	-	1.5
Lysophosphatidyl serine	0.18	-	+	-	2.2
Lysophosphatidyl choline	0.15	+	-	-	0.8
Origin		-	+	-	3.0

[a]Thin layer chromatography in chloroform:methanol:water (65:25:4, v/v).

of the total wt of the lipids was calculated.

The solvent system chloroform:methanol: water (65:25:4, v/v) was used to analyze polar lipids. Identification of the components was also done by comparison of their R_f with standards run on the same plate and with specific reactions (6,7). Once the polar lipids were chromatographied and identified, their phosphorus content was determined by the method of Barlett (8) modified by Doizaki and Zieve (9).

Fatty Acid Composition

The total lipids were saponified with alcoholic KOH during 45 min at 80 C. The unsaponifiable material was extracted with petroleum ether. The free fatty acids were recovered from the acidified medium. They were then esterified with methanol-HCl (10) purified by sublimation (11) and finally analyzed in a Pye gas-liquid chromatograph with an argon ionization detector. The columns were packed with 15% diethylene glycol succinate on Chromosorb W (80-100 mesh).

Identification of the fatty acids in the chromatogram was done by comparison of their retention times with standards, using the graphic procedure of the retention times vs. chain length (12), Ackman's separation factors I, II, and III (13-15), and Haken's factor (16). The percentage of each fatty acid was calculated by measuring the area of peaks. To confirm the chain length of the unsaturated fatty acids, the samples wery rechromatographied after hydrogenation (17).

RESULTS AND DISCUSSION

Lipid Fractions

Mesodesma mactroides contains an amount of lipids that changes from 0.8% to 1.9% depending on the season of the year (1). It is low in October (spring) and attains a peak in December-January (summer) (1). Fractionation of lipids with silicic acid between polar and nonpolar followed by TLC separated different lipid classes. The components of the nonpolar fraction are shown in Table I. Triglycerides and alkoxyglycerides (neutral plasmalogens) are the most abundant. Lipids tentatively identified

TABLE III

Distribution of Total, Polar, and Nonpolar Lipids of Total Soft Tissues and Hepatopancreas of *Mesodesma mactroides*

	Total tissues			Hepatopancreas		
	Total lipids (g/100g tissue)	Nonpolar (g/100g TL[a])	Polar (g/100g TL)	Total lipids (g/100g tissue)	Nonpolar (g/100g TL)	Polar (g/100g TL)
September (spring)	1.19	67.0	33.0	3.10	69.1	30.9
January (summer)	1.90	78.0	22.0	5.10	84.5	15.5
April	1.16	62.1	37.9	2.00	70.0	30.0

[a]TL = total lipids.

as alkoxyglycerides (R_f 0.70) run faster than triglycerides (R_f0.51) in petroleum ether (60-90 C):ethyl ether:acetic acid (90:10:1, v/v) and were stained by iodine and the 2.4 dinitrophenylhydrazine-HCl reaction. The position of the spots was similar to that found by Nakamura and Privett (18) in similar conditions.

Free (R_f 0.12) and esterified (R_f 0.87) sterol spots stained by $FeCl_3$ were also recognized. Because the spot of R_f 0.12 in petroleum ether (60-90 C):ethyl ether:acetic acid (90:10:1, v/v) was also stained by iodine, it was fractionated by TLC with petroleum ether (60-90 C):ethyl ether:acetic acid:methanol (90:50:2:5, v/v) and the presence of diglycerides recognized. The presence of other sterols generally found in invertebrates (19,20), besides cholesterol, was not investigated.

Free acids (R_f 0.30), reduced amounts of pigments, and phospholipids were also found in the TLC of the nonpolar fraction separated with silicic acid.

Both triglycerides and alkoxyglycerides store fatty acids in sea vertebrates and probably play the same role in invertebrates.

The polar lipid spectrum of the yellow clam is similar to that of the sea organisms in general (21-24). The main phospholipid identified (Table II) is phosphatidyl choline. Phosphatidyl ethanolamine and phosphatidyl serine are also present. The spot corresponding to phosphatidyl serine may also contain ceramideaminoethylphosphonate, characterized by a covalent union carbon-phosphorous. Ceramideaminoethylphosphonate has a similar R_f according to data published by Itasaka et al. (25). Besides, it may be stained by ninhidrine but it contains phosphorus, not detectable by the Barlett method (8). Ceramideaminoethylphosphonates have been recognized in molluscs, sea animona, and protozoa (26-28).

In Table II, there are also tentatively identified minor amounts of phospholipids, common to the rest of the water molluscs:cardiolipin, sphingomyelin, sphingoethanolamine, lysoderivatives, and phosphorylated glycolipid.

The amount of total lipids extracted from hepatopancreas and other soft tissues is shown in Tables III and IV. The lipid content was markedly higher in the hepatopancreas. The season of the year not only modified the amount of lipids but also the relative proportion of polar and nonpolar fractions and their distribution in the different tissues (Table III). In January (summer), the amount of total lipids was higher and was evoked by an increase in nonpolar lipids.

Fatty Acids

The fatty acid composition of the yellow

TABLE IV

Distribution of Total, Polar, and Nonpolar Lipids in Different Tissues of *Mesodesma mactroides* (September)

Total soft tissues	Lipids		
	Total (g/100g tissue)	Polar (g/100g TL[a])	Nonpolar (g/100g TL)
Total soft tissues	1.19	33.0	67.0
Hepatopancreas	3.10	30.9	69.1
Mantle and gills	0.40	36.8	63.2
Muscle (foot)	2.00	62.3	37.7
Gonads	3.00	29.0	71.0

[a]TL = Total lipids.

TABLE V

Percent Fatty Acid Composition of the Lipids of Total Soft Tissues of *Mesodesma mactroides*

Fatty acids	September	January	April		
	Total lipids	Total lipids	Total lipids	Nonpolar	Polar
14:0	9.2	4.7	5.8	7.5	5.5
14:1	2.2	0.5	2.1	2.0	1.6
15:0	6.6	1.0	2.6	1.8	1.6
15:1	---	0.5	---	---	---
16:0	22.6	15.2	23.9	29.5	22.0
16:1ω7	7.5	9.8	12.7	9.3	20.0
16:2ω7	2.4	1.7	---	---	---
17:0	---	---	1.6	---	2.8
16:2ω7?	1.0	2.8	2.4	2.5	1.6
18:0	8.0	2.8	7.4	8.3	4.5
18:1ω9	6.0	19.3	7.3	8.7	5.5
18:2ω9	---	3.6	1.7	1.0	1.0
18:2ω6	2.3	1.9	1.7	2.3	0.8
18:3ω6	---	1.1	1.7	2.2	1.0
20:0	---	---	1.5	1.7	0.4
18:3ω3	---	1.0	0.6	2.0	---
20:1ω9	3.4	0.7	1.1	2.0	1.0
20:2ω9	3.2	2.5	6.5	6.0	4.8
20:2ω6	0.7	---	1.9	1.9	1.0
20:4ω6	---	0.7	2.8	2.7	2.7
20:5ω3	12.9	15.9	10.4	6.6	13.7
22:4ω6	1.8	0.7	---	---	0.6
22:4ω3	---	---	---	---	0.2
22:5ω3	1.6	0.7	---	---	1.0
22:6ω3	8.6	12.9	4.3	2.0	6.7

clam is shown in Table V. The acids 16:0, 16:1ω7, 18:1ω9, 20:5ω3, and 22:6ω3 are major components. The general composition is similar to that observed in other aquatic molluscs (28-32). However, the amount of fatty acids of the linoleic (ω6) family found in the clam was lower than the values published by Gruger et al. (29) and Ackman et al. (30) in other molluscs.

Effect of Diet

The total lipid intake from food by the clam varies along the year, depending on the relationship phytoplankton:detritus. Besides, both food components have different fatty acid compositions. Diatoms, for example, present high percentages of 14:0, 16:0, and 20:5ω3 acids and small amounts of saturated and unsaturated acids of 18 carbon atoms and 22:6ω3 acid (33,34). The contribution of detritus, on the other hand, consists mainly in saturated and monounsaturated acids of 14, 16, and 18 carbons (35).

Taking this into consideration, the fatty acid composition of the clams in January, April, and September was compared to the food available in the same periods.

In January, the clams feed abundantly, especially on phytoplankton (1), which provides most of the lipids and almost all the poly-

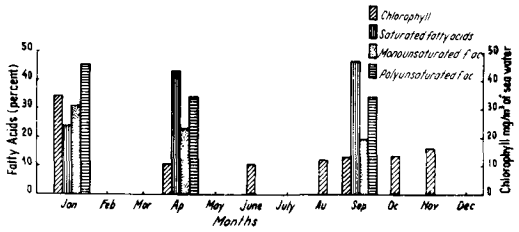

FIG. 1. Available phytoplankton in "Mar Azul" (1) and distribution of saturated, monounsaturated, and polyunsaturated acids in total lipids of *Mesodesma mactroides* collected in January, April, and September.

unsaturated fatty acids. The clam stores its lipids, principally as fats, in the hepatopancreas (Table III), with high concentrations of 18:1ω9, 20:5ω3, and 22:6ω3 (Table V).

In April, less food is available and it is mostly detritus. Accordingly, the total lipids in hepatopancreas and in total soft tissues decrease compared to their values in January (Table III). The total amount of saturated fatty acids almost doubles that of January and shows a remarkable decrease in the percentages of 18:1ω9, 20:5ω3, and 22:6ω3 acids (Table V).

In September, more food is available than in April, and the phytoplankton:detritus ratio is also increased (1). Therefore, the content of total lipids and fat in the soft tissues and in the hepatopancreas is slightly increased with respect to the April values (Table III). The percentages of the polyunsaturated acids (20:5ω3 and 22:6ω3 are intermediate between those of January and April (Table V). As a result, particularly the phytoplankton available to the clam makes an important contribution to the fatty acid composition of the lipids.

Figure 1 summarizes the information obtained. It confirms that in January the great amount of polyunsaturated fatty acids is correlative to an increased phytoplanktonic biomass in the environment measured as chlorophyll. In April, with scarce phytoplankton in the food, there is a decrease in the level of mono- and polyunsaturated fatty acids, and, therefore, an increase in saturated acids. Finally, in September, the histogram is similar to that of April, but with internal differences in the percentages of fatty acids, especially the mono- and polyunsaturated acids. The content of the acids 20:1ω9, 20:5ω3, and 22:6ω3 had increased, whereas the acids 16:1ω7, 18:2ω9, and 20:2ω9 had decreased.

The above data, in particular, indicate that there is a direct correlation between the amount of the acids 20:5ω3 and 22:6ω3 found in the tissues of the clam and the amount of phytoplankton in the food. These acids would be selectively incorporated from the diet, as can be deduced from the fact that the ratio 20:5ω3/22:6ω3 is constant not only in the total lipids, but also in the fats or phospholipids of the clam (Table V), and the same ratio is observed in the total lipids of the diatoms (1). Besides, an increase in both acids in the lipids of the clam was found when there was an increase in stored fat.

Effect of Temperature

It has been observed that at low environmental temperatures there is generally an increase in unsaturation of the fatty acids in aquatic organisms (36-42).

The yellow clam is an eurithermic species. The temperature of the coastal waters that wash the clam banks ranges between 8.5 C in August and 22 C in February (1). Accordingly, enhanced unsaturation of the fatty acids of clam could be expected in September compared to January, but this is not the case. It is supposed that the polyunsaturated fatty acids from the diet, because of their amount, mask the effect of temperature, provided this effect exists in the clam. Gardner and Riley (43) reached the same conclusion studying the cirripedium *Balanus balanoides* and noted the necessity of isolating both variables, diet and temperature, to establish their influences separately. Besides, de Torrengo et al. (44) found that the fresh water fish, *Pimelodus maculatus,* kept in a tank for 15 days, did not increase the relative proportion of unsaturated acids when the temperature of the water was decreased from 30 C to 15 C. However, the enzymatic activity of the Δ6 desaturase was increased.

Effect of Sexual Stage

The sexual cycle of *Mesodesma mactroides* follows the same pattern as other marine animals. In January, most of the population showed their gametes in the first stages of maturation. Full maturation was only achieved in September. Analysis of different parts of the body of clams collected in September (Table VI) showed characteristics that were also similar to those observed in marine organisms in general (45-46): a remarkable amount of the polyunsaturated fatty acids stored in the gonads in the period corresponding to sexual maturity. However, it must also be emphasized that high percentages of polyunsaturated fatty acids were also found in the hepatopancreas.

It has been reported (41,42) that bivalve molluscs (47,48) store the lipids in the hepatopancreas and then transfer them to the gonads. A similar transference takes place, apparently,

TABLE VI

Percent Fatty Acid Composition of Different Tissues of *Mesodesma mactroides* (September)

Fatty acid[a]	Mantle + gills	Gonads	Hepatopancreas	Foot
14:0	3.7	7.4	12.2	5.8
14:1	1.1	1.1	1.1	2.1
15:0	1.5	1.9	1.1	2.6
16:0	36.0	31.5	32.6	34.8
16:1ω7	6.3	12.8	10.0	7.2
16:2ω7	3.0	2.1	1.6	4.2
18:0	18.8	10.9	9.1	16.3
18:1ω9	9.5	6.1	7.0	10.9
18:2ω6	3.5	3.7	3.0	3.1
20:1ω9	3.8	2.7	2.7	2.3
20:2ω9	4.5	3.1	3.2	2.4
20:2ω6	1.1	0.5	---	0.4
20:5ω3	7.2	16.2	16.4	7.9

[a]Only fatty acids of chain length up to 20 carbons were analyzed.

in *Mesodesma mactroides.* In January, the organism receives more lipids from the environment than necessary for its economy and they are preferentially stored in the hepatopancreas. In September, when the gonads reach full maturation, the lipids are distributed principally between the gonads and the hepatopancreas. The lipids in the gonads will be used for the normal reproduction process.

Regulation of Synthesis and Storage of Polyunsaturated Fatty Acids

The fatty acid composition observed in the lipids of the clam (Table V) suggests the existence of a regulatory mechanism similar to that found in other animals.

Administration of faty free diets or diets supplemented with only some fatty acids to rats and other animals has shown that the amount of unsaturated fatty acids of one family stored in the tissues depends on the presence and amount of acids of other families. Thus, the acids of α-linolenic acid family displace the acids of linoleic family in the tissues, and these replace those of the oleic family (49-53). In this bivalve, it was observed that the lipids are regulated by a similar mechanism. In April, when the diet consists mainly of detritus and is therefore deficient in polyunsaturated fatty acids (of the ω3 family), there was a decrease in these acids concomitant with an increase in saturated and unsaturated acids of other families, with the exception of oleic acid. Therefore, *Mesodesma mactroides* shows a typical lipidic composition of sea organisms in which the fatty acid composition is mainly regulated by diet and to a lesser extent by other mechanisms.

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Extraction of Lipids from Yeast[1]

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ABSTRACT

Several methods for the extraction of lipids from intact yeast cells have been compared. Extraction of intact cells with methanol followed by methanol:benzene (1:1, v/v) and benzene resulted in the recovery of equal or greater amounts of polar and nonpolar lipids than obtained by other methods. A preparative method involving preincubation of cells with aqueous KOH followed by the treatment of the cellular residue as described above yielded slightly more steryl esters than was extracted from broken cell preparations.

INTRODUCTION

Application of the classical procedures of Folch et al. (1) and of Bligh and Dyer (2) for the extraction of lipids from yeast generally are of limited success, apparently because of the rigid cell wall of yeast (3). Several procedures have been employed for the extraction of lipids from intact yeast cells (4-7) but, in general, interest has focused only on the extraction of phospholipids. In addition, the procedures developed are cumbersome, making the simultaneous extraction of multiple samples difficult.

Recently, this laboratory has been examining the effects of several hypocholesteremic drugs on lipid metabolism in yeast. To evaluate the effect of different concentrations of drug at various stages of culture growth, it is necessary to extract and analyze many samples simultaneously. A simple, fast extraction method would facilitate these studies greatly. However, to insure that all of the drug effects on lipid metabolism are monitored, such simple extraction procedures should be complete and reproducible. Since it was anticipated that sterol biosynthesis would be inhibited at one or more sites by the hypocholesteremic compounds under study, it was especially important to insure completeness of extraction of both free sterols and of steryl esters.

This paper will describe a comparison of several methods of extraction of polar and nonpolar lipids from intact yeast for analysis of multiple samples. Preparative methods for optimum recovery of sterols and steryl esters for subsequent identification of individual components will be evaluated also.

[1]From a dissertation to be submitted to the Graduate School, University of Maryland, by Michael T. Sobus, in partial fulfillment of the requirements for the PhD degree in biochemistry.

MATERIALS AND METHODS

Reagents

(1-^{14}C) Sodium acetate (57.8 mCi/mmol) was obtained from New England Nuclear (Boston, MA). Chromatographic reference standards were obtained from the following sources: squalene (Calbiochem, Oak Grove Village, IL); lanosterol, ergosterol, cholesterol oleate, methyl oleate, triolein, oleic acid, phosphatidyl choline, and phosphatidyl ethanolamine (Applied Science Laboratories, State College, PA). All solvents, except Skellysolve B (Skelly Oil Co., Kansas City, MO), were reagent grade. Both diethyl ether and Skellysolve B were redistilled before use.

Cell Growth Conditions

Cultures of *Saccharomyces cerevisiae*, strain MY 306, were maintained on 2% agar slants in a medium containing 0.1% NH_4Cl, 1.1% K_2HPO_4, 1.85% KH_2PO_4, 2% yeast extract, and 2% glucose. To prepare an inoculum, one loopful of yeast from an agar slant was added to 5 ml of sterile medium in a 18 x 150 mm test tube and incubated for ca. 17 hr at 28 C. Two percent (by vol) of this 17 hr culture was added to either 500 or 250 ml Erlenmeyer flasks containing 50 or 25 ml of sterile medium, respectively, and the indicated amount of (1-^{14}C) sodium acetate. The cultures were incubated with shaking (50 rpm) at 28 C for the indicated time. At the time of cell collection, absorbance at 640 nm (A_{640}) was determined with a spectrophotometer (Bausch and Lomb Spectronic 20). The amount of protein was assayed by a modified procedure of Lowry et al. (8), which consisted of incubating the cell pellet obtained from an aliquot of culture with 0.1 ml 1M NaOH at 80 C until a clear solution was obtained. After cooling, protein was assayed in the normal manner except that the 2% $NaCO_3$ (w/v) solution was prepared in distilled water rather than in 0.1M NaOH.

Cells were collected in most cases by addition of an aliquot of culture to an equal volume

of 10% trichloroacetic acid (TCA), and centrifugation for 5 min at a setting of "5" on a table top centrifuge (International) yielded a pellet of cells. In some experiments, the aliquot was centrifuged directly. The supernatant was removed by aspiration, and the pellet was washed twice with distilled water (0.5 of original culture volume). In all cases, duplicate samples were taken for extraction and analysis.

Extraction Methods

Fung (9): Unless otherwise indicated, wet cells were mixed 3 times with methanol (0.2 of original culture volume) continuously for 10 min, followed by centrifugation at a setting of "5" with a table top centrifuge for 5 min. All extracts were pooled.

Deierkauf and Booij (4): Lipids were serially extracted from intact cells using 0.2 culture volumes of methanol, chloroform:methanol (1:1, v/v), and chloroform. After continuous mixing for 10 min, the extracts were separated from the cells by filtration. The extracts were pooled.

Pederson (5): Lipids were extracted from intact cells 3 times with 0.2 culture volumes of chloroform:methanol (1:1, v/v) for 10 min. The extracts were separated from the cells by filtration and were pooled.

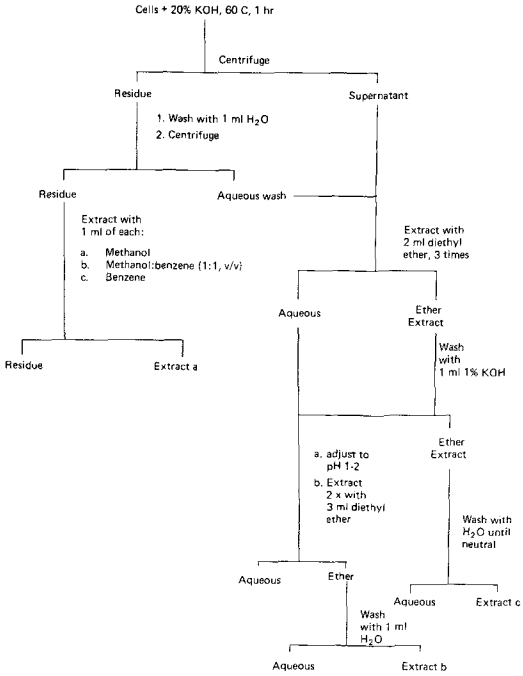
Folch et al. (1): Lipids were extracted from intact cells once with at least 1 culture volume of chloroform:methanol (2:1, v/v). The extract was separated from the cells by filtration and was washed with 0.2 culture volumes of 0.1M KCl according to the method of Folch et al. (1).

Bligh and Dyer (2): Intact cells were suspended in 0.2 culture volumes of chloroform and 0.4 culture volumes of methanol. After the contents were mixed for 10 min, 0.2 culture volumes of both chloroform and distilled water were added. The contents were filtered and the filtrate was centrifuged until the phases clarified. The bottom phase was collected as the lipid extract.

Letters (6): Intact cells were extracted with 4 culture volumes of 95% ethanol, twice with 1 culture volume of chloroform:methanol (2:1, v/v), and once with 1 culture volume of chloroform:methanol:hydrochloric acid (125:65:1, v/v/v). The acidified extract was neutralized with 2N NaOH. The extracts were separated from the cells by filtration and pooled.

Itoh and Kaneko (10): Intact cells were suspended in 0.08 culture volumes of methanol and 0.12 culture volumes of glass beads in a 15 ml conical centrifuge tube, cooled in an ice bath, and sonicated for 10 min with an ultrasonic disintegrator (Megason) equipped with a micro tip. Chloroform (0.16 culture volumes) was added after sonication. Filtration through a sintered glass filter separated the disrupted cells from the extract. Residual cells and debris were suspended in 0.2 culture volumes of chloroform:methanol (2:1, v/v) for 10 min and then filtered. This procedure was repeated 3 times, and the extracts were pooled.

Aqueous alkaline hydrolysis (KOH): The cell pellet obtained from 5 ml of culture was suspended in 0.2 culture volumes of 20% KOH (w/v) containing ca. 3 mg of pyrogallol, and the suspension was heated at 60 C for 1 hr. The extraction proceeded as follows:



Extracts a, b, and c were analyzed separately and after pooling.

Lipid Analysis

Aliquots of the pooled extracts were counted in 5 ml of scintillation solvent consisting of 0.4% 2,5-diphenyloxazole (w/v), 0.01% bis(0-methylstyryl)-benzene (w/v), 66% toluene (v/v), and 34% cellosolve (v/v) in a scintillation spectrophotometer (Intertechnique). The counting efficiency for all samples counted was 90 ± 2%. The reported cpm values are averages for duplicate samples.

Individual lipid components were analyzed by thin layer chromatography (TLC). Aliquots of the pooled extracts were spotted on precoated silica gel thin layer plates (Quantum, Fairfield, NJ). Reference standards of squalene, ergo-

TABLE I

Extraction of ^{14}C Lipids from Whole Cells of *Saccharomyces cerevisiae:* Comparison of Several Methods

Method of extraction	Cell condition	Total ^{14}C in pooled extracts (cpm x 10^{-4} /mg protein[d])
Fung[a]	wet	2.28 ± 0.34
	freeze-dried	2.75 ± 0.14
	acetone-dried	1.61 ± 0.01
Deierkauf and Booij[a]	wet	2.62 ± 0.05
	freeze-dried	1.67 ± 0.04
	acetone-dried	1.94 ± 0.17
Pederson[a]	wet	1.57 ± 0.10
	freeze-dried	1.26 ± 0.44
Fung[b]	wet	4.45 ± 0.21
Fung (washed)[b,c]	wet	3.21 ± 0.21
Folch, et al.[b]	wet	0.60 ± 0.04
Bligh and Dyer[b]	wet	1.40 ± 0.05

[a]*S. cerevisiae* was grown in the presence of (1-^{14}C) sodium acetate (0.2 μCi/ml) for 4 hr. A_{640} = 3.5; protein = 1 mg/ml. Cells were collected directly without addition of trichloroacetic acid.

[b]*S. cerevisiae* was grown in the presence of (1-^{14}C) sodium acetate (1.0 μCi/ml) for 17 hr. A_{640} = 9.6; protein = 3.35 mg/ml. Cells were collected with trichloroacetic acid.

[c]Pooled extracts were washed according to method of Folch et al. (1).

[d]Results for the extraction of duplicate samples are presented as the mean ± standard error.

TABLE II

Extraction of Nonpolar Lipids from *Saccharomyces cerevisiae*[a]: Comparison of Deierkauf and Booij Procedure with Use of Other Solvents[b]

Extraction procedure	Total ^{14}C extracted[d]	Lipids recovered from thin layer chromatograms[c]			
		Ergosterol[d]	Unknown sterol[d]	Lanosterol[d]	Steryl ester[d]
Deierkauf and Booji	19.8 ± 0.60	1.03 ±0.11	0.248 ± 0.05	0.246 ± 0.05	1.96 ± 0.10
Fung	16.0 ± 0.10	0.72 ± 0.06	0.186 ± 0.01	0.179 ± 0.017	0.36 ± 0.01
Skellysolve B[b]	17.1 ± 0.20	0.94 ± 0.07	---	---	0.327 ± 0.04
Acetone[b]	21.8 ± 0.40	0.94 ± 0.09	0.272 ± 0.01	0.287 ± 0.005	1.44 ± 0.20
Diethyl ether[b]	17.9 ± 0.40	0.891 ± 0.040	---	---	1.04 ± 0.05
Ethyl acetate[b]	17.6 ± 0.10	0.933 ± 0.020	---	---	0.973 ±0.01

[a]*S. cerevisiae* was grown in the presence of (1-^{14}C) sodium acetate (2 μCi/ml) for 24 hr. A_{640} = 8.50; protein = 2.9 mg/ml.

[b]Duplicate aliquots of culture (2.5 ml) were collected with trichloroacetic acid amd extracted twice with methanol and once with the indicated solvent.

[c]Tentative identification of substances after thin layer chromatography with System I.

[d]Mean cpm x 10^{-4}/mg protein ± standard error.

sterol, lanosterol, cholesterol oleate, methyl oleate, triolein, and oleic acid were spotted for analysis of the nonpolar lipids. Cholesterol, phosphatidyl ethanolamine, phosphatidyl choline, and lysophosphatidyl choline were spotted as reference standards for the analysis of the polar lipids. Thin layer plates for the analysis of the nonpolar lipids were developed to 10 cm twice with benzene:ethyl acetate (5:1, v/v) and once to 17 cm with Skellysolve B:diethyl ether:glacial acetic acid (95:5:1, v/v/v), System I, or were incubated in NH_3 vapor for 5 min followed by development once to 10 cm in Skellysolve B:diethyl ether (60:40, v/v) and once to 15 cm with the same solvents (95:5, v/v), System II. Plates for the analysis of polar lipids were developed to 15 cm once with chloroform:methanol:glacial acetic acid:water (170:25:25:6, v/v/v/v), System III. In all cases, reference standards were detected by exposure to I_2 vapors.

Radioautographs of the developed thin layer chromatograms were prepared according to the method of Holmlund (11). Sections of the

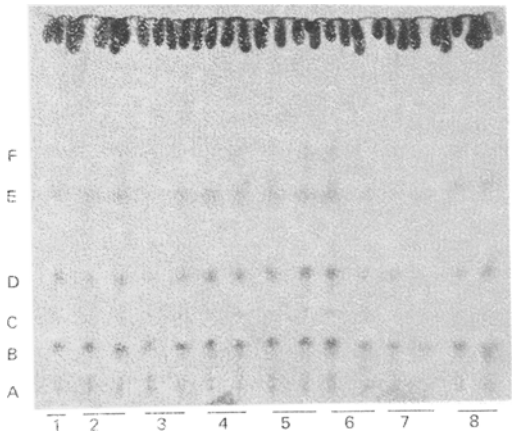


FIG. 1. Comparison of the extraction of polar lipids from whole cells of *Saccharomyces cerevisiae*. The culture was grown and extracted as indicated in Table III. Duplicate samples 1-5 were extracted serially with methanol, with methanol:indicated solvent (1:1, v/v), and with the indicated solvent. Extracts were chromatographed with System III and radioautographed. The duplicate of 1 was lost. 1 = acetone; 2 = ethyl acetate; 3 = diethyl ether; 4 = tetrahydrofuran; 5 = benzene; 6 = samples were extracted once with methanol and twice with methanol:acetone (1:1, v/v); 7 = samples were extracted serially with methanol, methanol:acetone (1:1, v/v), and diethyl ether; 8 = Deierkauf and Booij procedure. Zones B, D, and F migrated like lysophosphatidyl choline, phosphatidyl choline, and phosphatidyl ethanolamine, respectively.

chromatograms corresponding to the darkened areas on the film, which in turn related to the reference standards, were scraped and the labeled components were counted in 5 ml of scintillation solvent.

RESULTS AND DISCUSSION

Extraction of Lipids from Intact Cells

Table I shows that the extraction of wet, intact cells of *S. cerevisiae* with methanol according to Fung's procedure yielded more total ^{14}C material than the extractions according to the procedures of Pederson (5), Folch et al. (1), and Bligh and Dyer (2) and approximately as much as the Deierkauf and Booij (4) procedure. When this methanol extract was washed according to the procedure of Folch et al. (1), 25% of the total counts and 50% of the dry wt were lost. The material in the wash had the following characteristics: it was not mobile in the thin layer chromatographic Systems I and III: it did not react with I_2 vapor, H_2SO_4-$K_2Cr_2O_7$ spray (12). or the phosphatide spray of Dittmer and Lester (13); however, it did react with ninhydrin spray (14). This would indicate that the material was not lipid in nature. No further attempts were made to identify it.

Treatment of cells by freeze-drying or acetone drying did not substantially improve the extraction of ^{14}C material by the procedures of Fung, Deierkauf and Booij, and Pederson.

In Table II, analysis of the extracted lipids is shown. While the Fung extraction was nearly comparable to the Deierkauf and Booij method in the extraction of ^{14}C material, the Fung method yielded only 18% of the ^{14}C steryl ester extracted by the Deierkauf and Booij procedure. Substitution of methanol in the last extraction step with other solvents, notably acetone, improved the extraction of ^{14}C steryl ester to 75% of the amount extracted by the Deierkauf and Booij procedure. Differences in the "ergosterol," other unidentified sterols, and "lanosterol" zones caused by the variation of extracting solvents were not pronounced.

To improve the extraction of steryl esters, further modifications of the extraction procedure were attempted as shown in Table III. The extraction method utilizing methanol: benzene extracted more ^{14}C material (129%) and more ^{14}C steryl ester (122%) than the Deierkauf and Booij procedure. It was as effective in the extraction of the other nonpolar lipid components as the procedures utilizing other solvents. In addition, the ^{14}C material extracted was comparable in quantity to that obtained by the extraction procedure of Letters (6). The solvent combination of methanol: tetrahydrofuran was comparable to the combination of methanol:benzene in the ability to extract nonpolar lipids. A more complete extraction of polar lipids was also provided by the methods utilizing methanol:benzene and methanol:tetrahydrofuran than by the Deierkauf and Booij procedure (Fig. 1 and Table IV).

Letters (6) has utilized a procedure involving a sequential extraction of intact cells with ethanol, chloroform:methanol (2:1, v/v), and finally with chloroform:methanol:hydrochloric acid (124:65:1, v/v/v). The last extract is then neutralized with 2N NaOH. The author claims that this procedure will extract as much lipid as could be obtained from broken cell preparations, but provided data only for phospholipids. Removal of salts by washing of the extract by the procedure of Folch et al. (1) is required before TLC. This additional treatment is not required with the methanol:benzene extraction method. Moreover, in our hands, it was difficult to obtain reproducible results when utilizing the Letters procedure on small samples.

The preferred procedure, among those tested, for the extraction of polar and nonpolar lipids from intact yeast cells involves sequential

TABLE III

Extraction of Nonpolar Lipids from *Saccharomyces cerevisiae*[a]: Modifications of the Deierkauf and Booij Procedure

Extraction procedure	Total ^{14}C extracted[e]	Lipids recovered from thin layer chromatograms[c]				
		Ergosterol[e]	Unknown sterol[e]	Lanosterol[e]	Triglyceride[e]	Steryl ester[e]
Acetone[b]	48.8 ± 1.7	2.24 ± 0.10	---	---	5.48 ± 0.16	10.8 ± 0.6
Ethyl acetate[b]	51.9 ± 1.5	2.50 ± 0.01	---	---	5.48 ± 0.34	10.5 ± 0.2
Diethyl ether[b]	43.0 ± 3.8	2.25 ± 0.26	---	---	5.14 ± 0.25	8.9 ± 1.8
Tetrahydrofuran[b]	52.1 ± 0.2	2.70 ± 0.08	1.16 ± 0.05	0.422 ± 0.02	5.18 ± .60	11.8 ± .3
Benzene[b]	53.2 ± 0.36	3.00 ± 0.05	1.08 ± 0.17	0.505 ± 0.001	5.09 ± .06	12.1 ± 1.5
Methanol:acetone (1:1)[d]	52.4 ± 0.2	2.71 ± 0.15	---	---	4.36 ± 0.30	10.5 ± 0.04
Deierkauf and Booij	47.1 ± 0.3	2.72 ± 0.01	1.10 ± 0.06	0.400 ± 0.027	4.43 ± 0.68	10.0 ± 0.5
Letters	51.3					

[a] *S. cerevisiae* was grown in the presence of (1-^{14}C) sodium acetate (2.0 μCi/ml) for 6 hr. A_{640} = 4.60; protein = 1.0 mg/ml culture.

[b] Cells from duplicate aliquots of culture (2.5 ml) were collected with trichloroacetic acid and extracted serially with methanol, with methanol-indicated solvent (1:1, v/v), and with the indicated solvent.

[c] Tentative identification of substance after thin layer chromatography with System I.

[d] Equal aliquots of culture were extracted once with methanol and twice with methanol:acetone (1:1, v/v).

[e] Mean cpm x 10^{-4}/mg protein ± standard error.

TABLE IV

Extraction of Polar Lipids from *Saccharomyces cerevisiae*[a]: Modification of the Deierkauf and Booij Procedure

Extraction procedure[a]	Lipids recovered from thin layer chromatograms[b]					
	A	B[c]	C	D	E	F[c]
	(cpm x 10^{-3}/mg protein)					
Tetrahydrofuran	4.22 ± 0.19	17.0 ± 0.4	1.64 ± 0.12	8.99 ± 0.59	8.73 ± 0.45	3.21 ± 0.40
Benzene	3.98 ± 0.22	16.4 ± 1.2	1.88 ± 0.18	9.52 ± 1.45	9.21 ± 0.75	3.96 ± 0.29
Deierkauf and Booij	2.64 ± 0.43	13.1 ± 2.4	1.54 ± 0.23	7.60 ± 1.30	5.72 ± 0.43	3.68 ± 0.44

[a] *S. cerevisiae* was grown and extracted as indicated in Table III.

[b] Zones corresponding to darkened region of autoradiograph (Fig. 1) were scraped and counted. Results are expressed as mean ± standard error.

[c] Zones B and F migrated like lysophosphatidyl choline and phosphatidyl ethanolamine, respectively.

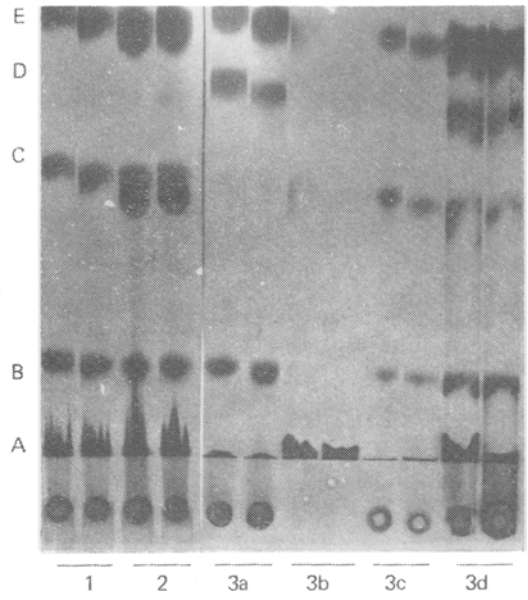


FIG. 2. Comparison of extractions of nonpolar lipids from whole and broken cell preparations of *Saccharomyces cerevisiae*. The culture was grown in the presence of (1-^{14}C) sodium acetate (1.0 μCi/ml) for 6 hr. A_{640} = 6.3, protein = 1.9 mg/ml culture. Duplicate 5 ml aliquots of culture were collected directly (Material and Methods). Extracts of duplicate samples were chromatographed with System II and radioautographed. 1 = Serial extraction of whole cells with methanol:benzene. 2 = Extraction of disrupted cells by procedure of Itoh and Kaneko. 3 = Cell suspensions were subjected to hydrolysis with aqueous KOH and then extracted; (a) extract of residue, (b) extract of acidified supernatant, (c) extract of alkaline supernatant, (d) aliquots of a-c were chromatographed together. Migration of zones: A like oleic acid, B like ergosterol, C like triolein, D like methyl oleate, and E like cholesteryl oleate. For further details, see the Materials and Methods section.

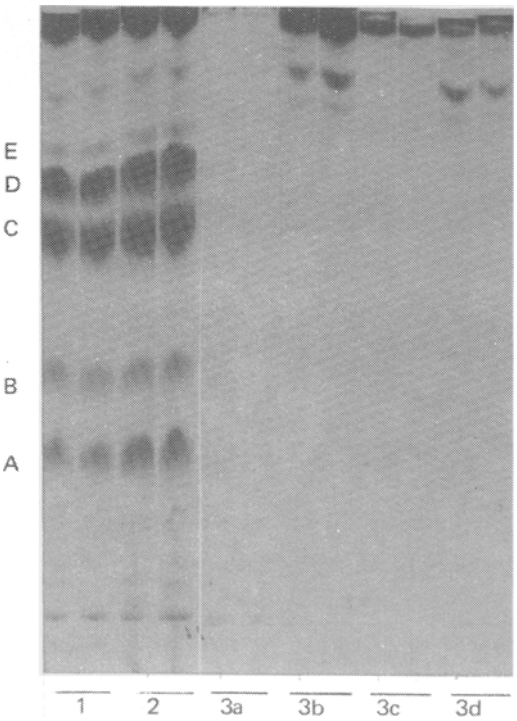


FIG. 3. Comparison of extractions of polar lipids from whole and broken cell preparations of *Saccharomyces cerevisiae*. The culture was grown and extracted as indicated in Figure 2. Extracts were chromatographed with System III and radioautographed. 1 = Serial extraction of whole cells with methanol: benzene. 2 = Extraction of disrupted cells by procedure of Itoh and Kaneko. 3 = Cell suspensions were subjected to hydrolysis with aqueous KOH and then extracted; (a) extract of residue, (b) extract of acidified supernatant, (c) extract of alkaline supernatant, (d) aliquots of a-c were chromatographed together. Zones A, C, and E migrated like lysophosphatidyl choline, phosphatidyl choline, and phosphatidyl ethanolamine, respectively. For further details, see the Materials and Methods section.

extraction with methanol, methanol:benzene (1:1, v/v), and benzene. The cell pellet can be separated from the extract by centrifugation followed by removal of the extract, since the cells do not float in the solvents employed. In addition, the methanol:benzene procedure is more complete in lipid extraction and more reproducible in that the filtration steps which are most subject to handling losses are not necessary.

Extraction of Lipids from Intact and Broken Cell Preparations

Itoh and Kaneko (10) recently described a procedure wherein lipids were extracted from yeast by homogenizing wet cells in a chloroform:methanol (1:2, v/v) suspension. No information was provided concerning the efficiency of cell rupture. Sterols are customarily recovered from yeast by refluxing in an aqueous alcoholic solution of sodium or potassium hydroxide. Although this procedure permits extraction of total sterol, estimation of the fraction originally present in the ester form is impossible. It seemed likely that alkaline hydrolysis in the absence of alcohol might spare steryl esters and yet cause disruption of permability barriers by hydrolysis of proteins so that subsequent extraction of nonsaponifiable lipids could be effected more efficiently. Application of such a procedure (Materials and Methods) provided a total of 13.8 mg as "ergosterol" from 0.5 liter of culture based on the absorbance of the extract at 282 nm. TLC revealed the presence of steryl esters, triglycerides, sterols, and fatty acids in the combined extracts. Moreover, when the extracted residue

TABLE V

Extraction of Lipids from *Saccharomyces cerevisiae*[a]: Comparison of Extractions from Intact and Broken Cell Preparations

Nonpolar lipid zones scraped from thin layer chromatogram[b]	Extraction procedures[d]		
	Methanol:benzene	Itoh and Kaneko	Aqueous KOH
	(cpm x 10^{-3}/mg protein)		
Steryl esters	20.2 ± 2.8	43.2 ± 2.2	50.4 ± 4.03
Methyl esters	0.25 ± 0.03	1.65 ± 0.46	16.1 ± 3.4
Triglycerides	10.6 ± 0.53	46.6 ± 2.3	8.26 ± 1.6
Sterols	12.1 ± 2.2	11.6 ± 0.35	12.16 ± 1.5
Polar lipid zones scraped from thin layer chromatogram[c]			
E	0.567 ± 0.01	1.02 ± 0.031	---
D	7.55 ± 0.37	12.0 ± 0.84	---
C	7.66 ± 0.061	11.6 ± 0.46	---
B	0.397 ± 0.083	1.05 ± 0.03	---
A	1.98 ± 0.012	3.32 ± 0.10	---

[a]*S. cerevisiae* was grown in the presence of (1-^{14}C) sodium acetate (1.0 μCi/ml) for 17 hr. A_{640} = 6.3; protein = 1.7 mg/ml. Cells were collected directly without addition of trichloroacetic acid.

[b]Tentative identification of substances after thin layer chromatography with System II.

[c]Zones counted corresponded to those in Figure 2.

[d]For further details, see the Materials and Methods section. Results are presented as the mean ± standard error.

resulting from aqueous saponification was subsequently subjected to saponification by refluxing for 1 hr with 20% KOH (w/v) in 80% ethanol (v/v), only an additional 0.4 mg of "ergosterol" was recovered. Thus, it appears that extraction of yeast after aqueous saponification yields at least 97% of the total sterol recoverable by refluxing with alcoholic KOH. Moreover, as additionally determined by separate experiments, no hydrolysis of steryl esters occurred in the absence of alcohol.

Aliquots of a culture of yeast prelabeled with (1-^{14}C) sodium acetate were extracted (a) by the methanol:benzene procedure involving intact cells, (b) by a modified Itoh and Kaneko procedure, and (c) by the method described in the Materials and Methods section, subsequent to aqueous alkaline digestion of the cells. Extracts were analyzed by TLC employing Systems II and III (Figs. 2 and 3). Labeled zones were analyzed for ^{14}C material (Table V). All three extraction methods yielded essentially the same amounts of free sterol, whereas prior aqueous saponification of the cells provided ca. 16% more ^{14}C steryl ester than recoverable by the Itoh and Kaneko method, and more than twice that obtained by methanol:benzene extraction of intact cells. Aqueous alkaline hydrolysis of cells followed by extraction would appear, therefore, to be a preparative method for recovery of steryl esters. This procedure apparently does cause complete hydrolysis of phospholipids (Fig. 3) but incomplete hydrolysis of triglycerides. Anomalous production of fatty acid methyl esters also appears to occur.

Extraction of intact cells with methanol:benzene provided ca. 50% of the steryl esters and ca. 60% of the phospholipids recovered by the Itoh and Kaneko procedure. Extent of recovery of individual phospholipids varied somewhat from fraction to fraction.

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Nutritional Effects of the Cyclic Monomers of Methyl Linolenate in the Rat

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ABSTRACT

Low levels (0.0075, 0.0225, and 0.15%) of cyclic fatty acid methyl esters (>98% pure) were incorporated into diets of weanling rats fed different levels of protein. Animals on low protein diets (8-10% casein) exhibited decreased wt gains and feed consumption with increasing levels of cyclic esters in their diets after 6 weeks. Liver enlargements due to a significant ($P<0.01$) accumulation of liver lipid were noted in animals receiving 0.15% cyclic fatty acid esters in their diets.

INTRODUCTION

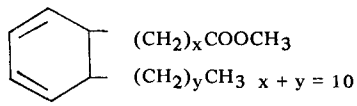
Many studies have been conducted in which a heat-abused fat or fractions from such fats were fed (1-12). Such heated oils produced adverse nutritional and physiological conditions when fed to laboratory animals as parts of normal diets (8-12). Moreover, the non-urea adduct forming fatty acids, a concentrate of the cyclic fatty acids and other polar materials present in such oils, caused a high number of deaths among rats fed this material (11). Repeated studies of the non-urea adduct forming fraction from heated oils have identified disubstituted aromatic and alicyclic fatty acids as major and minor components of these fats (13-16).

Although studies of others (8-12) have shown that cyclic fatty acids in diets cause adverse physiological effects when fed to rats, these diets contained only uncharacterized concentrated fractions fed at relatively high levels rather than the pure cyclic fatty acids themselves. In the present work, the nutritional effects of low levels of highly purified isomeric ω(2-alkyl cyclohexadienyl) carboxylic acids in nutritionally adequate diets containing different levels of protein (casein) were determined.

MATERIALS AND METHODS

Synthesis of Cyclic Fatty Acids

Cyclic fatty acids were synthesized by a modification of the method used by Scholfield and Cowan (17). The crude cyclic fatty acids were converted to the corresponding methyl esters which were further purified by argentation chromatography on silicic acid, with 2% diethyl ether in hexane as eluant. This procedure eliminated all of the dimeric or polymeric material formed and most of the isomers of conjugated linolenic acid as determined by thin layer chromatography. Subsequent gas chromatography after hydrogenation indicated that the isomeric ω(*o* alkyl cyclohexadiene) carboxylic acid methyl ester mixture made up ca. 90% of the sample; the remainder of the sample was the corresponding aromatic isomer mixture, the general structure of which is:



Experimental Animals and Diets

Male weanling SPF Albino rats (40-50 g) of Sprague Dawley descent, obtained from Murphy Breeding Laboratories (Plainfield, IN), were used for all experiments and housed in galvanized iron wire cages with mesh bottoms. The composition of the diets used (in g/100 g diet) was as follows: salt mixture w (a modification of the Osborne Mendel salt mix containing only inorganic constituents [18]), 3.5; vitamin premix,[3] 1.0; choline chloride, 0.15; corn oil containing either 0, 0.05, 0.15, or 1.0% cyclic fatty acid methyl ester, 15; casein, 8, 10, or 15; dextrose, to 100. All diets were made up in 1 kg batches and were isocaloric containing 4.72 calories/g. Thus, each diet contained 0.0075, 0.0225, and 0.15% cyclic fatty acid methyl ester. The diets were stored under nitrogen in the cold between feedings. Each group of animals was fed the diets for 6 weeks.

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[3] Vitamin mix (mg vitamin/kg diet): retinyl acetate, 20,000 IU; calciferol, 2,000 IU; α tocopherol, 100 IU; ascorbic acid, 22; inositol, 112; menadione bisulfite, 48; para-amino benzoic acid, 22; niacin, 100; Ca Panthothenate, 67; riboflavin, 22; pyridoxine HCl, 22; thiamine HCl, 100; biotin, 0.45; folic acid, 4; cyanocobalamin, 0.03.

Lipid extractions were accomplished according to the method of Folch et al. (19).

Statistical Treatment of Data

Data mean values are expressed as standard error of the mean (SEM). The F test for significance was employed (20).

RESULTS

Average wt gain, total and average feed consumed, and feed and energy efficiency data for animals in the dietary study are presented in Table I. In diets containing 8 and 10% protein, the average wt gained and feed consumed exhibited a progressively decreasing trend for animals in groups fed with increasing amounts of cyclic fatty acid methyl esters. There is one exception to the trend (group 6) where average wt gains and feed consumption tended to be less than that of a group with a higher content of cyclic esters in the diet. Group 8 gained significantly less wt ($P>0.05$) and consumed less food than did other groups fed the same protein level. Wt gains and feed consumption of animals fed 15% protein diets showed no signficant influence of cyclic monomer level.

The action of the cyclic monomer in the diet can also be observed in the values obtained for energy efficiency. These values measured the efficiency of utilization of food for growth; an increase indicated that the food was not utilized as well for growth. The modified protein efficiency ratios (PER) obtained (Table I) indicated that the lowest ratios were obtained for groups 4 and 8 compared with other groups consuming the same amount of protein. There was not a significant trend for animals fed 15% protein. Values reported for these modified PERs seem to lie on a curve similar to those obtained by Bunce and King (21) in which a maximum PER was obtained between 10-14% protein (casein). Lower PER values were obtained for lower and higher levels of protein.

Liver and Liver Lipid Wts

The percentages of liver:body and lipid:liver wt ratios of rats fed different levels of protein and cyclic fatty acids for different lengths of time are indicated in Table II. Certain experiments took longer to complete than others, and thus the liver:body wt ratios represent average ratios obtained during the period the experiments were conducted. Animals fed the 8% protein diets were fed the diets longer because of their small size upon completion of the 6-week dietary study. Therefore no tests of significance were applied. There were, however, small differences among liver:body wt ratios of animals fed different levels of cyclic acids and protein.

The lipid:liver wt ratios showed a large (probably significant) difference for rats that consumed 0.15% cyclic fatty acids in their diets in groups 1, 2, and 3. The amount of lipid in the liver of animals consuming 0.15% cyclic fatty acids in their diets was slightly higher than the others. The livers removed from rats that consumed 0.15% cyclic fatty acids (groups 1, 2, and 3) were spongy to the touch and white-yellow in color. It was clearly seen that certain lobes exhibited more pronounced fatty infiltration than others.

DISCUSSION

The feed consumption and wt gain data presented in Table I, especially with respect to animals fed 8 and 10% protein, showed trends which were observed by others who reported wt gains among animals fed various heated unfractionated fats (1-3,11,12). Altered organ wts, modified enzyme activities, and an acceleration in the formation of certain types of abnormal tissue have been reported in animals fed heated fats (22). Gottenbos and Thomasson (23) fed rats fish oils which contained low levels of aromatic cyclic fatty acids. Lower wt gains and feed consumption trends were observed in those animals fed increasing levels of cyclized fish oils. In a study comparing body wt changes in toxicity tests, Frazer (24) showed that wt gain and food intake tended to parallel each other. Animals fed small amounts of known toxic compounds also displayed differences in behavior and scattered significantly more food. Animals in the present dietary study also exhibited differences in behavior, and those fed 8% protein were clearly more tense and excitable than rats fed higher levels of protein. Animals fed different levels of protein and increasing amounts of cyclic fatty acids in their diets did not scatter any more food than the control animals. Lower wt gains achieved by these animals were not due to palatability, since preliminary studies in our laboratory indicated that diets containing cyclic compounds seemed to be preferred to diets without them.

A number of investigators have reported wts of and lipid content of livers from experimental animals fed heated fats (1,2,4-6,25,26). The results of liver wts reported varied from highly significant wt differences (5) to no wt changes for rats on heated and fresh oil diets (2,25,26). The protein content in the experimental diets ranged from 18% (5,25) to 30% (4), while others (1,2) provided diets in which the exact

TABLE I

Wt Gained and Feed Consumed by Rats Fed Different Levels of Cyclic Fatty Acids and Protein for 6 Weeks

Group	Protein fed (% by wt)	Number of animals	Cyclic fatty acids in diet (%)	Average wt gain[a] (g)	Average feed consumed (g)	Energy[b] efficiency	Wt gain[c]/wt protein consumed
1	8	8	0.0000	78.6 ± 4.5	448.9 ± 15.4	25.5 ± 1.9	2.06 ± 0.11
2	8	8	0.0075	79.0 ± 4.0	433.6 ± 19.2	25.2 ± 0.9	2.23 ± 0.07
3	8	8	0.0225	70.1 ± 3.2	415.5 ± 13.6	26.5 ± 0.8	2.06 ± 0.08
4	8	8	0.1500	62.0 ± 6.0	382.2 ± 21.6	27.2 ± 0.6	1.96 ± 0.13
5	10	10	0.0000	123.9 ± 10.4	488.0 ± 30.5	19.0 ± 0.7	2.49 ± 0.08
6	10	10	0.0075	102.7 ± 5.8	437.8 ± 11.4	20.0 ± 1.1	2.34 ± 0.11
7	10	10	0.0225	110.9 ± 5.5	451.6 ± 27.9	19.3 ± 0.4	2.44 ± 0.06
8	10	10	0.1500	89.0 ± 7.1[d]	400.9 ± 19.3	21.8 ± 1.3	2.21 ± 0.11
9	15	9	0.0000	213.4 ± 5.2	616.5 ± 8.4	13.5 ± 0.4	2.30 ± 0.06
10	15	10	0.0075	218.1 ± 5.5	640.4 ± 9.0	13.8 ± 0.3	2.26 ± 0.04
11	15	10	0.0225	219.6 ± 7.0	621.7 ± 14.8	13.4 ± 0.3	2.35 ± 0.05
12	15	10	0.1500	212.5 ± 6.6	602.5 ± 10.9	14.9 ± 0.8	2.34 ± 0.07

[a]Mean values ± SEM.

[b] $= \frac{\text{Caloric intake}}{\text{wt gain}}$

[c]A modified protein efficiency ratio.

[d]Significant at $P<0.05$ from groups fed the same protein level.

TABLE II

Effect of Protein and Cyclic Fatty Acid Level on Liver Size and Liver Lipid Content in the Rat

Cyclic fatty acid in diet (%)	Liver wt (g)	Lipid wt (g)	Lipid wt / Liver wt (%)	Liver wt / Body wt (%)
	Group 1: 8% protein[a]			
0.0000	6.49 ± 0.40	0.40 ± 0.14	6.09 ± 0.39	2.91 ± 0.22
0.0075	6.21 ± 0.28	0.39 ± 0.15	6.35 ± 0.83	2.87 ± 0.23
0.0225	6.42 ± 0.16	0.38 ± 0.04	5.86 ± 0.83	3.10 ± 0.04
0.1500	6.31 ± 0.35	0.61 ± 0.22	10.30 ± 1.08	3.18 ± 0.07
	Group 2: 10% protein[b]			
0.0000	6.54 ± 0.55	0.38 ± 0.05	6.14 ± 0.48	3.02 ± 0.09
0.0075	5.65 ± 0.27	0.32 ± 0.02	6.30 ± 0.58	2.98 ± 0.65
0.0225	5.98 ± 0.39	0.43 ± 0.07	7.70 ± 1.17	2.97 ± 0.09
0.1500	5.60 ± 0.21	0.51 ± 0.50	10.40 ± 0.15	3.29 ± 0.11
	Group 3: 15% protein[c]			
0.0000	8.36 ± 0.48	0.47 ± 0.06	5.52 ± 0.44	3.65 ± 0.16
0.0075	9.45 ± 0.36	0.51 ± 0.05	5.36 ± 0.55	3.74 ± 0.56
0.0225	8.94 ± 0.98	0.49 ± 0.05	5.61 ± 0.32	3.67 ± 0.14
0.1500	10.50 ± 0.78	0.89 ± 0.02	8.46 ± 0.36	4.08 ± 0.15
	Group 4: 15% protein[d]			
0.0000	11.60 ± 0.54	0.53 ± 0.03	4.51 ± 0.29	3.32 ± 0.07
0.0075	12.14 ± 1.02	0.57 ± 0.02	4.80 ± 0.46	3.50 ± 0.19
0.0225	12.29 ± 0.43	0.62 ± 0.05	5.06 ± 0.35	3.54 ± 0.15
0.1500	13.00 ± 1.04	0.68 ± 0.09	5.20 ± 0.30	3.44 ± 0.12

[a]Mean ± SEM (8 rats fed diets 9-11 weeks).
[b]Mean ± SEM (10 rats fed diets 6-8 weeks).
[c]Mean ± SEM (4 rats fed diets 5-6 weeks).
[d]Mean ± SEM (5 rats fed diets 7-11 weeks).

protein content was not reported.

The practice of measuring lipid content and composition of livers of rats fed heated oil diets has been repeatedly employed; however, none of the values reported in the literature have been as high as 8-10% of the liver wts as obtained in this study. While not determined in the present study, previous work has indicated that the major lipid accumulated in the liver of rats fed heated fat diets was triglyceride (27,28).

ACKNOWLEDGMENT

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SHORT COMMUNICATION

Gas Chromatographic Separation of Wax Esters Based on the Degree of Unsaturation

ABSTRACT

The wax esters of sperm whale head oil have been characterized by gas-liquid chromatography on an APOLAR 10C column according to their carbon number and number of double bonds. The novel technique permits the direct quantitative analysis of saturated and unsaturated wax esters.

INTRODUCTION

Introduction of the new thermally stable cyanosiloxane liquid phase has permitted gas-liquid chromatography (GLC) of lipid components of higher mol wt based on their degree of unsaturation as indicated in the analysis of diacylglycerols (1). This report describes an application of this technique to the analysis of wax esters.

Usually, GLC of wax esters has hitherto been performed on a nonpolar column, and the results have been presented in the compositions based on the carbon number only (2-6). The analysis of wax esters based on their carbon number and degree of unsaturation has been reported in a few papers: open-tubular GLC of short chain wax esters of carbon numbers < 18 in the jaw fat of the Atlantic bottle-nosed dolphin (7); GLC with a nonpolar column on saturated, monoenoic, and dienoic fractions obtained by $AgNO_3$-silicic acid column chromatography of wax esters in sperm whale head oil (8); and GLC of wax esters in some lantern fish lipids on a diethylene glycol succinate polyester column (9).

This paper presents a simple GLC technique for the resolution of saturated and unsaturated wax esters having carbon numbers up to 40 on a thermally stable polar column.

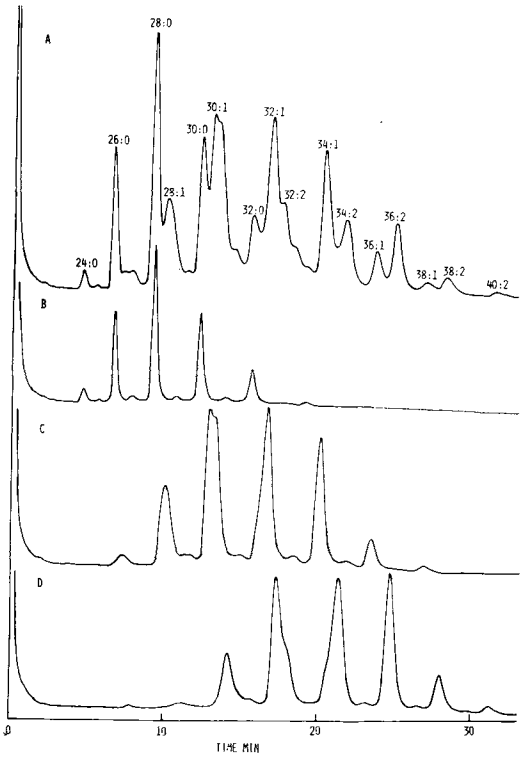

FIG. 1. Gas-liquid chromatographic resolution of wax esters in sperm whale head oil on APOLAR 10C. (A) Total wax esters. (B) Saturated wax esters. (C) Monoenoic wax esters. (D) Dienoic wax esters. The fractions B, C, and D were obtained by $AgNO_3$-thin layer chromatography of the original wax esters.

EXPERIMENTAL PROCEDURES

Wax esters were obtained by silicic acid column chromatography of the head oil of the sperm whale captured at the Antarctic Ocean. The GLC packing material containing 5% APOLAR 10C on Gas Chrom Q (100-120 mesh) was obtained from Applied Science Laboratories (State College, PA). GLC was carried out with a Yanagimoto GC-80 instrument equipped with dual glass columns (150 cm length, 4 mm inside diameter) and a flame ionization detector. The columns were conditioned at 260 C for 10 hr. Detector and injection heaters were operated at 280 C. The column temperature was programmed from 200 to 260 C at the rate of 2 C/min. Difficulties in determining peak areas due to the abnormal shapes of the peaks were overcome by the use of a digital integrator (Shimadzu ITG-4AX). Thin layer chromatographic (TLC) separation of wax esters based on their degree of unsaturation was carried out by developing with benzene:hexane (1:1 v/v) on a $AgNO_3$-silicic

TABLE I

Compositions Percent and ECL Values of Wax Esters[a]

Carbon number	Polar column										Nonpolar column[d]
	Saturated		Monoenoic			Dienoic			Total		
	GLC[b]	TLC-GLC[c]	ECL	GLC	TLC-GLC	ECL	GLC	TLC-GLC	GLC	TLC-GLC	GLC
24	0.6	0.8	-	-	-	-	-	-	0.6	0.8	0.5
26	4.6	5.6	26.9	0.7	1.1	-	-	-	5.3	6.7	5.9
28	10.5	11.6	28.6	6.7	6.6	29.5	0.9	0.5	18.1	18.7	18.1
30	7.4	7.5	30.5	15.2	15.3	31.4	2.2	2.4	24.8	25.2	25.5
32	4.7	3.4	32.8	12.1	12.6	33.9	6.5	5.9	23.3	21.9	22.0
34	1.2	0.5	34.7	9.2	8.1	35.5	6.0	5.8	16.4	14.4	15.0
36	-	-	36.6	2.9	2.0	37.3	4.6	4.7	7.5	6.7	6.8
38	-	-	-	1.2	0.5	-	1.6	1.2	2.8	1.7	2.0
40	-	-	-	-	-	-	0.2	0.3	0.2	0.3	1.0
Others[e]	1.0	1.1	-	-	2.0	-	-	0.5	1.0	3.6	3.2
Total	30.0	30.5		48.0	48.2		22.0	21.3	-	-	-

[a]ECL = equivalent chain length, GLC = gas-liquid chromatography, TLC = thin layer chromatography.

[b]Average of the triplicate analysis in direct GLC. Peaks were identified by comparison of the saturated, monoenoic, and dienoic fractions isolated by $AgNO_3$-TLC with appropriate reference standards.

[c]The fractions isolated by $AgNO_3$-TLC were subjected to GLC on APOLAR 10C. Total percents of each fraction were calculated from their wts.

[d]Column 75 cm length, 0.3 cm inside diameter packed with 2% OV-17 on Chromosorb W 80-100 mesh, temperature programmed 230-330 C at 4 C/min.

[e]Odd chain wax esters from C_{25} to C_{41} and other minor components.

acid (Wakogel B-10) plate. Spots were detected under ultraviolet light after spraying with 2′,7′-dichlorofluorescein reagent.

RESULTS AND DISCUSSION

The gas chromatograms of the total wax esters of sperm whale head oil and the subfractions obtained by $AgNO_3$-TLC on APOLAR 10C are shown in Figure 1. The average of the separation factors, monoene/saturate and diene/monoene, are 1.15 and 1.16, respectively. In spite of the appropriate separation factors, partial overlapping of a few peaks occurs as shown in Figure 1A. This can be attributed to the broadening of the unsaturated ester peaks due to small differences in the retention times of wax esters having the double bonds in the various positions of the acid and alcohol moieties. The double bonds are distributed in positions 5, 7, 9, 11, and 13 in the acids and alcohols of the sperm whale oil (10). The compositions obtained by GLC of the original wax esters on APOLAR 10C and by GLC of the subfractions separated by $AgNO_3$-TLC are compared in Table I. The good agreement indicates the satisfactory accuracy for direct GLC on APOLAR 10C. It was reported previously that the observed compositions of wax esters in sperm whale head oil were in reasonable agreement with those calculated under the assumption of random esterification (8). Random esterification is borne out by the results of the present study.

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Effects of Dietary Saturated and *trans* Fatty Acids on Cholesteryl Ester Synthesis and Hydrolysis in the Testes of Rats

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ABSTRACT

Studies were made of the enzymic synthesis and hydrolysis of cholesteryl esters in rat testes. Weanling rats were fed for 14 weeks diets containing 5% by wt of hydrogenated coconut oil (HCO), a concentrate of ethyl elaidate and linolelaidate (TRANS), devoid of essential fatty acids (EFA), or safflower oil (SAFF). Cholesterol esterifying activity was localized in the soluble fraction, and cholesteryl ester hydrolase activity was distributed in both particulate and soluble fractions obtained from tissue homogenates. The optimum pH was 6.0 for esterification and 6.9-7.0 for hydrolysis. Neither esterifying nor hydrolytic activity was affected by freezing and thawing, but both reactions were inhibited by heat or sonication. The animals of both the HCO and TRANS groups had developed an EFA deficiency before they were sacrificed. The EFA deficiency produced upon feeding the HCO diet had no apparent effect on the synthesis and hydrolysis of cholesteryl esters in rat testes. The TRANS diet influenced the development of the testes as judged by their size, and cholesterol esterifying and cholesteryl ester hydrolyzing activities were suppressed in the testes of the animals of this group. A major difference in the effects of the HCO and TRANS diets on the lipids of the testes was the relatively minor amount of eicosatrienoic acid (20:3) and the elevated level of docosapentaenoic acid (22:5) in the cholesteryl esters of the testicular lipids of the TRANS group.

INTRODUCTION

Among the major metabolic aberrations of an essential fatty acid (EFA) deficiency in rats is impairment of testicular function (1-4). Docosapentaenoic acid is believed to play an important role in spermatogenesis because it is the major EFA of the testis (4,5). Because the testes cholesteryl esters contain relatively large amounts of docosapentaenoic and arachidonic acids (6-8), the effect of an EFA deficiency on testicular function may be due, in part, to a role of these fatty acids in cholesterol metabolism. The presence of cholesterol esterase has been detected in testes (9), and a survey of lecithin:cholesterol acyltransferase (LCAT) activity in rat tissues (10) indicates that this enzyme system also may be present in this organ. However, synthesis and hydrolysis of cholesteryl esters in testes have not been extensively investigated.

The effects of feeding weanling rats diets containing either hydrogenated coconut oil (HCO), or a concentrate of ethyl elaidate and linolelaidate (TRANS) devoid of EFA, as the sole source of dietary fat, on the synthesis and hydrolysis of cholesteryl esters in testes are reported here.

MATERIALS AND METHODS

Animals and Diets

Male Sprague-Dawley rats purchased from Dan Rolfsmeyer Co. (Madison, WI) were used in all experiments. For studies of the properties and localization of enzyme activity, 300-400 g animals fed Purina Lab Chow were used. For studies of effects of diets containing either saturated or *trans* fatty acids, weanling animals weighing 50-60 g were divided into three groups and fed for 14 weeks a fat-free diet containing minerals and vitamins in the required amounts (11) and 5% by wt of safflower oil (SAFF), hydrogenated coconut oil (HCO), or a concentrate of ethyl elaidate and linolelaidate (TRANS) prepared from elaidinized safflower oil ethyl esters (12). The fatty acid composition of the TRANS fat was 21.3% palmitic, 9.1% stearic, 20.8% elaidic, and 47.5% linolelaidic acids. Data on the fatty acid composition of the sera, hearts, livers, and kidneys, and growth of the animals are reported elsewhere (12).

Enzyme Preparation

The rats were killed by decapitation and the testes were immediately ecised. After removal of the capsule and visible blood vessels, the testes were minced and fractionated by a combination of the methods of Vanha-Perttula (13) and Kennan et al. (14) as follows: The testes of

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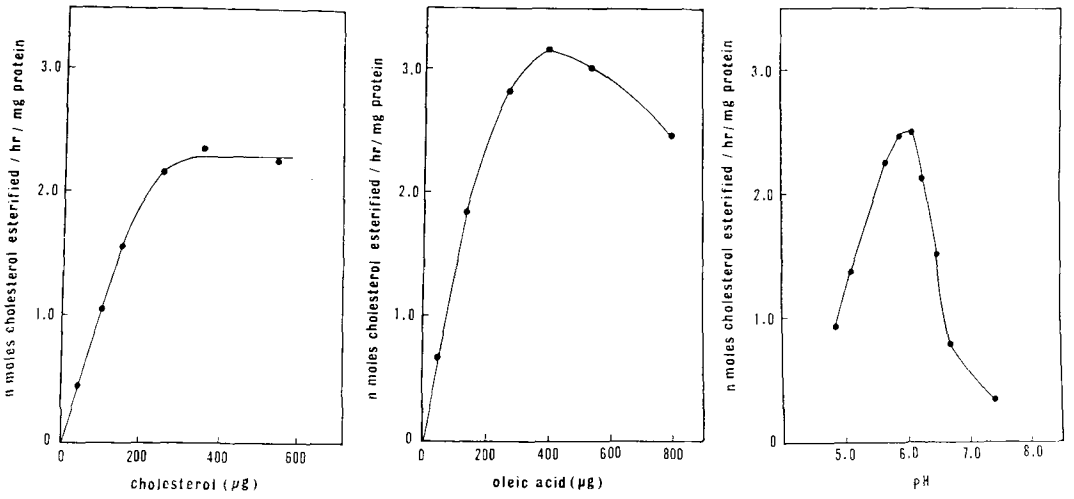

FIG. 1. Effect of substrate concentration, cholesterol and oleic acid, and pH on cholesterol esterifying activity of rat testicular tissue. Except for the factor being varied (concentration of substrates or pH), each incubation was carried out at 37 C for 2 hr with an incubation mixture of 0.9 ml of 0.1 M potassium phosphate buffer and 0.1 ml of enzyme preparation, Fraction II (see text for method of preparation), containing 250 μg of cholesterol with or without 0.05 μCi of 4-^{14}C-cholesterol and 400 μg of oleic acid with or without 0.05 μCi of 1-^{14}C-oleic acid.

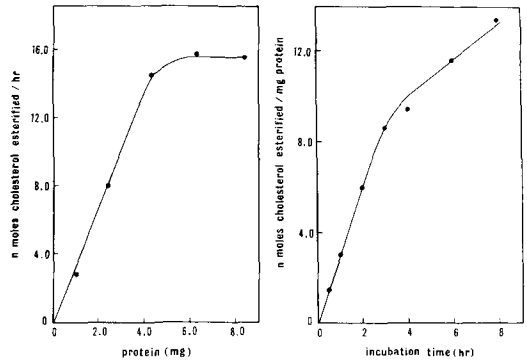

FIG. 2. Effect of enzyme concentration (mg protein/ml) and time course on cholesterol esterifying activity of rat testicular tissue. Except for the factors being varied (enzyme concentration [mg protein/ml of incuabation mixture] and time course of reaction), each incubation was carried out at 37 C for 2 hr with an incubation mixture of 0.9 ml of 0.1 M potassium phosphate buffer and 0.1 ml of enzyme preparation, Fraction II (see text for method of preparation), containing 250 μg of cholesterol and 0.05 μCi 4-^{14}C-cholesterol and 400 μg of oleic acid at pH 6.0.

each animal were homogenized in 5 ml of 0.25 M sucrose using a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 750 x g for 10 min to remove cell debris and nuclei. The supernatant fraction was designated as Fraction I and was used as the enzyme source in some experiments. The mitochondrial fraction was prepared from this fraction by resuspending the pellet in 5 ml of 0.25 M sucrose and recentrifugation under the same conditions. The supernatant fraction obtained in the preparation of the mitrochondrial fraction was designated as Fraction II. This fraction was centrifuged at 105,000 x g for 1 hr to yield a pellet of the microsomal fraction. The supernatant fraction from this centrifugation was designated as the "soluble fraction." All procedures were carried out at O C. The protein content of all fractions was determined by the Lowry et al. method (15) using bovine serum albumin as a standard.

Substrates and Other Materials

1-^{14}C-Oleic acid (sp act 51.2 mCi/mmol), 4-^{14}C-cholesterol (sp act 52.0 mCi/mmol), and 4-^{14}C-cholesteryl linoleate (sp act 20.9 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, MA). Impurities were separated from these preparations by thin layer chromatography (TLC) using plates coated with Silica Gel H and a solvent system of petroleum ether:diethyl ether:acetic acid (85:15:1, v/v/v).

Bovine serum albumin (Fraction V), adenosine triphosphate (ATP; sodium salt), and Coenzyme A (CoA) were purchased from the Sigma Chemical Co. (St. Louis, MO). Oleic acid, cholesterol, and cholesteryl linoleate were obtained from the Lipids Preparation Laboratory of The Hormel Institue (Austin, MN).

Scintillation chemicals, 2,5-diphenyloxazole (PPO) and 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene (dimethyl POPOP),

TABLE I

In Vitro Cholesteryl Ester Synthesis in Rat Testicular Tissue[a]
(nmol cholesterol esterified/hr/mg protein)

Exogenous cofactors	Labeled substrates	
	4-^{14}C-cholesterol	1-^{14}C-oleic acid
Control (none)	2.59	2.87
Plus ATP[b]	2.40	1.81
Plus CoA[b]	2.58	2.80
Plus ATP and CoA[c]	2.01	1.27

[a]Standard assay conditions (see text for details).
[b]Five μmol/ml of incubation mixture. ATP = adenosine triphosphate.
[c]Five μmol each/ml of incubation mixture.

were obtained from Packard Instrument Co. (Downers Grove, IL).

Incubation Procedures

Benzene solutions of the substrates cholesterol (or cholesterol containing 0.05 μCi of 4-^{14}C-cholesterol) and oleic acid (or oleic acid containing 0.05 μCi of 1-^{14}C-oleic acid) or cholesteryl linoleate containing 0.05 μCi of 4-^{14}C-cholesteryl linoleate were evaporated in glass test tubes under a stream of nitrogen. The residue was dissolved in 50 μl of acetone which was dispersed rapidly in 0.9 ml of 0.1 M potassium buffer by mixing vigorously using a Vortex mixer. Then 0.1 ml of either whole homogenate or a subcellular fraction thereof was added as the enzyme source. The final solutions were incubated at 37 C with shaking in a water bath. Controls for each sample were made without the addition of the enzyme or without incubation.

All incubations were carried out in an atmosphere of air, and the reactions were stopped by the addition of 8 vol of choloroform: methanol (2:1, v/v). This mixture was allowed to stand overnight, and the upper water:methanol phase was filtered and the precipitate washed twice with fresh chloroform:methanol (2:1, v/v). The filtrates were combined and evaporated in vacuo at 30 C in a water bath. The residue was dissolved in ethyl ether and fractionated by TLC on plates coated with Silica Gel H with petroleum ether:diethyl ether:acetic acid (85:15:1, v/v/v). Cholesterol and cholesteryl ester spots were made visible by spraying the plate with 1% iodine in methanol. The plates were marked and, after evaporation of the iodine, the cholesterol and cholesteryl ester fractions were scraped into scintillation vials containing 15 ml of a solution containing 5 g of PPO and 0.3 g of dimethyl POPOP per liter toluene for determination of their radioactivities in a Packard Tricarb Model 3002 liquid scintillation spectrometer. Counting efficiency was 85% for the standards dissolved directly in the toluene solution and 71% for samples recovered from chromatoplates.

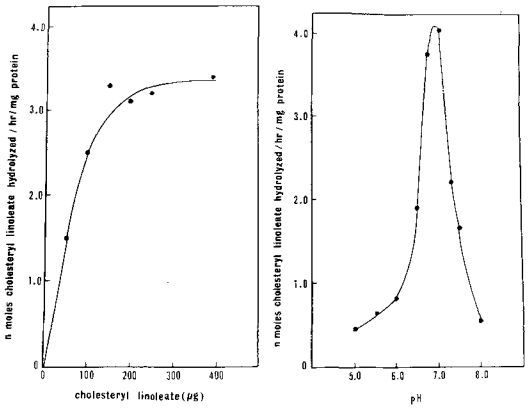

FIG. 3. Effect of substrate, cholesteryl linoleate, and pH on cholesteryl ester hydrolase activity of rat testicular tissue. Except for the factor being varied (substrate concentration and pH), each incubation was carried out at 37 C for 2 hr with an incubation mixture of 0.9 ml of 0.1 M potassium phosphate buffer and 0.1 ml of enzyme preparation, Fraction II (see text for method of preparation), and 200 μg of cholesteryl linoleate containing 0.05 μCi of 4-^{14}C-cholesteryl linoleate at pH 7.0.

The concentration of cholesterol or cholesteryl esters was determined in separate experiments by quantitative TLC, using pure compounds as standards, and the charring-photodensitometric technique (16). The amount of endogenous cholesteryl ester in the homogenates or the subcellular fractions was negligible compared to the amount of exogenous cholesteryl linoleate added to the incubation mixtures. All incubations were carried out in duplicate, and enzyme activities were expressed as nmol cholesterol esterified or cholesteryl ester hydrolyzed per hr per mg protein.

TABLE II

In Vitro Synthesis and Hydrolysis of Cholesteryl Esters in Rat Testicular Tissue[a]

Treatment	Effect of physical treatments			
	Esterification[b]		Hydrolysis[c]	
	Activity	% Inhibition	Activity	% Inhibition
Control	2.96	0	7.10	0
Heat: 56 C, 5 min	1.29	56.4	5.05	28.9
10 min	0.83	72.0	3.65	48.6
30 min	0.09	97.0	0.66	93.0
Sonication: 3 min at 4 C[d]	1.26	57.4	2.20	69.0
Freeze-thawing: -20 C for 48 hr	2.86	3.3	6.46	9.0

	Localization of activity			
	Experiment 1		Experiment 2	
	Esterification[b]	Hydrolysis[c]	Esterification[b]	Hydrolysis[c]
Whole homogenate	1.20	4.34	1.23	3.09
Fraction I		4.34		8.81
Mitochondria	1.80	3.03	1.19	3.56
Fraction II	2.54		2.56	
Microsomes	1.35	6.51	1.51	3.72
Soluble fraction	3.18	4.67	3.24	2.62

[a]See text for standardized assay conditions and fractionation procedure.

[b]nMol of cholesterol esterified/hr/mg protein.

[c]nMol cholesteryl linoleate hydrolyzed/hr/mg protein.

[d]See text for details.

Fatty Acid Analysis

The total lipid and individual lipid classes were converted to methyl esters using HCl as a catalyst and analyzed by gas liquid chromatography (GLC), as previously described (17). The conditions employed in these analyses, as well as the method for the extraction of the lipid and isolation of the individual lipid classes by TLC, are the same as those described in other studies on these animals (12).

RESULTS

Cholesterol Esterifying Activity

Conditions for the assay of cholesterol esterifying activity were developed from a study of the effect of pH and concentration of substrates shown in Figure 1. These results showed that the rate of esterification plateaued at a concentration of cholesterol of 250 μg. The reaction appeared to be sensitive to oleic acid concentration, with the optimum amount being 400 μg. The optimum pH of the reaction was relatively sharp at 6.0.

The linearity of the reaction relative to protein content of the enzyme preparation and the time course of the reaction are shown in Figure 2. The rate of the reaction increased linearly, with an increase in enzyme concentration up to 4 mg of protein, and the time course was linear for ca. 4 hr. Hence, the standard conditions developed for the assay of cholesterol esterifying activity consisted of carrying out the reaction with 2-3 mg enzyme protein, 250 μg of cholesterol, and 400 μg of oleic acid per ml of incubation mixture at a pH of 6.0 for 2 hr at 37 C.

The addition of ATP or CoA did not stimulate the reaction, as shown in Table I, using 4-^{14}C-cholesterol as the labeled substrate. When the assay was conducted with 1-^{14}C-oleic acid as the labeled substrate with added ATP or CoA, there appeared to be an inhibition of cholesterol esterification. However, this effect was attributed to the fact that oleic acid was utilized in other reactions in competition with its esterification with cholesterol.

Cholesteryl Ester Hydrolase Activity

Studies of the cholesteryl ester hydrolase activity showed that the optimum pH was between 6.9 and 7.0 and the reaction plateaued at a substrate concentration of 150-300 μg of cholesteryl linoleate (Fig. 3). Larger amounts of substrate had no inhibitory effect on the reaction. Experiments similar to those conducted for the esterification of cholesterol (Fig. 2) showed that the hydrolysis was linear up to a concentration of enzyme protein of ca. 5 mg

per ml incubation mixture for at least 4 hr. Hence, the standard conditions developed for the assay of cholesteryl ester hydrolase activity consisted of carrying out the reaction with 2-3 mg of enzyme protein and 200 μg of cholesteryl linoleate per ml of incubation mixture at pH 7.0 with a reaction time of 2 hr at 37 C.

Effects of Physical Treatments and Localization of Enzyme Activities

Using the standardized assay conditions, it was found that freezing the enzyme preparation (Fraction I) 48 hr at -20 C had no effect on either the synthesis or hydrolysis of cholesteryl esters as shown in Table II. However, both reactions were inhibited by heat and sonication (Table II). The sonication was carried out on the enzyme preparation (Fraction I) using Sonifier Cell Disruptor, Model W 140 D, equipped with a microtip (Ultrasonics, Inc., Long Island, NY) in six 30-sec treatments in an ice water bath.

Assay of the enzyme activities in the subcellular fractions (Table II) indicated that the esterifying activity was greatest in the soluble fraction. This fraction contained ca. threefold the activity of the whole tissue homogenate. However, some activity was also present in the mitochondrial and microsomal fractions. Hydrolase activity was greatest in the microsomal fraction.

Effects of Dietary Fats on Cholesteryl Ester Synthesis and Hydrolysis

Application of the assay methods to Fraction I showed that the EFA deficiency induced by the HCO diet had no effect on the synthesis or hydrolysis of cholesteryl esters (Table III). However, both reactions were suppressed in the testes of the animals fed the TRANS diet. The testes of the animals of this group were much smaller than those of the other groups (Table III).

The fatty acid composition of the testicular lipids of the animals of the three groups (Table IV) showed that the animals fed the HCO and the TRANS diets exhibited the characteristic pattern of an EFA deficiency, namely, a reduction of EFA with a corresponding increase in monoenoic and eicosatrienoic acids. The testicular lipid of the animals fed the TRANS diet also contained a small amount of linolelaidic acid, as might be expected. The fatty acid compositions of the individual lipid classes (Table IV) showed that marked differences existed between the cholesteryl esters of the testicular lipids of the animals fed the TRANS diet and those fed the HCO diet or the SAFF diet. The most striking of these were the relatively minor

TABLE III

Effect of Dietary Fat on the In Vitro Cholesteryl Ester Synthesis and Hydrolysis in Testicular Tissue of Rats 14 Weeks after Weaning

Diet[a] supplement	Testes wt	p[b]	Cholesterol (mg/g, wet wt)	Cholesteryl esters (mg/g, wet wt)	P	Cholesteryl esters			
						Synthesis[c]	P	Hydrolysis[d]	P
SAFF	3.5 ± 0.30[e]		1.34 ± 0.17	0.09 ± 0.005		4.42 ± 0.79		10.05 ± 1.98	
HCO	3.1 ± 0.15	NS	1.35 ± 0.35	0.11 ± 0.021	NS	3.28 ± 0.39	NS	9.49 ± 0.93	NS
TRANS	1.5 ± 0.37	<0.001	1.33 ± 0.30	0.28 ± 0.108	<0.025	1.00 ± 0.29	<0.001	2.31 ± 0.36	<0.001

[a]SAFF = safflower oil; HCO = hydrogenated coconut oil; TRANS = elaidinized safflower oil ethyl esters.
[b]Student's *t* test for significance different from SAFF group (NS = not significant).
[c]nMol cholesterol esterified/hr/mg protein under standard assay conditions (see text).
[d]nMol cholesteryl linoleate hydrolyzed/hr/mg protein under standard assay conditions (see text).
[e]Mean ± SD.

TABLE IV

Fatty Acid Composition of Testicular Tissue Lipid of Rats 14 Weeks after Weaning (% by wt)

Diet[a]	16:0	18:0	16:1	18:1	Linolelaidate	18:2	20:3	20:4	22:5
				Total lipid					
SAFF	27.5 ± 1.6[b]	5.8 ± 0.5	1.7 ± 1.0	13.6 ± 1.8	--	10.3 ± 3.0	--	14.4 ± 1.6	18.8 ± 2.3
HCO	30.2 ± 1.0	6.2 ± 0.3	3.9 ± 1.0	24.6 ± 2.8	--	0.4 ± 0.1	8.3 ±0.7	6.5 ± 0.4	9.7 ± 0.9
TRANS	21.3 ± 1.4	5.8 ± 0.6	10.8 ± 1.1	38.7 ± 2.1	4.9 ± 1.3	0.8 ± 0.2	2.5 ± 1.5	5.4 ± 1.0	4.9 ± 1.7
				Triglycerides					
SAFF	25.1 ± 2.4	2.5 ± 0.2	7.2 ± 0.9	21.2 ± 2.5	--	24.4 ± 3.6	--	2.8 ± 0.4	9.4 ± 2.6
HCO	28.6 ± 1.5	2.6 ± 0.2	13.0 ± 2.1	37.8 ± 3.2	--	trace	3.5 ± 1.0	0.9 ± 0.1	3.0 ± 0.8
TRANS	20.4 ± 1.3	2.1 ± 0.3	19.1 ± 0.6	45.0 ± 1.4	8.6 ± 1.4	trace	trace	trace	--
				Cholesteryl esters					
SAFF	19.6 ± 1.8	6.2 ± 0.6	3.2 ± 0.7	9.5 ± 2.1	--	8.3 ± 0.9	--	17.9 ± 3.2	17.1 ± 1.7
HCO	14.9 ± 3.5	7.2 ± 1.3	7.7 ± 2.1	24.0 ±1.6	--	3.8 ± 2.6	14.8 ± 1.4	6.1 ± 0.7	7.2 ± 3.3
TRANS	11.2 ± 0.1	4.9 ± 0.4	2.8 ± 0.7	29.3 ± 4.9	trace	trace	1.9 ± 0.7	2.9 ± 0.5	27.9 ± 5.0
				Phosphatidyl choline					
SAFF	40.8 ± 0.9	3.0 ± 0.2	0.7 ± 0.1	15.3 ± 0.2	--	2.3 ± 0.2	--	14.0 ± 0.5	20.1 ± 0.3
HCO	35.3 ± 0.7	3.4 ± 0.2	1.5 ± 0.2	19.2 ± 1.4	--	0.5 ± 0.1	12.0 ± 1.6	7.4 ± 0.5	13.6 ± 0.6
TRANS	38.3 ± 1.0	6.0 ± 1.1	1.3 ± 0.2	25.9 ± 3.5	trace	1.0 ± 0.3	5.3 ± 2.0	11.4 ±3.6	7.3 ± 1.6

[a]SAFF = safflower oil; HCO = hydrogenated coconut oil; TRANS = elaidinized safflower oil ethyl esters.
[b]Mean ± SD.

amount of 20:3 and the elevation of 22:5 in the cholesteryl esters of the animals fed the TRANS diet.

DISCUSSION

The present study indicates that cholesteryl ester synthesis and hydrolysis are suppressed in the testes or rats upon feeding *trans* fatty acids devoid of EFA. On the other hand, these reactions do not appear to be affected by an EFA deficiency produced upon feeding HCO as the sole source of fat in the diet.

The elevated level of 18:1 and low level of 20:3 in the testicular lipids of the animals fed the TRANS diet compared to those of the animals fed the HCO diet indicate that the interconversion of unsaturated fatty acids is also impaired in the testes of rats upon feeding *trans* fatty acids devoid of EFA as the sole source of dietary fat. The increased level of 22:5 in the cholesteryl esters of the testes of the animals fed the TRANS diet compared to those of the other groups may be attributed to impairment of hydrolase activity in the testes of these animals.

No attempt was made to delineate the enzyme systems involved in the cholesterol esterifying and cholesteryl ester hydrolyzing activities via isolation and purification techniques as recently described for rat aorta, for example (18). However, it is noteworthy that exogenous CoA and ATP did not stimulate either cholesteryl ester synthesis or hydrolysis. Glomset and Kaplan (10) reported a low level of LCAT activity in rat testes using ^{3}H-cholesterol as the labeled substrate under the assay conditions for this enzyme. However, because this observation could have been due to cholesterol esterifying activity, we tested for LCAT activity in rat testes in accessory experiments using 2-oleoyl-1-^{14}C-phosphatidyl choline as the labeled substrate. No radioactive cholesteryl esters were detected in these experiments. Hence, in view of the fact that radioactive cholesteryl esters in Glomset and Kaplan's assay could have been produced by cholesterol esterifying activity, inasmuch as this reaction takes place readily without exogenous ATP and CoA (18,19), LCAT does not appear to contribute to cholesteryl ester synthesis in rat testes.

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Lipids of *Klebsiella pneumoniae:* The Presence of Phosphatidyl Choline in Succinate-Grown Cells[1]

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ABSTRACT

The carbon and energy source for aerobically grown cultures of *Klebsiella pneumoniae* profoundly influenced the total lipid content and phosphatide composition. Glucose-grown cells contained 13% lipid, 56% of which was phospholipids. Succinate-grown cells contained 8% lipid, 66% of which was phospholipids. The predominant phosphatides of glucose-grown cells were phosphatidyl ethanolamine, 82%; phosphatidyl glycerol, 4.5%; phosphatidic acid, 5%; cardiolipin, 6.5%; phosphatidyl serine; and trace amounts of unidentified phosphatides. Phosphatides of succinate-grown cells were phosphatidyl ethanolamine, 38%; diphosphatidyl glycerol, 14%; phosphatidyl glycerol, 13%; phosphatidyl choline, 14.5%; phosphatidyl serine, 6%; phosphatidic acid, 4%; and 10% unknown lipids. No trace of phosphatidyl choline was found in glucose-grown cells.

INTRODUCTION

It has long been established that phospholipids and lipid-synthesizing enzymes in bacteria are associated with the cell membrane (1). The culture and growth conditions influence the biosynthesis and activity of membrane-bound enzymes (2-4) with concomitant variation in the content and the composition of lipids (5-9). Succinic dehydrogenase is the only enzyme of the tricarboxylic acid cycle (TCA) that is membrane bound in *Escherichia coli* (10). Changes in the environment may consequently affect respiratory activity, functioning of the TCA, and composition of lipids. Therefore, it was of interest to study the lipid content and composition of *Klebsiella pneumoniae* grown aerobically on both succinate and glucose as the only carbon and energy source.

MATERIALS AND METHODS

Organism and Methods of Culture

The strain of *K. pneumoniae* used throughout this study is a spontaneous smooth mutant of the wild encapsulated type. Isolation and characterization of this mutant will be discussed in another publication. Cells used for inoculation were grown aerobically overnight at 35 C in 200 ml growth medium containing (g/liter): substrate (glucose or succinate), 5; NH_4NO_3, 6; K_2HPO_4, 5; KH_2PO_4, 2; 0.5 ml of 1% $CaCl_2$-$2H_2O$ solution in water; 1 ml of trace elements solution containing (g/liter): $MnCl_2 \cdot 4H_2O$ 0.004; $CuSO_4 \cdot 5H_2O$, 2.0; $ZnSO_4 \cdot 7H_2O$, 2.2; and ammonium molybdate, 0.22; pH was adjusted to 6. After autoclaving, 1.0 ml of 1.0% solution of Fe $SO_4 \cdot 7H_2O$ in water was added. This culture was used as an inoculum for all subsequent experiments.

For large batch experiments, 31 media were inoculated with 50 ml inoculum in 31 carboys fitted with compressed air lines and incubated at 35 C. Cells were harvested in their mid-log phase (Klett reading of 160 and 280 for glucose and succinate grown cells, respectively) by centrifugation at 10,000 x g for 10 min. Four hundred milliliters of supernatant media were retained for lipid extraction. Cells were washed twice with 0.1 M Tris-HCl buffer, pH 7.0, resuspended in 10 ml of water, and lipids were extracted.

A solution containing 25 μc of L [^{14}C-methyl] methionine (56.4 mc/mmol) was added to *K. pneumoniae* cells grown to their mid-log phase in glucose or succinate media, and incubation was contained for 3 hr. Cells were harvested by centrifugation and washed as described above. Lipids were extracted (11,12), and suitable aliquots were counted by a Packard Tri-Carb Liquid Scintillation Spectrometer using 10 ml of scintillation counting cocktail (omniflour, New England Nuclear, Boston, MA, 4.5 g/liter in toluene). Labeled lipids separated by paper chromatography (13) were autoradiographed and analyzed as described by Wassef et al. (13) and Kates et al. (14).

Dry Weight Determinations

Cells of *K. pneumoniae* were grown in 50 ml of the defined media for glucose and succinate, inoculated with 0.1 ml of the corresponding cell inoculum, and incubated at 35 C with shaking. Aliquots of 5 ml were withdrawn at different time intervals and determined Klett reading. Cells were then retained on a pre-

[1]Paper 75-11-170 of the Kentucky Agricultural Experiment Station.

TABLE I

Separation of Lipids of *Klebsiella pneumonia* into Polarity Groups by Silicic Acid Column Chromatography

Fraction and eluting solvent[a]	Percent of total lipids Cells grown on		Percent of tissue Cells grown on	
	Glucose	Succinate	Glucose	Succinate
Chloroform (S_1)	15.3	8.2	1.8	0.89
Chloroform:acetone (1:1 v/v) (S_2)	22.0	20.4	2.7	1.8
Acetone (S_3)	7.1	5.6	0.88	0.41
Methanol (S_4)	55.6	65.8	6.9	4.7

[a]See ref. 15 for fraction composition.

[b]Oven dry-wt basis.

TABLE II

Percent Composition of Glycolipids in *Klebsielka pneumoniae*

Glycolipid composition	Percent composition Cells grown on	
	Succinate (%)	Glucose (%)
Lipids of monoglycosyldiglyceride polarity	28.9	38.1
Lipids of diglycosyl diglyceride polarity	56.0	61.9
Chromatographic origin	15.1	0

weighed millipore filter, washed twice with 0.1 M Tris-HCl buffer at pH 7.0, followed by water, dried overnight, and weighed.

Extraction and Fractionation of Lipids

Lipids were extracted by the method of Bligh and Dyer (11) as modified by Kates et al. (12). The total lipid extracts were freed of non-lipid contaminants by passage through a Sephadex G-25 column and were separated into polarity groups by silicic acid column chromatography according to the procedure described by Rouser et al. (15). Wts were determined on Fraction 1 from the Sephadex column (F1) and on all four fractions of the silicic acid column by an electrobalance procedure (15). Techniques for preventing autooxidation of lipids were carried out according to Rouser et al. (15).

Analytical Procedures

Qualitative analyses of the total lipid extracts (F1) and silicic acid column fractions were made by one- and two-dimensional thin layer chromatograph (TLC) (15-17), and the silicic acid-impregnated Whatman 3 MM paper chromatographic procedure of Marinetti (18), as described by Kates (19). Specific spray and dip reagents were used according to Kates (19,20). TLC plates were traced with a Brinkmann photocopier (Brinkmann Instruments, Inc., Westbury, NJ).

The identity of the Phospholipids was established by comparison of R_f values and co-chromatography with those of appropriate standards (obtained from Supelco Inc., Bellefonte, PA) and by the mild alkaline hydrolysis procedure of Dawson (21) as modified by Chang and Kennedy (22). The phosphoryl products were separated by paper chromatography (23,24) and detected by specific reagents (20,25).

Glycolipids separated on TLC were syphoned off the plates and measured by the procedure described by Dittmer and Wells (26). Corresponding results for glycolipid content were also produced using the method described by Kates (20). Total phosphorus was determined as described by Rouser et al. (27) on TLC and as described by Wassef et al. (28) on paper chromatograms. Ester bonds were determined on TLC as described by Kates (20).

RESULTS

Overall Lipid Analysis

When *K. pneumoniae* cells were grown aerobically in glucose as the only energy and carbon source, extractable total lipids amounted to 13.0% of the dry wt of the cells. This value was only 8.1% when succinate was used as the only energy and carbon source. Fractionation of total lipids from cells grown on either source, according to their polarity

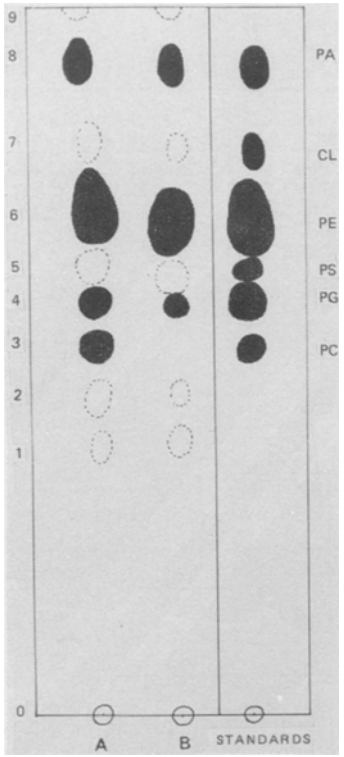

FIG. 1. Paper chromatogram of phospholipids from *Klebsiella pneumoniae* cells grown on (A) succinate and (B) glucose media. Chromatogram was run on silicic acid impregnated Whatman 3 MM paper in diisobutylketone:acetic acid:water (40:25:5 v/v) and stained with Rhodamine 6G. Similar chromatograms were treated with Periodate-Sciff, ninhydrine, and choline stains. Staining behavior and quantitative composition are given in Table III. Compounds present in higher and lower concentrations are represented by shaded and dotted lines, respectively. PA = phosphatidic acid, CL = diphosphatidyl glycerol (Cardiolipin), PE = phosphatidyl ethanolamine, PS = phosphatidyl serine, PG = phosphatidyl glycerol, PC = phosphatidyl choline.

using silicic acid column chromatography, is shown in Table I. Phospholipids constituted 55.6% and 65.8% of the total lipids extracted from cells grown on glucose or succinate, respectively, whereas glycolipids, Fractions 2 and 3, made up ca. 26-29% of the total lipids (Table I). It seems that cells grown on glucose produce twice as much neutral lipids, 15%, compared with 8%, as cells grown on succinate. On a cell dry wt basis, phospholipids constituted 6.9% and 4.7%, glycolipids formed ca. 3.6% and 2.2%, and neutral lipids made up 1.8% and 0.9% for glucose- and succinate-grown cells, respectively (Table I).

After *K. pneumoniae* cells were grown to mid-log phase and harvested by centrifugation, 400 ml of the supernatant medium was extracted by the procedure of Bligh and Dyer (11) as modified by Kates (12). No appreciable amount of lipid was found in this medium.

Neutral Lipid Analysis

The neutral lipid fraction was separated on a silicic acid column (S1) and chromatographed on TLC using the solvent system described by Kates and Paradis (17). Diglycerides appear to be the predominant neutral lipid component for cells grown on succinate or glucose. They composed over 95% of the total S1. Traces of monoglycerides, free fatty acids, and some minor unknown components were also present.

Glycolipid Analysis

When glycolipid fractions were separated on two-dimensional TLC (15) and the sugar content was determined (20), succinate-grown cells contained 28.9% of their glycolipids as monoglycosyl diglyceride and 56.0% as the diglycosyl isomer; whereas these values were 38.1% and 61.9% for glucose grown cells (Table II). There was ca. 15% of unknown sugar-containing lipids at the origin in case of succinate-grown cells that could not be resolved either by paper chromatography or TLC.

Phospholipid Analysis

The phospholipid fraction separated on a silicic acid column (S4) was chromatographed both on paper and on two-dimensional TLC in three different solvents. Figure 1 shows a paper chromatogram of the phospholipid composition of *K. pneumoniae* cells grown on succinate and on glucose; staining behavior and quantitative composition are given in Table III. It is clear that the phospholipid profile for cells grown on either substrate is similar except for the presence of a phosphatide in the cells grown on succinate that is absent in the cells grown on glucose (Fig. 1). That extra phosphatide present in the lipids of succinate-grown cells had a mobility similar to that of standard phosphatidyl choline (PC) and gave positive reactions when the paper chromatogram was treated either with the phosphomolybdic acid procedure or the Dragendorff reagent for choline (20). The aqueous soluble products obtained after mild alkaline hydrolysis of the phosphatides of glucose- and succinate-grown cells were examined by paper chromatography in two solvents. It was evident from the migration and staining behavior of the glycerophosphoryl lipids that succinate-grown cells contained a component that was not present in glucose grown cells, and that the migration of this com-

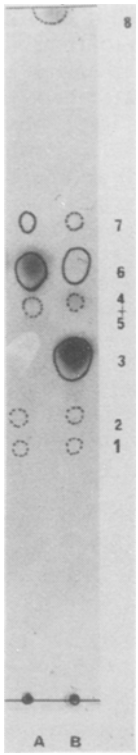

FIG. 2. Chromatogram and autoradiography of total lipids of *Klebsiella pneumoniae* cells incubated with [^{14}C-CH_3] methionine and grown on glucose A and succinate B. Chromatographic conditions and spot identification as in Figure 1.

ponent was similar to that of the glycerophosphoryl choline standard.

Further evidence for PC production was derived from the incorporation of [^{14}C-methyl] methionine into lipids of *K. pneumoniae* (Fig. 2). Over 90% of the ^{14}C-activity incorporated by succinate-grown cells was associated with one component (Fig. 2, Spot 3, Lane B) which co-chromatographed with standard PC. Acid hydrolysis and milk-alkaline hydrolysis were performed on Spot 3 (Fig. 2, Lane B). The mild-alkaline hydrolysis produced a phosphoryl component that had the same paper chromatographic mobility of standard glycerophosphoryl choline. Acid hydrolysis of the eluate of Spot 3 produced a water-soluble base that corresponded to authentic choline when examined by paper chromatography.

Lipids of glucose-grown cells incubated with [^{14}C-methyl] methionine (Fig. 2, Lane A) contained over 90% of the radioactivity in phosphatidyl ethanolamine (PE). Upon mild alkaline hydrolysis of eluted PE (from paper chroma-

TABLE III

Paper Chromatographic Analysis of Phospholipid Fraction of Lipids of *Klebsiella pneumoniae*

Spot number[a]	Ninhydrin spray	Periodate schiff	Choline stain	Phosphorus stain	Lipid-P (%) Cells grown on Succinate	Glucose	Spot identification[b]
origin	trace	-	-	trace	trace	trace	
1	-	+	-	+	trace	trace	unidentified
2	-	-	-	+	10.0	trace	unidentified
3	-	-	+	+	14.5	-	PC
4	-	+	-	+	12.8	4.5	PG
5	+	-	-	+	5.9	1.9	PS
6	+	-	-	+	38.1	82.1	PE
7	-	-	-	+	14.4	6.5	CL
8 + 9	-	-	-	+	4.3	5.0	PA + neutral lipids

[a]See Figure 1.

[b]PC = phosphatidyl choline, PG = phosphatidyl glycerol, PS = phosphatidyl serine, PE = phosphatidyl ethanolamine, CL = diphosphatidyl glycerol (Cardiolipin), PA = phosphatidic acid.

togram), the radioactivity was associated almost entirely with the fatty acid fraction. Although fatty acids were not examined, cyclopropane fatty acids are suspected to contain almost all the radioactivity. This is in view of the fact that cyclopropane fatty acids are biosynthesized by the addition of a C1 unit (from methionine) across the double bond of *cis*-vaccinic acid (29) or palmitoleic acid (30). This active donor of the C1 unit used to form the ring was shown to be derived from S-adenosylmethionine (31).

Studies on the production and biosynthesis of PC by *K. pneumonia* have recently been reported by Wassef (unpublished data).

These results show that *K. pneumoniae* grown on succinate-containing medium produces PC as a major phosphatide in addition to PE. Glucose-grown cells, on the other hand, do not produce PC, and PE is the major phosphatide, a situation similar to that of other gram-negative bacteria (1).

DISCUSSION

The content and composition of lipids in bacteria have long been known to vary with the composition of the culture medium (1). The increase found in the lipid content of glucose-grown *K. pneumoniae* cells agrees with the findings of Geiger and Anderson (32). Furthermore, the increase in lipid content of glucose-grown cells is only apparent in the neutral lipid fraction of these cells. The major component of the neutral lipid in *K. pneumoniae* is a diglyceride. The composition of the individual fatty acids was not examined, and their determination is warranted.

The widespread distribution of glycolipids in bacteria is now firmly established (33). Although the glycolipid fraction comprised 25-29% of the total lipids extracted from *K. pneumoniae,* the exact composition was not determined. However, the general trend is that the diglycosyl diglyceride and lipids of the same polarity are present in twice the concentration of the monoglycosyl diglyceride group. There is some unknown sugar-containing lipid that could not be resolved or identified in cells grown on succinate.

PC is known to be a major phosphatide in animal, plant, and fungal lipids. By contrast, PC appears to occur in only relatively limited groups of bacteria (34). *K. pneumoniae* contained ca. 15% of its total phosphatides as PC when the cells were grown on glucose. Since the phospholipids are known to be located in the cell membrane of gram-negative bacteria (1,34), this difference in the composition of one of the main phospholipids of *K. pneumoniae* would be expected to result in significant differences in electron transport, permeability, and other surface properties in the same organism grown on glucose or succinate. Reviewing bacterial phospholipids, Ikawa (35) observed that PC is present in bacteria requiring highly efficient electron transport, and he also speculated that lecithin-containing bacteria might be the more advanced form in evolution. Hagen et al. (36) suggested a correlation between bacterial lecithin and intracytoplasmic membrane structure, and demonstrated PC to be present in Hyphomicrobium and *Nitrosocystis oceanus,* organisms which have complex structures (36,37). Therefore, it may be of great interest to determine whether there are major differences between *K. pneumoniae* grown on media containing glucose, succinate, or other organic acids with regard to the electron transport system, intracellular structures, or permeability; and whether the enzyme(s) capable of producing PC are not present, inhibited, or catabolite repressed.

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Autoxidation of Polyunsaturated Fatty Acids: II. A Suggested Mechanism for the Formation of TBA-Reactive Materials from Prostaglandin- like Endoperoxides

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ABSTRACT

The nature and mechanism of formation of the thiobarbituric acid (TBA)-reactive material produced in the autoxidation of polyunsaturated fatty acids (PUFA) or their esters has been studied. On the basis of chemical studies and spectroscopic evidence, it is concluded that the TBA test detects malonaldehyde which arises at least in part from the acid-catalyzed or thermal decomposition or endoperoxides (2,3-dioxanorbornane compounds). These endoperoxides have structures related to those of the endoperoxides produced in the biosynthetic sequence leading to prostaglandins. A mechanism is proposed in which these endoperoxides are formed in a free radical cyclization process operating in competition with hydroperoxide formation during the autoxidation of PUFA or their esters containing three or more double bonds. When 20:3 or 20:4 PUFA undergo autoxidation, some of the natural, physiologically active prostaglandins would be produced, although in very low yield, along with many other stereo- and positional isomers. Thus, it is possible that some of the complex symptoms of lipid peroxidation in vivo could be due to nonenzymatically produced prostaglandins or their stereoisomers.

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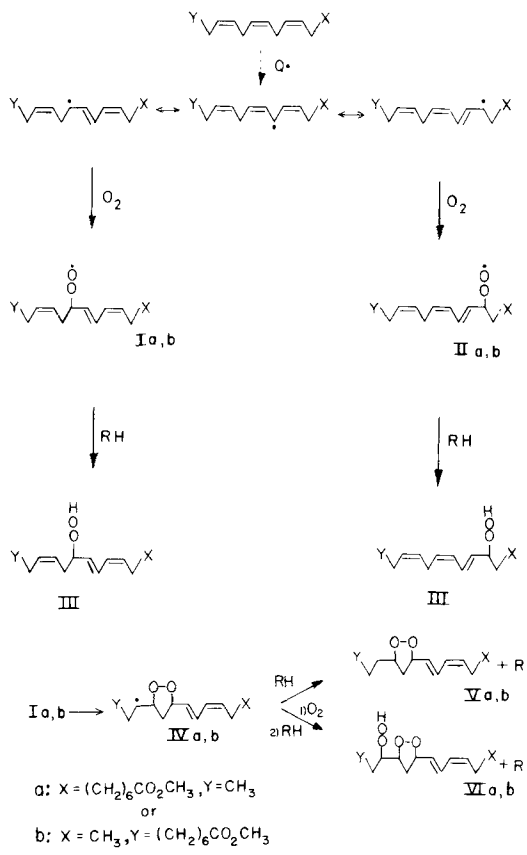


FIG. 1. Dahle et al. (5) mechanism for the formation of thiobarbituric acid-reactive material from triene systems.

INTRODUCTION

In a previous study of the ozone-initiated autoxidation of polyunsaturated fatty acids (PUFA), we observed (1-3), in agreement with published reports (4-12), the production of thiobarbituric acid (TBA)-reactive material from the autoxidation of methyl linolenate (18:3) but not from methyl linoleate (18:2). This TBA-reactive materials is quite frequently referred to as malonaldehyde (6,7,10,13), although it has been known for some time (4,5) that the material which is formed is predominantly a nonvolatile substance and, therefore, not malonaldehyde. This nonvolatile TBA-reactive material apparently decomposes under the conditions of the TBA test to produce malonaldehyde which then reacts with TBA (4,5).

The nature of the nonvolatile precursor of malonaldehyde is of considerable interest for a number of reasons. The TBA test is the most frequently used analytical measurement for the occurrence of lipid peroxidation both in vivo

and in vitro, in spite of numerous papers that warn of the limitations of the test, the uncertainties in the identity of the TBA-reactive material, and the fact that TBA numbers are treacherous measures of the extent of autoxidation (4-12). Furthermore, malonaldehyde itself is an important and damaging product of lipid peroxidation because of its ability to crosslink proteins (8,10). Finally, the manner by which either malonaldehyde, or a molecule that is capable of decomposing to give it, is produced from a PUFA is an interesting mechanistic problem which has received little study. Any mechanism that is suggested must explain the fact that only lipids containing three or more double bonds give appreciable amounts of TBA-reactive material.

In 1962, Dahle et al. (5) suggested an extremely clever mechanism, shown in Figure 1, which they suggested accommodated the then known facts of malonaldehyde production during autoxidation. Abstraction of a hydrogen atom from a triene system was envisioned to take place at one of the positions between two double bonds; each such hydrogen abstraction would produce a radical which can react with oxygen to produce one of the two conjugated peroxy radicals, I and II. Radicals I and II then abstract hydrogen from the hydrocarbon to form hydroperoxides, the main autoxidation products, as indicated. Dahle et al. (5) suggested, however, that radical I, having a double bond β-γ to the carbon bearing the peroxy group, could cyclize to form the 5-membered ring cyclic peroxides IV. (Similar cyclization of II would give a 5-membered ring with the odd electron on the central carbon within the ring, instead of external to the ring as in IV, a possibility Cahle et al. neglected.)

Dahle et al. (5) proposed that radical IV could abstract a hydrogen atom from a hydrocarbon to produce the cyclic peroxide V (14). However, it appears more probable that IV would react with oxygen to produce a peroxy radical, since reaction of alkyl radicals with oxygen is much faster under autoxidation conditions than reaction with hydrocarbons (15,16). The peroxy radical would ultimately abstract a hydrogen atom to yield VI. Dahle et al. (5) proposed that V is the nonvolatile precursor of malonaldehyde and that V decomposes under the conditions of the TBA test as shown in Figure 2. It appears even more likely that VI might decompose in that manner (Fig. 2). The malonaldehyde produced from either V or VI would than condense with 2 mol of TBA as shown in Figure 2 to form the highly colored TBA adduct which is measured spectrophotometrically (4).

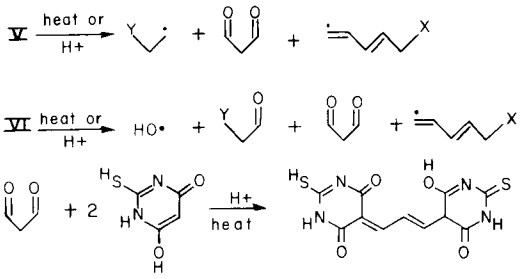


FIG. 2. Formation of malonaldehyde and the thiobarbituric acid (TBA)-malonaldehyde adduct under the conditions of the TBA test. X and Y as in Figure 1.

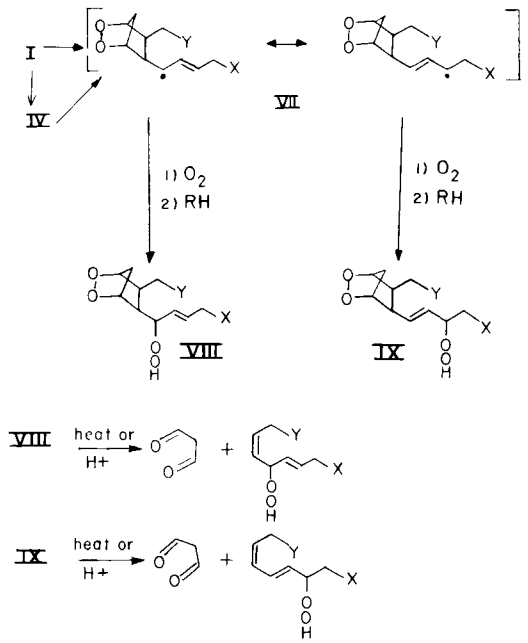


FIG. 3. Prostaglandin-like endoperoxide mechanism for the formation of thiobarbituric acid-reactive material from triene systems. X and Y as in Figure 1. No particular stereochemistry is implied for the structures shown. (Possible stereochemical consequences for these reactions are discussed in the Discussion section.) However, double bonds which were stereochemically equilibrated by becoming part of an allylic radical system are shown in their thermodynamically preferred *trans* form.

For reasons to be discussed later, we do not feel that the Dahle et al. mechanism adequately explains the failure of diene systems (e.g., 18:2) to produce TBA-reactive material. A more attractive mechanism in our view is one in which the precursor of malonaldehyde is a bicyclic endoperoxide analogous to the endoperoxide formed in the biosynthesis of prostaglandins (17-26) (Fig. 3). Peroxy radical I, most likely in

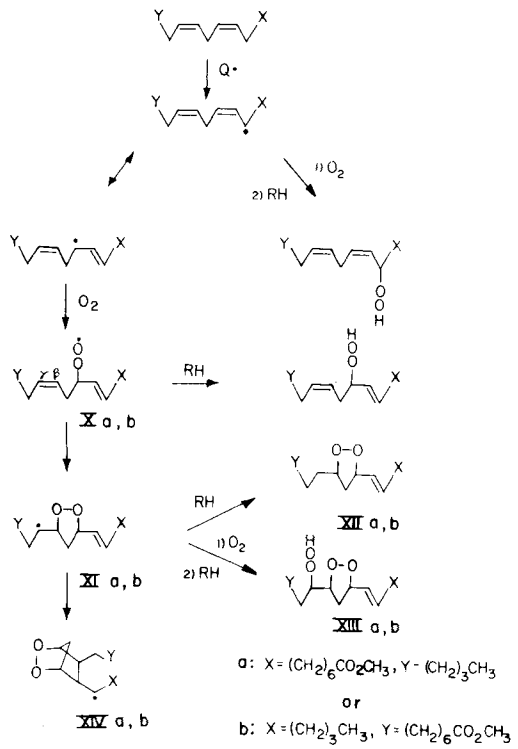


FIG. 4. Dahle et al. mechanism for the formation of thiobarbituric acid-reactive material, applied by current authors to a diene system.

a stepwise process via radical IV, undergoes ring closure to produce the bicyclic endoperoxide radical VII, an allyl radical, which is subsequently transformed into prostaglandin-like endoperoxide compounds VIII and IX. Malonaldehyde is a known by-product in the biosynthesis of prostaglandins (17-20,24,26,27) and is thought to be produced from an endoperoxide which has a ring system analogous to that of structures VIII and IX as shown in Figure 3. In the biosynthesis of prostaglandins, of course, the cyclization of I to IX is effected by enzymes, and only one of the possible endoperoxides is produced; in a nonenzymatic system, a number of positional and stereoisomers would be expected (see discussion below).

As mentioned above, TBA-reactive material is produced in substantial amounts only from PUFA containing three or more double bonds (5), and any mechanism must account for this fact. Dahle et al. (5) reasoned that only peroxy radicals containing a double bond in the position β-γ to the carbon bearing the peroxy group (radicals such as I) can cyclize into a 5-membered ring. However, they only consider radicals derived from the abstraction of a hydrogen from a PUFA at a position *between* two double bonds, and with that stipulation, only triene systems can give a peroxy radical in the proper position (Fig. 1). However, hydrogen abstraction by even relatively selective peroxy radicals would be expected to occur not only at methylene positions between two double bonds but also at the allylic positions on the *ends* of the alkene system (28), as shown in Figure 4. Abstraction of a hydrogen at this position (which would be statistically favored in a diene system such as 18:2) can lead to peroxy radical X. Since X is β,γ-unsaturated, as is radical I, it would be predicted by the Dahle et al. mechanism to cyclize to radical XI, which could subsequently be converted into XII (or, more likely, XIII). Product XIII would, in turn, be expected to give a TBA test in the same way that product VI does. Thus, rationalizing the failure of a diene PUFA to give a strong TBA test is difficult in terms of the Dahle et al. mechanism.

On the other hand, in the endoperoxide mechanism, this difficulty can be avoided. It can be seen from Figure 3 that the critical step in the endoperoxide mechanism is the formation of the carbon-carbon bond of the bicyclic system and that, whether the reaction occurs directly from radical I or stepwise through radical IV, the product radical VII is an allylic radical if produced from a triene. However, as is seen in Figure 4, in the diene system, formation of the analogous bicyclic radical XIV from X (via intermediate formation of XI) produces a secondary radical which is not allylic. Since the difference in stability between the secondary allylic radical produced in the triene system (VII) and the secondary radical produced in the diene system (XIV) is ca. 10 kcal/mol (29), one would expect a very much smaller yield of the endoperoxide in the diene system, as is observed. Interestingly, enzyme systems which produce prostaglandins from triene and tetraene systems are unable to form the endoperoxide from dienoic PUFA (26,30,31).

EXPERIMENTAL PROCEDURES

Materials and methods for purification, autoxidation, and product analysis were as described previously (3). The procedure for the TBA test (3,13) and $SnCl_2$ reduction (17,18,25) have been described. The prostaglandin E (PGE) test in the literature (22,32,33) was modified for use with autoxidation reaction solutions. In our solutions, several species that have absorbance near 278 nm are produced. Thus, it is necessary to calibrate autoxidation solutions by addition of

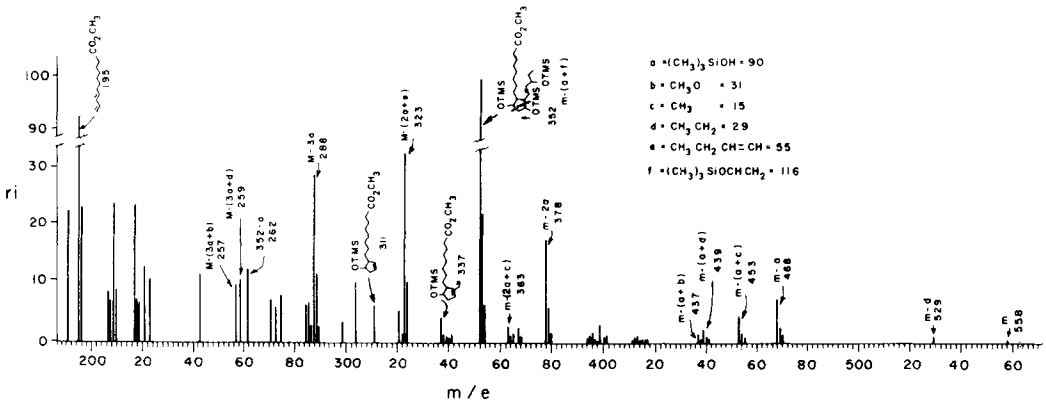


FIG. 5. Mass spectrum of the trimethylsilyl derivative of fraction 1B. ri = relative intensity. Structural assignment of various peaks made assuming the fraction contains a mixture of structures XV-XVIII, and are based in large part on the analyses or corresponding spectra of prostaglandin $F_{1\alpha}$ of Granstrom et al. (34).

known amounts of authentic PGE (a generous gift of the Upjohn Co.). Our test involves the addition of 15 μl of concentrated ethanolic NaOH to ultraviolet (UV) cells containing PGE (final base concentration 0.04 *M*) and recording the spectra both before base addition and after equilibrium is reached (20-40 min). A flat base line is drawn as a line tangent to the absorbance curve at 255 and 300 nm, and the difference in the distance from this base line to the curve itself at 278 nm before and after base addition is taken as the absorbance of PGE. An absorbance of 1.0 corresponds to 59.3 μg of PGE-type materials (from 18:3).

The thermal decomposition rates of various products in a mixture of autoxidized 18:3 were measured in the neat reaction mixture by heating the mixture in a stream of He (to remove any volatile material) at 80 C and periodically measuring the concentration of total peroxide, TBA-, and PGE-test reactive materials. Malonaldehyde was found in the He stream. We had previously determined that ca. 90% of the TBA-reactive material in autoxidizing 18:3 is not malonaldehyde and is nonvolatile (3). Only ca. 10% of the TBA-reactive material produced in the autoxidation of 18:3 could be blown off in a stream of He, at room temperature, although authentic, added malonaldehyde was rapidly volatilized from our solutions.

Product isolation was accomplished as follows: Purified neat methyl linolenate was subjected to ozone-catalyzed (0.35 ppm) autoxidation until the concentration of total peroxidic material was ca. 15% (3). The reaction mixture was dissolved in ether and reduced immediately with aqueous $SnCl_2$. The reduced mixture was dried, the ether evaporated, and the residue dissolved in hexane. The unreacted 18:3 was separated from the more polar products by chromatography on a silica gel column, using cyclohexane as solvent until all of the unreacted 18:3 was eluted. The solvent was then changed to ether and finally to methanol and the oxidized products collected. The products were then subjected to preparative thin layer chromatography (TLC) (0.25 g per 20 x 20 cm plate, 2 mm thickness of PF-254 silica gel) using the organic layer of ethyl acetate:iso-octane:water (50:50:100) as solvent. Five bands were obtained: Band 1 at rf = 0.05, 2 at rf = 0.16, 3 at rf = 0.37, 4 at rf = 0.63, and 5 at rf = 0.74. Fraction 5 was determined to be unreacted starting material not removed by the column chromatography. The relative amounts of the recovered products after elution from the plate were: Fraction 1, 19.9%; 2, 14.5%; 3, 15.2%; and 4, 50.4%. Fraction 4 was determined by infrared (IR), UV, nuclear magnetic resonance (NMR) and mass spectroscopy (MS) to be a mixture of mono-hydroxy esters of 18:3, mainly conjugated. Fractions 2 and 3 have not been identified as yet. Fraction 1 was subjected to further preparative TLC separation using 50:50 $CHCl_3$:ethyl acetate as the solvent and yielded four fractions. The rf values and % by wt of Fraction 1 were: Fraction 1A, rf ca. 0.0, 16%; 1B, rf = 0.22, 81%; 1C, rf = 0.43, 2%; 1D, rf = 0.89, 1%.

Trimethylsilyl and acetyl derivatives of fraction 1B were prepared as described by Samuelsson et al. (17,18,34). Gas chromatography-mass spectroscopic (GC-MS) studies were conducted on a Perkin Elmer-990GC connected to a Perkin Elmer RMS-4MS, using a 2 ft silinized 3% SE-30 column at 200 C and 30 lb He. The GC trace of the TMS derivative of 1B indicated a number of minor components and a major

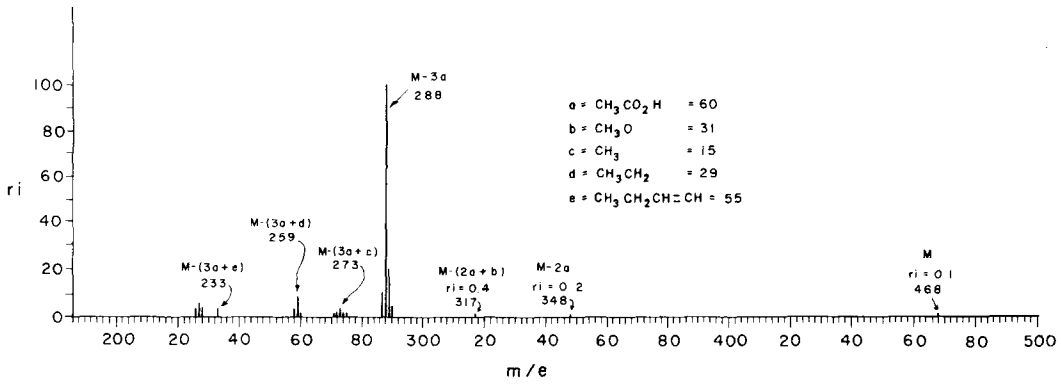


FIG. 6. Mass spectrum of the acetate derivative of fraction 1B. ri = relative intensity. Structural assignments of various peaks made assuming the fraction contains a mixture of structures XV-XVIII, and are based in large part on the analyses of corresponding spectra of prostaglandin $F_{1\alpha}$ of Granstrom et al. (34).

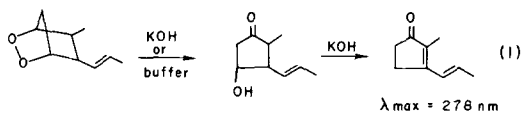
peak amounting to ca. 80% of the total with a retention time of 45 min; this major peak was analyzed by MS, and the spectrum is shown in Figure 5. The acetate derivative was treated similarly, and its spectrum is shown in Figure 6.

RESULTS

As a starting point for our studies, we adopted the working hypothesis that the TBA-reactive materials produced in the autoxidation of 18:3 are a mixture of endoperoxides VIII and IX. It appeared probable that VIII and IX would decompose thermally or in the presence of aqueous acid (17-26) to yield, among other products, malonaldehyde, and we have attempted to obtain evidence of various kinds that this is indeed the case. Many of our experiments aimed at obtaining evidence for intermediates (VIII and IX) have been patterned on the elegant studies of Hambert et al. (17,18), Samuelsson (19), Woldawer and Samuelsson (20), and Nugteren et al. (21-23) on the prostaglandin endoperoxides.

Chemical Similarities of TBA-Reactive Material and Prostaglandin-like Endoperoxides

Our first indication that the malonaldehyde precursor is an endoperoxide came from comparisons of the responses of autoxidized solutions of 18:3 to the TBA test and a test developed for prostaglandins. The chemical test for PGE-type compounds involves the formation of UV absorption at 278 nm upon addition of alcoholic base (22,32,33). The test probably is relatively unspecific, but it is believed to convert PGE compounds into conjugated dienones such as prostaglandin A and B (PGA and PGB) by dehydration and rearrangement of the double bonds (32). Since base is known to rapidly decompose secondary dialkyl peroxides to form ketones and alcohols (35-37), we expected that endoperoxides could be decomposed, at least partially, into PGE-type compounds, which would then react further with the base and give rise to the PGB chromophore and a positive PGE test (Eq. 1):



(1)

Indeed, Nugteren and Hazelhof found that the endoperoxide obtained enzymatically from all *cis*-8,11,14-eicosatrienoic acid (20:3) is 90% converted at pH 10 into PGE, PGD, and PGA plus PGB (23); even higher yields of PGE and PGD were produced in neutral conditions (23). We expected, therefore, if endoperoxides such as IX are formed during autoxidation of 18:3, that autoxidized 18:3 would give a positive test for PGE-like structures. It appeared likely a priori that autoxidation of 18:3 would produce endoperoxides because the biosynthetic pathway for production of the prostaglandin endoperoxides involves a radical cyclization (17-22); furthermore, Nugteren et al. (38) have shown that autoxidation of 8,11,14-eicosatrienoic acid in an aqueous solution gives prostaglandins.

Indeed, autoxidized 18:3 does give a PGE test. This is true regardless of whether the oxidation is spontaneous (i.e., effected by pure air), or is initiated by ozone or NO_2. On the other hand, 18:2, autoxidized under the same conditions as 18:3 and to the same degree of reaction as judged by the percent of total peroxide formed, does not give appreciable yields of nonvolatile compounds which give either TBA or PGE tests.

When the formation of total peroxidic material, conjugated dienes, TBA-, and PGE-test reactive materials are followed as a function of time for autoxidizing 18:3 (in the presence of vitamin E to produce a substantial induction period [1-3]), curves such as shown in Figure 7 are obtained. It can be seen that the rates of formation of both the TBA- and the PGE-test reactive materials are essentially zero during the induction period and then they both increase together. Note also that the rates of formation of the total peroxide and conjugated diene are considerably greater than those for TBA- and PGE-test reactive materials, especially during the induction period. These data indicate that the TBA- and the PGE-test reactive materials probably have common precursors, namely endoperoxides, which are different from the main peroxidic and conjugated diene products.

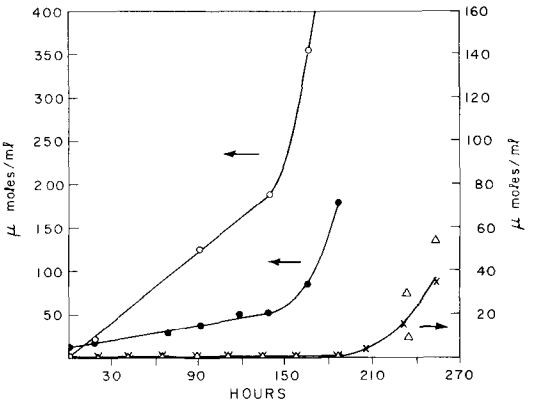


FIG. 7. Formation of total peroxide (○), conjugated dienes (●), thiobarbituric acid (TBA)-test reactive material (x), and prostaglandin E (PGE)-test reactive material (Δ) with time, during the autoxidation of methyl linolenate containing 1 mg vitamin E/ml of ester, exposed to 1/4 liter/min flow of air containing 1.5 ppm O_3. Left ordinate for peroxide and conjugated dienes; right ordinate for TBE-test reactive material and PGE-test reactive material.

Peroxidic Nature of the Malonaldehyde Precursor

We have obtained evidence that the precursor of the TBA-test reactive material produced from autoxidizing 18:3 is peroxidic. Aqueous $SnCl_2$ rapidly reduces peroxides of various kinds upon shaking; this treatment has been used (17,23,24) to convert the natural endoperoxide into $PGF_{1\alpha}$. Shaking an ether solution of autoxidized 18:3 with aqueous $SnCl_2$ not only destroys the peroxidic material (determined iodometrically), but also destroys both the TBA- and the PGE-test reactive material. In fact, partical reduction indicates that the TBA-test reactive material is reduced faster than is the total peroxidic material.

We also have followed the rates of the thermal decomposition in an inert atmosphere of total peroxidic material, TBA-, and PGE-test reactive materials in a sample of autoxidized 18:3. Each of these materials is found to disappear by a first order process, and the following rate constants (in sec^{-1}) were obtained at 80 C: total peroxide, $3.4 \pm .6 \times 10^{-5}$; TBA, $5.5 \pm 2 \times 10^{-5}$; and PGE, $5.8 \pm 1 \times 10^{-5}$. Although these rate constants are not as precise as might be wished, it does appear that both the TBA- and the PGE-test reactive materials are thermally more labile than the major hydroperoxide product from autoxidation. The fact that the TBA- and the PGE-test reactive materials disappear with approximately the same rate constants again indicates the two materials may have the same precursor.

Nugteren and Hazelhof (23) observed the disappearance of the endoperoxide to have a half-life at 20 C of 2.7 hr in organic solvents and 30 min in aqueous solution at pH 3-8, whereas we observe a half-life of ca. 3.3 hr at 80 C in methyl linolenate solvent. The more stable nature of our intermediate may be due to our use of a less polar solvent (which Nugteren and Hazelhof indicated contributes to the stability of endoperoxides), the presence of traces of metals or other impurities in the enzyme system, the difference between the ester in our system and the free acid in theirs (23), or a greater average stability for the endoperoxide stereoisomers formed in our nonenzymatic system relative to the natural prostaglandin endoperoxide. It appears likely that the thermal stability of a 2,3-dioxanorbornane ring system is substantial; endoperoxide of biological origin may contain trace metal impurities which catalyze its decomposition.

Shaking the endoperoxide derived from enzyme preparations with water results in its conversion into mainly PGE and PGD compounds (17,23). Thus, the precursor of TBA test material would be expected to be destroyed by shaking with water, whereas the PGE test values should not be greatly affected since water converts the prostaglandin endoperoxide into PGE-type compounds. Shaking our reaction mixture with water for 1 hr was found to destroy 25% of the PGE-like material and 53% of the TBA-reactive material. The fact that some PGE-like material was destroyed implies that not all of the endoperoxides destroyed by the water treatment are converted into PGE-type compounds. (VIII should not give PGB whereas IX could.) The fact that not all of the TBA-test reactive material was destroyed in 1 hr again indicates that some of the endoper-

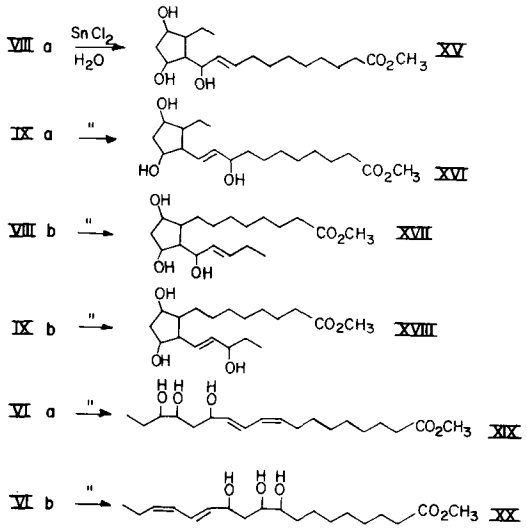


FIG. 8. Structures of the products expected from the stannous chloride reduction of endoperoxide intermediates and of the Holman cyclic intermediate. See Figure 1 for a and b.

oxide structures produced in our autoxidizing system are more stable than the endoperoxide produced in enzyme systems.

Product Studies

The thermal decomposition of autoxidized 18:3 at 80 C in an inert atmosphere produces genuine, volatile malonaldehyde which can be recovered from a trap. A similar but considerably faster decomposition of the autoxidized 18:3 product mixture occurs in dilute aqueous acid at 100 C, and this acid-catalyzed decomposition also produces malonaldehyde.

The most obvious way to prove the existence of endoperoxide intermediates such as VIII and IX would be to isolate and identify them. However, to date, no one has isolated such an endoperoxide, and the experience of Nugteren and Hazelhof (23) and of others (17-20,25) indicates that such endoperoxides could not be subjected to any of the extraction and chromatographic methods required for isolation of an endoperoxide from our system. Furthermore, the identification of such a substance as a relatively minor component of a mixture containing large amounts of other peroxidic materials seemed too unlikely to attempt. (We obtain at least 20 major GC peaks on autoxidation of 18:3). In work with solutions containing the prostaglandin endoperoxide intermediate produced from an enzymatic system (17,18,20,25), a TLC spot believed to be the endoperoxide was obtained which, when the solution was shaken with aqueous $SnCl_2$, was transformed into a substance of considerably more polar character and smaller rf value. We have found that the reaction mixture from autoxidized 18:3 gives four broad and slightly overlapping bands when spotted directly on silica gel plates and developed in 50:50 iso-octane:ethyl acetate. However, if the reacted material is first shaken with $SNCl_2$, a new band near the origin is obtained in addition to the original bands. Hamberg et al. (17) and Woldawer and Samuelsson (20) identified the low rf value material as $PGF_{1\alpha}$ from their mixtures; thus, $SnCl_2$ converts the relatively nonpolar endoperoxide hydroperoxide into the more polar $PGF_{1\alpha}$ triol. Accordingly, if endoperoxides VIII and IX are present in our reaction mixture, similar treatment with $SnCl_2$ should convert them into products XV-XVIII, as shown in Figure 8.

We have utilized a separation based largely on the work of Hamberg et al. (17) and Woldawer and Samuelsson (20), which is designed to reduce the reaction mixture predominantly to alcohols and to allow isolation of the triol fraction. This procedure, described in detail in the Experimental section, involves reduction with $SnCl_2$, column chromatography to separate polar reaction products from unreacted 18:3, followed by two successive preparative TLC separations. This procedure yielded a fraction, 1B, representing ca. 20% of the total isolated product fraction, which was investigated by UV, IR, and NMR spectroscopy. This fraction showed no appreciable absorbance above 220 nm, indicating that the material is not a conjugated diene. The IR spectrum reveals very strong alcohol OH stretching, very little if any vinyl hydrogen stretch, and a slightly broadened carbonyl peak. The NMR spectrum indicates that the material is a mixture, and the spectrum could not be fully interpreted nor were the relative areas of the various absorbances exactly integral numbers. Nevertheless, the spectrum is consistent with a PGF_1-type compound being a major component. Specifically, relative to the three hydrogens of the terminal methyl group or the methoxy group of the ester, the integral areas indicate 1.1 unsubstituted allyl hydrogens (consistent with structures XVI and XVIII but not XV, XVII, XIX, or XX in Fig. 8) and 2.1 vinyl hydrogens (consistent with structures XV-XVIII but not XIX or XX in Fig. 8).

Trimethylsilyl and acetyl derivatives (17,18,36) were prepared from fraction 1B and the mixture subjected to GC-MS analysis as described in Experimental Procedures. The mass spectra, shown in Figures 5 and 6, were found to correspond reasonably well to spectra

obtained by Hamberg et al. (17,18) and Granstrom et al. (34) for similar derivates of $PGF_{1\alpha}$ (after correcting for the fact that they used 8,11,14-eicosatrienoic rather than 9,12,15-octadecatrienoic as substrate), considering that our spectra result from several stereoisomeric and even structural isomeric endoperoxides. Our spectra also correspond reasonably well with those of Porter (private communication) and Porter and Funk (39) obtained from similar derivates of endoperoxides produced from their radical cyclization process. Suggested assignments of some of the high mass peaks are shown in Figures 5 and 6.

All of the spectroscopic evidence obtained is consistent with fraction 1B containing, as the major component, a mixture of the prostaglandin-like structures XV-XVIII; however, because of the small amount of material available and the fact that the material is impure, or at least a mixture of positional and stereoisomers, we do not consider this evidence to be entirely conclusive. However, it seems clear that the corresponding structures XIX and XX (Fig. 8), formed from the monocyclic peroxide intermediate, are not the material in fraction 1B. The mass spectra are consistent only with a structure, of the ones considered here, with either two double bonds, one double bond and one ring, or two rings. (The carbonyl of the ester group is not counted here.) The IR and NMR spectra are consistent only with a molecule with only one double bond, and the UV spectrum clearly indicates that the material is not conjugated.

More conclusive proof of the structure of the major component of fraction 1B will require isolation of compounds like XV-XVIII, which was impossible with the small sample sizes we have worked with here. Our present work is aimed at this goal, using modified experimental designs.

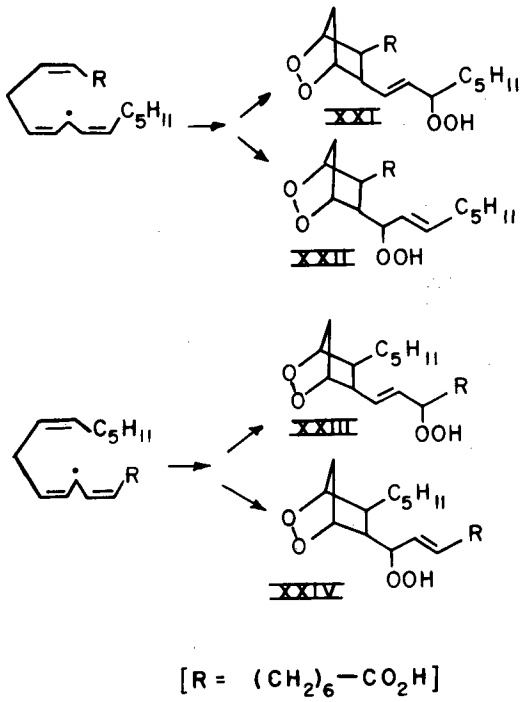


FIG. 9. Possible positional isomers of prostaglandin endoperoxides produced from 8,11,14-eicosatrienoic acid.

DISCUSSION

There are two major conclusions from the work reported here. The first is that autoxidation of methyl linolenate, and, by extension, esters of other PUFA containing three or more double bonds, produces endoperoxides. This finding complements that of Nugteren et al. (38), who studied the autoxidation of 8,11,14-eicosatrienoic acid in aqueous media and utilized both chemical and biological tests to show that prostaglandins were produced. The second conclusion is that the nonvolatile precursor of malonaldehyde produced from autoxidation of PUFA containing three or more double bonds is an endoperoxide. Malonaldehyde is produced during the enzyme-catalyzed production of prostaglandins and is believed to come from the decomposition of endoperoxides; however, this is the first suggestion that an endoperoxide is the source of the TBA-reactive materials in autoxidation systems.

The enzyme systems which form prostaglandins utilize the free acids. It appears likely, a priori, and our work together with that of Nugteren et al. (38) demonstrates, that autoxidation can convert either the free acid or esterified PUFA to endoperoxides and, hence, to prostaglandins. It is one of the basic tenets of free radical biology that autoxidation of PUFA in vivo, and particularly in lipids in membranes, is responsible for important biological consequences (40). The extent to which endoperoxide formation may be involved in this process obviously warrants considerable further research effort.

Some discussion of the stereochemistry of the nonenzymatic cyclization of PUFA to endoperoxides is warranted. Free radical reactions most often are not stereospecific and produce all possible positional and stereoisomeric isomers; however, certain factors may give rise to nonstatistical distributions of the various possible isomers (28).

Clearly, the cyclization of a trienoic PUFA will lead to the same isomeric possibilities for either the 18- or 20-carbon systems. Figure 9 outlines the possible structural and stereochemical isomers produced from autoxidation of 8,11,14-eicosatrienoic acid. The top half of the figure shows the enzymatically produced C-13 radical and the bottom half the unnatural C-10 radical; both can cyclize. For each of the four structures shown, there are four independent chiral centers and a double bond, leading to 32 isomers per structure or 128 total isomers.

Despite the usual lack of stereospecificity in free radical reactions (28), some stereoselectivity is not uncommon (28) and would be expected. In this case, it is unlikely that all 128 of the possible isomers are produced in equivalent yields. The enzymatically produced endoperoxide, of course, has the R group in XXI α (*endo*), the ring junction *trans*, the peroxy bridge α, and the double bond *trans*. Even if the nonenzymatic reaction strongly favors all four of these conformational possibilities, there will be 16 total possible isomers. Since the endoperoxide is not the major product of autoxidation, the yield of the endoperoxide with the all-natural structure and stereochemistry would be $< 1\%$ of the total product. This agrees with the results of Nugteren et al. (38).

In summary, although 128 possible endoperoxide isomers can be formed from the autoxidation of trienoic acids, some stereochemical preferences may occur which favor the production of structures analogous to the naturally occurring prostaglandins, a result with important consequences in free radical biology and pathology. In addition, the physiological properties of the nonnatural endoperoxides and their derivatives are worth further study.

ACKNOWLEDGMENT

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A Radical Scavenging Reaction of α-Tocopherol with Methyl Radical[1]

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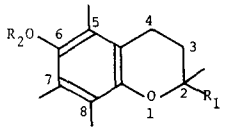
ABSTRACT

To study a radical scavenging reaction of α-tocopherol, it was reacted with methyl radical in dimethyl sulfoxide. Two main products, a geminal dimethyl cyclohexadienone and methyl ether of α-tocopherol, were obtained and these structures were determined by ^{13}C nuclear magnetic resonance spectroscopy. The radical methylation data of α-tocopherol suggested that a delocalized radical species would be an intermediate.

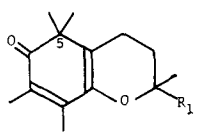
INTRODUCTION

It has been considered that tocopherols (vitamin E) are active as antioxidants and radical scanvengers in vivo (1-3), and that one of their roles is protection of membrane lipids from some radicals (4). However, only a few studies concerning the action of α-tocopherol against a radical attack were investigated (5). Recently some adducts of α-tocopherol to radical species such as an oxidized unsaturated fatty acid and cyanopropyl radical have been reported (6-9). These observations prompted us to survey radical additions of α-tocopherol, and we found radical methylation yielding a new geminal dimethyl cyclohexadienone and a methyl ether of α-tocopherol.

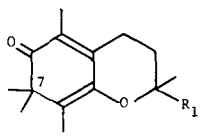
[1]TMIG-RI No. 3.



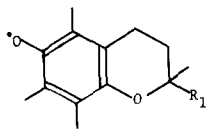
[I] $R_1=C_{16}H_{33}$, $R_2=H$
[II] $R_1=C_{16}H_{33}$, $R_2=CH_3$
[V] $R_1=R_2=CH_3$
[VI] $R_1=CH_3$, $R_2=H$



[III] $R_1=C_{16}H_{33}$
[IV] $R_1=CH_3$



[VII] $R_1=C_{16}H_{33}$



[VIII] $R_1=C_{16}H_{33}$ or CH_3

FIG. 1. Numerical key and structures of substrates, reaction products, and an intermediate.

MATERIALS AND METHODS

dl-α-Tocopherol was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). dl-γ-Tocopherol was prepared in our laboratory according to the usual method (10). dl-α-Tocopherol-5-CH_3-d_3 was synthesized from dl-γ-tocopherol and formaldehyde-d_2 in the presence of deuterohydrochloric acid and $SnCl_2$ in isopropyl ether (11). Formaldehyde-d_2 and deutrohydrochloric acid were obtained from E. Merck AG (Darmstadt, Germany). Silica Gel C-200 and dimethyl sulfoxide were purchased from Wako Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were obtained from common laboratory suppliers.

Methyl radical was generated by addition of 2.1 ml of 30% hydrogen peroxide to 3 ml of 4% ferrous sulfate solution in dimethyl sulfoxide (DMSO) (12) and allowed to react in situ with 2 mmol of dl-α-tocopherol (Fig. 1 [I]) at 60 C for 15 min. The reaction mixture was poured into ice-water and extracted with chloroform. After removal of chloroform, the extract was charged on a silica gel column and eluted with a mixture of n-hexane and benzene (5:1, v/v). Each fraction collected was analyzed by gas-liquid and thin layer chromatography.

^{13}C nuclear magnetic resonance (CMR) spectra were recorded on a Varian XL-100-12 WG nuclear magnetic resonance (NMR) spectrometer equipped with a Varian 620/L computer, operating at 25.2 MHz at ca. 30 C. Spectra were obtained in $CHCl_3$-d solution with tetramethylsilane (TMS) as an internal standard. Ten millimeter sample tubes were used for all CMR experiments. Mass, ultraviolet (UV), infrared (IR), and ^1H NMR (PMR) spectra were taken by the spectrometers as follows: Hitachi RMH-2, Cary 118c, Jasco IRA-2, and Varian XL-100-12 WG.

RESULTS AND DISCUSSION

Two main products (compound A and B) were isolated from the reaction mixture in yield

TABLE I

^{13}C Chemical Shifts[a]

Carbon number	α-Tocopherol [I]	α-Tocopherol methyl ether [II]	Compound B [III]
2	74.3	74.6	74.8
2a	23.8	23.9	23.3
3	31.6	31.3	31.2
4	20.8	20.7	18.5
4a	117.0	117.3	118.1 ○
5	118.5	125.4	46.7
5a	11.2	12.5	25.2 +
5b	---	---	25.7 +
6	144.4	149.3	204.6
7	121.0	127.5	127.7 ○
7a	12.1	11.8 ○○	14.1
8	122.3	122.7	141.1○
8a	145.4	147.6	146.8
8b	11.8	11.7 ○○	11.3
1′	39.8	40.1	39.7
2′	21.0	21.1	20.8
3′	37.5	37.4	37.4
4′	32.7	32.7 **	32.6 *
4′a	19.7	19.7	19.7
5′	37.5	37.4	37.4
6′	24.5	24.5	24.4
7′	37.5	37.4	37.4
8′	32.7	32.8 **	32.7 *
8′a	19.7	19.7	19.7
9′	37.5	37.4	37.4
10′	24.8	24.9	24.8
11′	39.4	39.4	39.4
12′	28.0	28.0	27.9
12′a	22.6	22.6 @	22.6
13′	22.6	22.7 @	22.6
OCH_3	---	60.2	---

[a]α-Tocopherol (1.0 mmol), α-tocopherol methyl ether (0.38 mmol), and compound B (0.27 mmol) were disolved in $CHCl_3$-d (1 ml), respectively: ○, ○○, +, *, **, @; tentative assignments.

of 8 and 42%, respectively. The compound A agreed spectrometrically with dl-α-tocopherol methyl ether (Fig. 1 [II]), which was synthesized from dl-α-tocopherol and dimethyl sulfate; mass: m/e (M^+) calculated for $C_{30}H_{52}O_2$: 444.3964, found 444.3970; analysis calculated for $C_{30}H_{52}O_2$: C 81.08, H 11.71, O 7.21, found: C 80.85, H 11.73, O 7.42; UV: λ_{max}^{EtOH} nm(ε) 281(2200), 287(2500), 290(2400); IR: ν_{max}^{neat} cm^{-1} 1015; PMR: $\delta_{TMS}^{CDCl_3}$ 0.90(d,12H,J=6.0Hz), 1.80(t,2H,J=6.5Hz), 2.11(s,3H), 2.15(s,3H), 2.20(s,3H), 2.59(t,2H,J=6.5Hz), 3.64(s,3H); CMR: $\delta_{TMS}^{CDCl_3}$ in Table I.

Mass, UV, IR, PMR, and CMR spectral analyses of the compound B are consistent with the nonaromatic structure of geminal dimethyl cyclohexadienone (Fig. 1 [III]); mass: m/e (M^+) calculated for $C_{30}H_{52}O_2$: 444.3964, found: 444.3960; analysis calculated for $C_{30}H_{52}O_2$: C 80.84, H 11.75, O 7.54; UV: λ_{max}^{EtOH} nm(ε) 245s(13000), 352(4800); IR: $\nu_{max}^{CHCl_3}$ cm^{-1} 1589, 1640; PMR: $\delta_{TMS}^{CDCl_3}$ 0.86(d,12H,J=7.0Hz) 1.65(t,2H,J=6.5Hz), 1.86(s,3H), 199(s,3H), 2.14(s,2H,J=6.5Hz); $\delta_{TMS}^{CDCl_3}$ (0.1 eq. Eu[fod]$_3$) 0.90(d,12H,J=7.0Hz), 1.39(s,3H), 2.29(s,3H), 2.49(s,3H), 2.91(s,3H); CMR: $\delta_{TMS}^{CDCl_3}$ in Table I and Figure 2A. The observation of the molecular ion peak (M^+; m/e 444.3960) demonstrated the introduction of a methyl group into α-tocopherol. IR, UV, and CMR data suggested the presence of a conjugated dienone and the loss of the aromatic structure in the molecule. The PMR and CMR spectra revealed the presence of the isoprenyl moiety and two methyl groups (CMR: 11.3 and 14.1 ppm) attached to olefinic carbon atoms in comparison with the spectra of α-tocopherol given in Table I (13,14). Furthermore, the presence of geminal dimethyl groups was

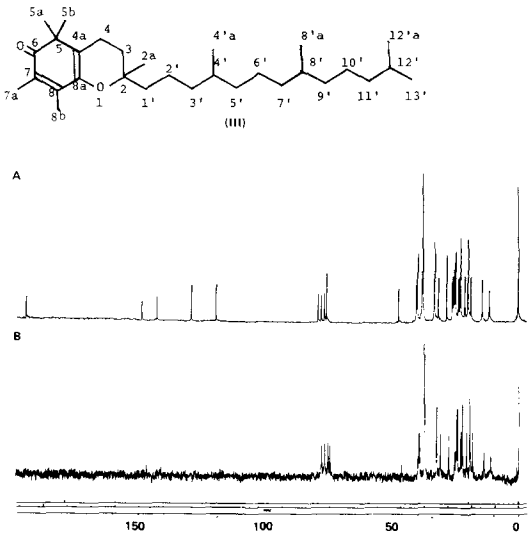


FIG. 2 (A) Proton noise decoupled (PND) natural abundance carbon-13 Fourier transform nuclear magnetic resonance spectrum of compound B [III] (120 mg in 1 ml $CHCl_3$-d containing 0.1 ml tetramethylsilane). Spectral condition: 7K pulses, 15° pulse, repetition rate 0.8s. (B) PND spectrum of the deuterium labeled compound B (30 mg in 0.6 ml $CHCl_3$-d containing 0.1 ml tetramethylsilane: 33K, 15°, 0.8s.).

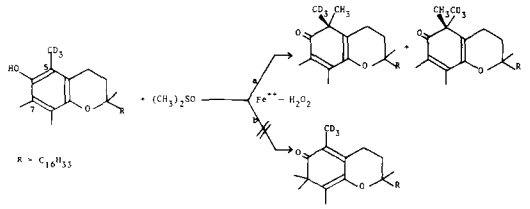


FIG. 3. Radical methylation of α-tocopherol-CH_3-d_3 at C-5.

proved by examining CMR spectra of the compound B prepared from either DMSO-d_6 and dl-α-tocopherol or DMSO and dl-α-tocopherol-5-CH_3-d_3. When DMSO-d_6 or dl-α-tocopherol-5-CH_3-d_3 was employed in place of each unlabeled substrate, the deuterium labeled product obtained was identical in the CMR spectrum, and the intensities of two peaks at 25.2 and 25.7 ppm were decreased by ca. 50%. In addition, a signal at 46.7 ppm observed as a singlet in the off-resonance spectrum was shifted to 46.6 ppm and diminished remarkably as shown in Figure 2B. These deuterium effects show that the two methyl carbon atoms (25.2 and 25.7 ppm) arose equivalently by the non-stereoselective attack of a methyl radical to C-5 of α-tocopherol and that they were bonded to a carbon atom at 46.7 ppm. The peak at 46.7 ppm was assigned to C-5 by the reason that, on using α-tocopherol-5-CH_3-d_3 and unlabeled DMSO as the substrates, the carbon atom at 46.7 ppm is bonded to a deuterated methyl group derived from a methyl group at C-5 of α-tocopherol-5-CH_3-d_3, and that two methyl groups attached to olefinic carbon atoms have no deuterium atom at all. The methylation occurred at C-5 (not at C-7) by route "a" in Figure 3. In the spectrum of the reaction product obtained from DMSO-d_6 and dl-α-tocopherol-5-CH_3-d_3, the signals at 25.2 and 25.7 ppm could not be found, the signal at 46.7 ppm was shifted to 46.5 ppm, and its intensity was greatly decreased. These large deuterium effects support the assignments of the two peaks at 25.2 and 25.7 ppm for geminal dimethyl groups and the one at 46.7 ppm for a quarternary C-5 atom bonded to them. These assignments also were confirmed by hetero nuclear selective decoupling (15) in $Eu(fod)_3$ shifted CMR and by selective enhancement of CMR signals from quarternary carbon atoms (16). The signals at 25.2 and 25.7 ppm were shifted to 28.0 ppm with 0.1 eq. $Eu(fod)_3$, and its shifted peak at 28.0 ppm became the highest sharp singlet when the single frequency decoupling radio-frequency field was centered at the frequency of a methyl proton absorption (PMR: 2.29 ppm) in the $Eu(fod)_3$ shifted PMR. The low power noise decoupling CMR spectrum for selective enhancement of the signals from quarternary carbon atoms gave seven strong sharp singlets assigned to C-2, 4a, 5, 6, 7, 8, and 8a.

It is surprising that the radical methylation occurs cleanly at C-5 (Fig. 1 [III]), with none observed at the nearly equivalent C-7 [VII] position. We also obtained a geminal dimethyl cyclohexadienone [IV] and methyl ether [V] derived from 2,2,5,7,8-pentamethyl chromanol (a model compound of α-tocopherol) [VI] under the same conditions of radical methylation.

A high resolution electron spin resonance (ESR) study has shown that chromanoxy radical [VIII] was produced from oxidation with diphenyl picryl hydrazil or photolysis of α-tocopherol and the model compound [VI] (17). In the radical methylation process, methyl radical would abstract a hydrogen atom from the hydroxyl group in α-tocopherol. The chromanoxy radical formed would be stabilized by radical delocalization, and the most reactive site of the delocalized radical seems to be the C-5 position.

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Comparative Antilipidemic Effects of Various Ethyl 5-Substituted Benzofuran-, 2,3-Dihydrobenzofuran-, and 3(2H)-Benzofuranone-2-Carboxylate Analogs of Clofibrate in a Triton Hyperlipidemic Rat Model

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ABSTRACT

The antilipidemic properties of certain benzofuran-, 2,3-dihydrobenzofuran-, and 3(2H)-benzofuranone-2-carboxylate analogs of clofibrate in a hyperlipidemic rat model are described. The hyperlipidemia was induced by intraperitoneal injection of Triton WR-1339. The results were analyzed in light of structural modifications as well as the lipid solubility of substituted compounds as assessed by a consideration of calculated log P values. Comparisons are made between the activity of these compounds and the activity of related cyclic analogs previously reported. Among the various compounds tested, only the 5-Cl and phenyl-substituted dihydrobenzofurans were selective against elevated serum cholesterol levels in this animal model. The data presented support the hypothesis that the cholesterol and triglyceride lowering activity of clofibrate related analogs in this animal model may be separated through a consideration of log P, conformational, and electronic changes. The proposal is advanced that relatively minor structural modification of clofibrate related analogs may lead to compounds which are not only selective in the Triton model but also to compounds which are likely to exert their effects by differing modes of action.

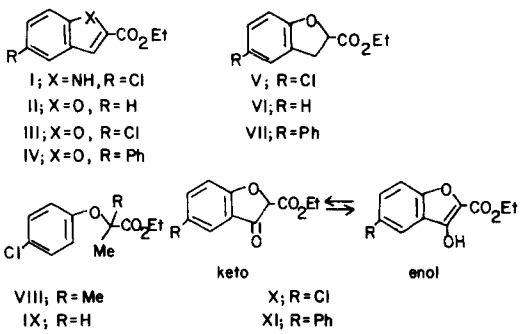

FIG. 1. Structures for cyclic analogs. I = ethyl 5-chloroindole-2-carboxylate; II = ethyl benzofuran-2-carboxylate; III = ethyl 5-chlorobenzofuran -2-carboxylate; IV = ethyl 5-phenylbenzofuran-2-carboxylate; V = ethyl 5-chloro-2,3-dihydrobenzofuran-2-carboxylate; VI = ethyl 2,3-dihydrobenzofuran-2-carboxylate; VII = ethyl 5-phenyl-2,3-dihydrobenzofuran-2-carboxylate; VIII = clofibrate; IX = ethyl α-(4-chlorophenoxy)propionate; X = ethyl 5-chloro-3(2H)-benzofuranone-2-carboxylate; XI = ethyl 5-phenyl-3(2H)-benzofuranone-2-carboxylate.

INTRODUCTION

Kariya and coworkers (1) have shown that ethyl 5-chloroindole-2-carboxylate (I) lowered plasma cholesterol levels without affecting plasma triglyceride levels in immature and mature Sprague-Dawley rats and in Wistar rats when it was mixed with their diets. We had previously observed that ethyl 5-chloro-2,3-dihydrobenzofuran carboxylate V selectively lowered elevated serum cholesterol levels without affecting serum triglyceride concentrations in Triton WR-1339 induced hyperlipidemic Sprague-Dawley rats, whereas the deschloro analog VI was inactive (2). Cyclic analog V is a conformationally constrained molecule related to ethyl α-(4-chlorophenoxy)propionate (IX), a desmethyl analog of clofibrate (VIII). The L(*S*) isomer of acyclic analog IX was observed to exhibit hypocholesterolemic activity in normal Swiss Webster rats whereas the D(*R*) enantiomorph was inactive (3).

To obtain leads for future drug design of selective hypolipidemic agents, we investigated the antilipidemic properties of certain 5-substituted-benzofurans (II-IV). The results are compared to those obtained for the 5-substituted-2,3-dihydrobenzofurans (V-VII) and the 3-keto analogs (X-XI) and are analyzed in light of the calculated log P values (4,5) for the corresponding carboxylic acid hydrolysis products (Fig. 1).

METHODS AND MATERIALS

Chemical Methods

Melting points were taken using a Thomas-

Hoover melting point apparatus. Infrared (IR) spectra were recorded using a Perkin-Elmer 257 grating spectrophotometer. The nuclear magnetic resonance (NMR) spectra were taken with a Varian-A-60A NMR spectrometer at 60 MHz using trimethylsilane as an internal reference. Chemical analyses were performed by Clark Microanalytic Laboratories, Urbana, IL.

Condensation of precursor 5-substituted-salicylaldehydes with diethylbromomalonate in the presence of anhydrous K_2CO_3 according to the methods of Kurkudar and Rao (6) afforded the respective ethyl 5-substituted-benzofuran-2-carboxylates (II-IV) with ethyl benzofuran-2-carboxylate (II) bp 99-100 C (0.005 mm); lit (7) bp 275 C (720 mm); yield = 65%. Ethyl 5-chlorobenzofuran-2-carboxylate (III) exhibited mp 64.5-65.5 C; lit (6) mp 65 C; yield = 70%.

Ethyl 5-phenylbenzofuran-2-carboxylate (IV) was prepared from 5-phenylsalicylaldehyde (0.99 g, 0.005 mol), diethyl bromomalonate (0.96 g, 0.004 mol), anhydrous K_2CO_3 (1.25 g, 0.009 mol), and 2-butanone (20 ml). The mixture was refluxed for 10 hr, and the solvent was removed under reduced pressure. The residue was cooled, poured into H_2O (100 ml), and extracted with ether. The ether layer was washed with cold 5% NaOH solution followed by H_2O and dried over anhydrous Na_2SO_4. The dried ether solution was filtered and concentrated under reduced pressure. The residue was recrystallized from ethanol affording 0.95 g (73%) of white crystals (mp 109-110 C; IR [$CHCl_3$] 1730 cm^{-1}; NMR [$CDCl_3$] δ 1.35 [t, 3H, CH_3], 4.38 [q, 4H, CH_2], 7.20-7.85 [m, 3H, aromatic + 1 vinylic H]; analysis calculated for $C_{17}H_{14}O_3$: C, 76.69; H, 5.26; found: C, 76.56; H, 5.44).

Hydrolysis of esters II and III, which served as precursors for the respective 2,3-dihydro compounds, was carried out according to Kurkudar and Rao (6), affording the respective free carboxylic acids. The constants for 5-chlorobenzofuran-2-carboxylic acid are found in Ref. 3. Benzofuran-2-carboxylic acid exhibited mp 192-193 C; lit (8) mp 192-193 C; yield = 60%.

5-Phenylbenzofuran-2-carboxylic acid was prepared from IV (1.3 g, 0.0084 mol) by refluxing for 4 hr in 50 ml of 10% alcoholic KOH. The solvent was removed under reduced pressure, and the residue was washed with ether. The basic solution was acidified with dilute HCl and extracted with ether. The ether layer was extracted with dilute $NaHCO_3$ solution. The aqueous solution was reacidified with dilute HCl and extracted with ether. The ether extract was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was crystallized from ethanol affording 1.1 g (95%) of a white solid (mp 220-221 C; IR [KBr] 1690 cm^{-1}; NMR [DMSO-d_6], δ 7.20-8.15 [m, 8H, aromatic + 1 vinylic H], 12.35 [broad, 1H, carboxylic acid H]; analysis calculated for $C_{15}H_{10}O_3$: C, 75,62; H, 4.23; found: C, 75.73; H, 4.44).

The 2,3-dihydrobenzofuran-2-carboxylic acids were obtained by reduction of the respective carboxylic acids derived from the hydrolysis of benzofurans II, III, and IV, through use of Na(Hg) according to the method described by Fredga (9). The physical properties for 5-chloro-2,3-dihydrobenzofuran-2-carboxylic acid are found in Ref. 3. 2,3-Dihydrobenzofuran-2-carboxylic acid exhibited mp 116.5-117 C; lit (10) mp 116.5 C.

5-Phenyl-2,3-dihydrobenzofuran-2-carboxylic acid was prepared from 5-phenylbenzofuran-2-carboxylic acid (5.0 g, 0.021 mol) in 10% aqueous NaOH solution (90 ml). The sodium salt separated. Sodium amalgam, prepared from Na (1.5 g, 0.065 g at) and Hg (50 g, 0.25 g at), was added with stirring during 1 hr to the NaOH mixture. After the addition, the mixture was stirred for 24 hr and allowed to stand at room temperature for an additional 24 hr. The Hg was separated, and the solution was neutralized with dilute HCl and extracted with ether. The ether solution was washed with dilute $NaHCO_3$ solution, and the aqueous layer was separated and reacidified with dilute HCl and extracted with ether. The ether solution was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residual solid was recrystallized from ethanol, affording 4.8 g (95%) of white crystals (mp 186-187 C; NMR [DMSO-d_6], δ 2.90-3.80 [m, 2H, CH_2], 4.90-5.40 [dd, 1H, methine], 6.0-6.5 [broad, 1H, carboxylic acid H], 6.72-7.90 [m, 8H, aromatic]; analysis calculated for $C_{15}H_{12}O_3$: C, 74.92; H, 5.04; found: C, 74.89; H, 5.30).

Fisher esterification of the above carboxylic acids resulted in the synthesis of the respective desired esters V, VI, and VII. Hence, ethyl 2,3-dihydrobenzofuran-2-carboxylate (VI) exhibiting bp 78-78.5 C (0.002 mm) (lit [11] bp 273 C [atmospheric pressure]) was prepared in 92% yield.

Ethyl 5-chloro-2,3-dihydrobenzofuran-2-carboxylate (V) was prepared from the corresponding carboxylic acid (9.9 g, 0.05 mol) in absolute ethanol (250 ml), toluene (100 ml), and concentrated H_2SO_4 (4 ml). The mixture was heated at reflux for 7 hr; H_2O was removed through use of a Dean-Stark trap. The reaction mixture was concentrated, and the residue was

dissolved in ether. The ether solution was washed with saturated $NaHCO_3$ solution and H_2O. The aqueous portion was extracted with ether, and the combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. Distillation of the residual high boiling liquid afforded 10.2 g (90%) of an oil (V) (bp 90-91 C [0.05 mm]; analysis calculated for $C_{11}H_{11}ClO_3$: C, 58.40; H, 4.91; Cl, 15.63; found: C, 58.31; H, 4.72; Cl, 15.54).

By a similar procedure, ethyl 5-phenyl-2,3-dihydrobenzofuran-2-carboxylate (VII) was prepared from the corresponding acid (2.5 g, 0.01 mol) by refluxing in a mixture of ethanol (60 ml), dry benzene (20 ml), and concentrated H_2SO_4 (2 ml), affording 2.65 g (95%) of colorless liquid (VII) (bp 180-181 C [0.35 mm]; NMR [$CDCl_3$], δ 1.16 [t, 3H, CH_3], 3.18-3.48 [m, 2H, benzylic CH_2], 4.12 [q, 2H, ester CH_2], 5.05 [dd, 1H, methine], 6.70-7.55 [m, 8H, aromatic]; analysis calculated for $C_{17}H_{16}O_3$: C, 76.10; H, 6.01; found: C, 76.46; H, 6.09).

Starting salicylaldehyde and 5-chlorosalicylaldehyde were purchased commercially. 5-Phenylsalicylaldehyde was prepared under Reimer-Tiemann Conditions (12,13) according to the following method. p-Phenylphenol (22 g, 0.13 mol) was dissolved in 95% ethanol; a solution of NaOH (40 g, 1 mol) in H_2O (80 ml) was rapidly added. The solution was heated to 75-80 C, at which point chloroform (20 ml) was added dropwise. The addition was at such a rate that gentle refluxing was maintained (added during 1 hr). After formation of a dark-red color (generally 5-15 min), further heating was unnecessary. Stirring was continued for 3 hr after all the chloroform was added. After cooling, the ehtanol and excess chloroform were removed under reduced pressure, and the resulting residue was cooled, poured into cold H_2O, acidified with dropwise addition of HCl, and extracted with ether. The ether solution was concentrated under reduced pressure, and the residue was poured into 2 vol of saturated $NaHSO_3$ solution and shaken vigorously (on a shaker) for 45 min. The semisolid (paste) bisulfite addition compound was allowed to stand for 1 hr, filtered in the dark, and washed with small portions of ethanol and ether (to remove the phenol). The bisulfite addition compound was decomposed with dilute H_2SO_4 by warming on a water-bath for 30 min. The cooled mixture was extracted with ether, dried (Na_2SO_4), filtered, and the solvent removed under reduced pressure. The residue was treated with activated charcoal and recrystallized from ethanol:H_2O, affording 12.4 g (48%) of yellow crystals (mp 98-99 C; lit [14] mp 102 C; analysis calculated for $C_{13}H_{10}O_2$: C, 78.77; H, 5.09; found: C, 78.49; H, 5.02).

The 3-keto analogs (X,XI) were prepared as follows: Ethyl 5-chloro-3(2H)-benzofuranone-2-carboxylate (X) was prepared from ethyl 4-chloro-2-carbethoxyphenoxyacetate (8.58 g, 0.03 mol), which in turn was prepared according to the method of Armarego (15). The phenoxyacetate was dissolved in 40 ml of dry benzene (16), and the solution was added dropwise with stirring to sodium ethoxide (2.1 g, 0.03 mol) in dry benzene (40 ml). Stirring was continued under reflux for 16 hr. After cooling to room temperature, the reaction mixture was poured, with stirring, into H_2O (200 ml) and made alkaline to litmus with dilute NaOH solution. The aqueous layer was made acidic with dilute HCl and extracted with ether. The organic layer was dried over anhydrous Na_2SO_4, filtered, and the solvent removed under reduced pressure. The resulting solid was recrystallized from ethanol, affording 6.4 g (88%) of white crystals (X) (mp 128-129 C; lit [17] mp 126-127 C; NMR [$CDCl_3$] δ 1.28-1.58 [t, 3H, CH_3], 4.25-4.68 [q, 2H, CH_2], 7.10-7.74 [m, 3H, aromatic and 1 H methine]).

Similarly, ethyl 5-phenyl-3(2H)-benzofuranone-2-carboxylate (XI) was prepared from 3.28 g (0.01 mol) of ethyl 4-phenyl-2-carbethoxyphenoxyacetate according to the method of Schroeder et al. (17) by dissolving the phenoxyacetate in dry benzene (50 ml) followed by dropwise addition of the solution to sodium ethoxide (0.23 g, 0.01 g at of Na in 15 ml of dry ethanol). After refluxing for 10 hr, the benzofuranone XI was isolated according to methods described for the isolation of X. Recrystallization of XI from ethanol afforded 1.8 g (68%) of crystals (mp 129.5-130.5 C; NMR [$CDCl_3$], δ 1.42 [t, 3H, CH_3], 4.48 [q, 2H, CH_2], 7.05-8.10 [m, 8H, aromatic and 1H methine]; analysis calculated for $C_{17}H_{14}O_4$: C, 72.34; H, 4.96; found: C, 72.22; H, 5.15).

Intermediate ethyl 4-phenyl-2-carbethoxyphenoxyacetate was prepared according to the general method of Armarego (15) in which ethyl 5-phenylsalicylate (6.05 g, 0.03 mol), ethyl bromoacetate (4.6 g, 0.03 mol), and anhydrous K_2CO_3 (13.8 g, 0.1 mol) were refluxed in 30 ml of dry acetone (16) for 20 hr. After cooling, the inorganic salt was separated by filtration, and the organic solvent was removed under reduced pressure. The residual oil was extracted (ether) and the ether layer was washed with cold dilute NaOH solution and H_2O. The organic layer was dried over anhydrous Na_2SO_4, and the solvent was removed

under reduced pressure. The resulting oil solidified on cooling and was recrystallized from ethanol, affording 5.6 g (68%) crystalline phenoxyacetate (mp 53-54 C; NMR [$CDCl_3$], δ 1.08-1.50 [m, 6H, $2CH_3$], 4.02-4.58 [m, 4H, 2 ester CH_2], 4.70 [s, 2H, $O\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-}$], 6.80-8.10 [m, 8H, aromatic]; analysis calculated for $C_{19}H_{20}O_5$: C, 69.51; H, 6.09; found: C, 69.69; H, 6.21).

Biological Methods

Compounds were tested in the Triton WR-1339-induced hyperlipidemic rat model (18) utilizing procedures virtually identical to those previously reported (2) for the evaluation of related analogs in our laboratories except that ten rather than six animals were employed in each group (Tables I and II). Compounds were dispersed in vehicle (0.25% aqueous methyl cellulose) to obtain a concentration of 8.33×10^{-3} mmol/ml. A total screening dose of 0.124 mmol/kg was administered to 270 ± 10 g rats. Plasma triglyceride was determined by the method of Eggstein (19); plasma cholesterol was measured by the method of Holub and Galli (20). Significant differences in plasma cholesterol and triglyceride concentrations between drug-treated and control groups were determined by Student's *t* tests on logarithms of individual data to allow the pooling of variances.

RESULTS

The effects of benzofurans II, III, and IV, 2,3-dihydrobenzofuran VII, and the 3-keto analogs X and XI on serum cholesterol (Table I) and triglyceride (Table II) concentrations were tested in normal and Triton WR-1339-induced (18) hyperlipidemic male Sprague-Dawley rats initially fed Purina Chow for a 4-5 day stabilizing period. As previously described (2), all rats, including controls, were fasted during analog evaluations. All compounds were evaluated at the same dosage (see Methods) which is equivalent to the dosage (0.124 mmol/kg) at which we previously evaluated clofibrate (2). To determine the effectiveness of these compounds in lowering cholesterol and triglyceride levels in hyperlipidemic animals, groups III (Triton hyperlipidemic) and IV (drug-treated Triton hyperlipidemic) were compared. Further, to determine whether the analog could lower hyperlipidemic serum levels of cholesterol and triglycerides to those found in normal animals, groups I (control) and IV were compared. Groups I and II (drug-treated control) were contrasted for significant differences to

TABLE I

Effect of Cyclic Clofibrate-Related Analogs on Plasma Cholesterol Levels (mg%) in Male Sprague-Dawley Rats

Compound	Control group (I)[a]	Drug-treated control (II)[a]	Triton hyperlipidemic (III)[a]	Drug-treated Triton hyperlipidemic (IV)[a]
Ethyl benzofuran-2-carboxylate (II)	88.1 ± 17.2	90.1 ± 15.8	310 ± 53.0	235 ± 79.2[b,c]
Ethyl 5-chlorobenzofuran-2-carboxylate (III)	90.5 ± 9.99	76.2 ± 16.9[d]	178 ± 36.1	108 ± 13.6[c,e]
Ethyl 5-phenylbenzofuran-2-carboxylate (IV)	77.0 ± 7.8	72.8 ± 7.7	178 ± 56.4	132 ± 60.2[c]
Ethyl 5-phenyl-2,3-dihydrobenzofuran-2-carboxylate (VII)	87.8 ± 11.4	80.4 ± 12.4	165 ± 36.8	94.3 ± 9.8[e]
Ethyl 5-chloro-3(2H)-benzofuranone-2-carboxylate (X)	87.4 ± 9.0	79.4 ± 12.3	165 ± 18.0	106 ± 13.9[c,e]
Ethyl 5-phenyl-3(2H)-benzofuranone-2-carboxylate (XI)	71.9 ± 13.5	79.0 ± 14.6	196 ± 57.3	149 ± 53.5[c]

[a]Mean ± SD; ten rats.
[b]Statistically significant $P<0.05$; comparison of groups III and IV.
[c]Statistically significant $P<0.01$; comparison of groups I and IV.
[d]Statistically significant $P<0.05$; comparison of groups I and II.
[e]Statistically significant $P<0.01$; comparison of groups III and IV.

TABLE II

Effect of Cyclic Clofibrate-Related Analogs on Plasma Triglyceride Levels (mg%) in Male Sprague-Dawley Rats

Compound	Control group (I)[a]	Drug-treated control (II)[a]	Triton hyperlipidemic (III)[a]	Drug-treated Triton hyperlipidemic (IV)[a]
Ethyl benzofuran-2-carboxylate (II)	30.9 ± 9.6	51.4 ± 14.1[b]	232 ± 83.9	140 ± 76.1[c,d]
Ethyl 5-chlorobenzofuran-2-carboxylate (III)	18.8 ± 4.7	32.1 ± 7.4[b]	115 ± 38.2	35.4 ± 12.5[c,d]
Ethyl 5-phenylbenzofuran-2-carboxylate (IV)	18.1 ± 2.6	23.7 ± 9.3[e]	132 ± 99.2	51.2 ± 46.6[c,d]
Ethyl 5-phenyl-2,3-dihydrobenzofuran-2-carboxylate (VII)	18.7 ± 6.4	18.1 ± 6.3	108 ± 28.3	90.1 ± 25.0[d]
Ethyl 5-chloro-3(2H)-benzofuranone-2-carboxylate (X)	26.3 ± 6.1	29.0 ± 5.9	77.4 ± 15.0	28.7 ± 7.9[c]
Ethyl 5-phenyl-3(2H)-benzofuranone-2-carboxylate (XI)	27.1 ± 8.2[f]	40.2 ± 13.5[e]	185 ± 100	78.7 ± 70.8[c]

[a]Mean ± SD; ten rats.
[b]Statistically significant $P<0.01$; comparison of groups I and II.
[c]Statistically significant $P<0.01$; comparison of groups III and IV.
[d]Statistically significant $P<0.01$; comparison of groups I and IV.
[e]Statistically significant $P<0.05$; comparison of groups I and II.
[f]Mean ± SD; nine rats.

determine whether these compounds exhibited hypolipidemic activity in normal (fasted) Sprague-Dawley rats under the conditions of this protocol (2).

Whereas the dihydrobenzofuran analog VI was previously observed to be inactive in hyperlipidemic and normal rats (2), benzofuran II, unsubstituted at the 5 position, exhibited marginal cholesterol and triglyceride lowering properties in hyperlipidemic animals. Insertion of a 5-Cl substituent afforded III, which is active, but could not return elevated cholesterol or triglyceride levels to normal. Further, the apparent selective activity (cholesterol lowering) of the structurally related dihydrobenzofuran V (2) was lost upon introduction of unsaturation into the heterocyclic ring (compound III). Introduction of the 5-phenyl substituent into the benzofuran series (compound IV) did not markedly affect the activity of the benzofuran as a triglyceride lowering agent and rendered the molecule virtually inactive against elevated cholesterol levels. The selective activity observed for the 5-phenyl-2,3-dihydrogenzofuran derivative VII is of particular interest because of its selective cholesterol lowering activity. This analog returned elevated serum cholesterol levels to those found in normal animals but did not affect elevated serum triglyceride levels.

When, however, the carbonyl function was inserted into position 3 of either V or VII, the respective benzofuranones X and XI exhibited no selective activity for lowering elevated serum cholesterol levels. In fact, the 3-keto analogs X and XI exhibited greater ability to lower elevated serum triglyceride levels.

DISCUSSION

Since both the 5-phenyl- and 5-chloro-2,3-dihydrobenzofurans exhibited selective activity against experimentally induced hypercholesterolemic levels, it seems to us that this heterocyclic ring system should be further investigated in an effort to develop compounds efficacious in the treatment of Type II hyperlipoproteinemia (21-26). Although additional evidence of selective activity obtained through use of other animal models (27) as well as through investigation of their action on cholesterolgenesis enzymes (28) is desirable, these preliminary data are of interest since clofibrate is mainly effective in the treatment of patients having hyperlipoproteinemia Types III, IV, and V (21-26). Further, it seems, at least in the Triton model, that the dual action of clofibrate (2) is subject to conformational control since the two constrained 5-substituted dihydroben-

TABLE III

Calculated log P (Octanol-Water Partition Coefficients) Values for the Free Acid Hydrolysis Products of the Tested Parent Esters

Free acid of the parent ester indicated	Values of substituents	Calculated log P = 1.51 + π values (experimentally determined log P values)[a]	
V	5-Cl = 0.6[b]	2.11	(2.11)[a]
VI	---	1.51	---
VIII	---		(2.57)[a]
II	Insertion of C=C = -0.48[c]	1.03	---
III	Insertion of C=C = -0.48[c] 5-Cl = 0.6[b]	1.63	---
IV	Insertion of C=C = -0.48[c] 5-C_6H_5 = 1.96[d]	2.99	---
VII	5-C_6H_5 = 1.96[d]	3.47	---
X	3-Keto oxygen = -1.21[e] 5-Cl = 0.6[b]	0.90	---
XI	3-Keto oxygen = -1.21[e] 5-C_6H_5 = 1.96[d]	2.26	---

[a]Taken from Ref. 33; experimentally determined at pH two units below the pKa.

[b]π value taken from Lewis, N.J., D.R. Feller, G.K. Poochikian, and D.T. Witiak, J. Med. Chem. 17:41 (1974).

[c]Estimated from the π substituent constant for substitution on benzene; CH_2-CH=CH_2 (1.10), CH_3 (0.56), CH_3CH_2(1.02); taken from Ref. 5; i.e., 1.10-0.56 = 0.54 for CH=CH_2; since CH_2CH_3 = 1.02, the CH_2CH_2 to CH=CH transformation = -0.48. However, since CH=CH has been experimentally determined to be 0.82 (Ref 5), this value for the CH_2CH_2 to CH=CH transformation likely is between -0.48 and -0.20.

[d]π value taken from Ref. 5.

[e]Estimated from the π substitutent constant for substitution on benzene; CH_3 (0.56), Ref. 4 or 5; CHO (-0.65), Ref. 5; i.e., -0.65 -0.56 = -1.21.

zofurans are mainly effective against elevated cholesterol levels.

Since phenyl and phenoxy analogs of clofibrate are generally good to very potent hypolipidemic agents (29-31) and insertion of phenyl groups increases lipid solubility, it seemed to us to be of interest to consider the biological results obtained with the phenyl, Cl, and H analogs in terms of the calculated log P values (Table III) for the corresponding free carboxylic acids, hydrolysis products. The log P values for the carboxylic acids, rather than the esters, were determined and used for correlation purposes since Thorp (32) had proposed that the free acid is the active form of clofibrate, and we have shown that several clofibrate analogs undergo rapid hydrolysis by serum esterase preparations (33). Calculated values were estimated by adding the substituent constants (π) to the experimentally determined (33) log P values for the unsubstituted free acid hydrolysis product of ethyl 2,3-dihydrobenzofuran-2-carboxylate (VI).

When attempts are made to correlate log P values with biological activity, it is important to recognize that changes in structure may, in fact, result in biological mechanism changes. Such changes in mechanism of action may or may not be a function of log P; steric and electronic properties of the functional group as well as the molecule as a whole may markedly affect binding to various enzyme systems. For example, we have previously observed that certain 6-substituted chromans have relatively good activity in the Triton-induced hyperlipidemic rat model (34). The calculated log P values for the substituted chromans are $\geqslant$ log P for clofibrate, which has also been shown to be very active in this assay (2,18). In the chroman series, analogs with large log P values (i.e., log P for the 6-cyclohexyl compound = 4.41) have selective activity against elevated triglyceride levels (34). However, ethyl 6-chlorochromone has a calculated log P = 0.81, a value much lower than the one observed for clofibrate, and, in the Triton model, this chromone was found to be more active than clofibrate (34) against both elevated cholesterol and triglyceride levels. It was suggested that this apparent lack of correlation of log P with antilipidemic activity is a reflection of changes in mechanism of action which, in fact, may be a function of the marked differences in the electronic character of chromones and chromans (34). Indeed, unpublished results recently obtained in our laboratories show that the 6-chloro-chromone is inactive in normal Sprague-Dawley rats, whereas clofibrate and the 6-phenyl- and 6-cyclohexyl-chromans lower cho-

lesterol levels and block HMG-CoA reductase upon chronic drug administration.

It would appear that relatively minor structural modification in the dihydrobenzofuran series also affords compounds exhibiting antilipidemic activity by differing modes of action, and further work will be necessary to substantiate this hypothesis. Nonetheless, this proposal seems likely for the following reasons: (a) The dihydrobenzofurans (V and VII) exhibit selective activity for reducing elevated cholesterol levels in the Triton-induced hyperlipidemic rat model. Insertion of Ph (VII) affords an analog with a higher log P value and increased selective activity when compared to the Cl analog V. Replacement of Cl by H (i.e., VI) lowers the log P value below the one observed for clofibrate and renders the compound inactive (2). (b) On the other hand, insertion of a C=C into the heterocyclic ring of the dihydrobenzofurans yields a second series of compounds (II, III, and IV) with loss of selective activity for elevated cholesterol levels. In fact, the 5-phenyl-benzofuran IV exhibits selective activity for elevated serum triglyceride levels and, for this activity, it appears that both the Cl and phenyl analogs (III and VII) which have the larger log P values exhibit greater potency than the unsubstituted compound II (Table II). (c) Insertion of a carbonyl function at position 3 also affords compounds (X and XI) which no longer exhibit selective activity against cholesterol. In this case, the chloro analog (X), having the lowest log P value, seems to be most effective against elevated triglyceride levels. Since 3-keto analogs exist in equilibrium with their enol forms (35), these compounds are expected to have markedly different electronic properties than the corresponding dihydrobenzofurans. Enol formation is in part stabilized owing to the generation of a π-excessive heteroaromatic ring system not unlike the hetero ring found in the benzofurans. The Cl and phenyl substituted benzofurans (III and IV) exhibit antilipidemic activity rather more similar to those observed for the respective 3-keto analogs (X and XI) than for those observed for the corresponding dihydrobenzofurans V and VII.

In conclusion, relative to the Triton model, the data presented in this paper support the hypothesis that the cholesterol and triglyceride lowering activity of clofibrate related analogs may be separated through a consideration of log P, conformational, and electronic parameters. Among the various analogs investigated in our laboratories (2,33,34), only the dihydrobenzofurans (V and VII) exhibited selective activity against elevated serum cholesterol levels. This selective activity is likely to be a reflection of conformational restriction of rotation rather than a change in log P since other derivatives having log P values either higher or lower than those calculated for V and VII lose selectivity.

ACKNOWLEDGMENT

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Free Radical Reactions of Peroxidizing Lipids with Amino Acids and Proteins: An ESR Study[1]

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ABSTRACT

Free radical transfer from oxidizing methyl linoleate to amino acids and proteins was studied in dry model systems incubated for periods up to 20 days. Electron spin resonance was used to study free radical production. Free radicals were detectable in the amino acids lysine, arginine, histidine, tryptophan, and cysteine. Reduced glutathione and, to a limited extent, cystine also gave free radical signals. Free radicals produced in proteins primarily showed central singlet lines, attributable to carbon-centered radicals, with $g = 2.004 \pm 0.001$. Sulfhydryl proteins also exhibited downfield shoulders at $g \cong 2.015$ and 2.023 that were essentially identical to peaks observed in cysteine and reduced glutathione. The field positions of sulfur resonance in cysteine and proteins suggested a sulfur-oxygen complex rather than thiyl radicals.

INTRODUCTION

Lipid peroxidation, a radical chain reaction, has been shown to exert radiomimetic effects on proteins in a variety of model systems (1). Typical biochemical changes in proteins exposed to peroxidizing lipids are similar to those induced by ionizing radiation and include: (a) loss of enzyme activity (2); (b) destruction of individual amino acids (3), particularly methionine, lysine, histidine, cystine, and tryptophan; and (c) polymerization, crosslinking, or scission (4,5), depending on water activity of the system and the nature of the protein (2). Such characteristics have led to the proposal (6) that reactions of free radicals arising during lipid oxidation are as important as those of peroxide breakdown products, such as malonaldehyde.

Loss of enzyme activity in aqueous model systems containing peroxidizing lipids has been shown to coincide with the appearance of fluorescent products caused by reactions between peroxide breakdown products and the protein. However, Roubal (7) subsequently noted that amino acid destruction paralleled free radical production in model systems of oxidizing lipids and proteins, whereas fluorescence did not. He thus proposed that free radicals are the major damaging species of peroxidizing lipids.

The probability of interactions between peroxidizing lipids and other system components has long been recognized. In the past several decades, however, studies of interaction mechanisms have been directed almost exclusively towards reactions involving lipid oxidation products, reactions which (unlike free radical reactions) may be studied with relative ease with ordinary physico-chemical methods. The recent adaptation of special techniques in electron spin resonance (ESR) spectroscopy has made possible the detection and direct identification of free radical species in many experimental situations and thus more practicable the study of free radical reactions in complex biological systems.

Roubal's have been the only reported ESR studies (8,9) of lipid free-radical interactions with proteins. He detected singlet signals with occasional downfield shoulders that he ascribed to lipid peroxy radicals stabilized in the protein matrix. However, doubt remains as to the identity of the radicals responsible for the observed ESR signals.

To further characterize free radical transfer from oxidizing lipids to proteins, we used ESR to examine free radical production in dry model systems (lyophilized emulsions and direct chemical mixtures) of oxidizing methyl linoleate (ML) and various proteins and amino acids (10,11). These model systems simulate conditions in dry foods. Although such solid model systems are not directly analogous to biological tissues, they facilitate room temperature ESR investigations by stabilizing free radical intermediates. Aqueous model systems can be studied via fast-flow and/or low-temperature ESR techniques, but with some loss in detail of resolution. In the solid systems, moreover, the components and physical form of the model systems can be easily controlled and manipulated to investigate the effects of various physical and biochemical parameters (e.g., amorphous vs. crystalline protein, anti-

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oxidant involvement) on the radical transfer reactions. Although our solid systems were designed to simulate dry foods, we believe that they provide useful and valid models for the study of free radical interactions that may also occur in tissues and membranes.

We have shown that as the water content of the lyophilized model emulsion systems is increased, the rates of radical recombination increase, and detectable radical concentrations decrease correspondingly (12). ESR signals from lysozyme reacted in such systems were singlets with g = 2.0051 ± 0.005 and line widths of 11 ± 3 G, which were identical to those obtained from pure lysozyme irradiated in air with γ-rays (11). Downfield resonance suggestive of sulfur radicals was detected in lysozyme only when the disulfide bonds were reduced before exposure to the oxidizing linoleate (10,11). Lysozyme signals were then quite similar to the signals reported by Roubal (8,9).

We report here evidence of free radical transfer to amino acids and to a number of proteins with a range of sulfhydryl/disulfide contents, and probable evidence that the "downfield shoulder" signals noted by Roubal arise from radicals centered on the sulfhydryl groups. We also propose a mechanism for free radical interactions between oxidizing lipids and proteins or amino acids, based on data obtained in the dry model systems.

EXPERIMENTAL PROCEDURE

Materials

Model systems were prepared by mixing methyl linoleate (Hormel Institute, Austin, MN; Nu-Check-Prep, Inc., Elysian, MN) directly with crystalline proteins or amino acids. Wt proportions were 1:5 (lipid:protein) and 1:8 (lipid: amino acid).

Proteins included lysozyme, bovine serum albumin, catalase, α-lactalbumin, and horse heart myoglobin (Nutritional Biochemicals, Cleveland, OH); casein, ovalbumin, and gliadin (Mann Research Labs, New York, NY). Amino acids studied were L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-cystine, L-glutamine, L-glutamic acid, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, and L-valine (Nutritional Biochemicals); L-methionine (Calbiochem, Oak Grove Village, IL), and glycine (Fisher Scientific, Fairlawn, NJ). Peptides included glycylglycine (Schwarz-Mann, Orangeburg, NY) and reduced glutathione (Nutritional Biochemicals).

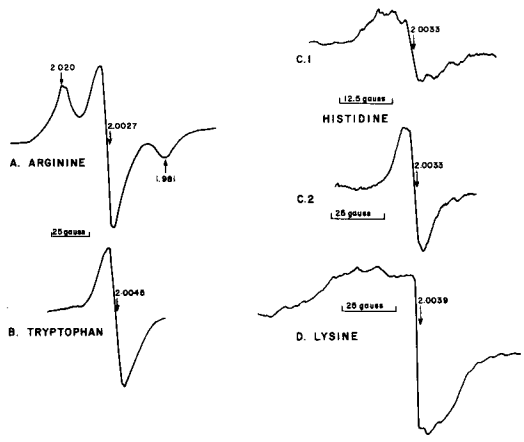

FIG. 1. Electron spin resonance spectra from amino acids exposed to oxidizing methyl linoleate. (A) Arginine: 1 day incubation (relative sensitivity 62.5, power 2 mW). (B) Tryptophan: 7 days incubation (relative sensitivity 6.4, power 10 mW). (C) Histidine: 1. 3 days incubation (relative sensitivity 10, power 2 mW); 2. 7 days incubation (relative sensitivity 10, power 2 mW). (D) Lysine: 3 days incubation (relative sensitivity 6.4, power 10 mW). Relative sensitivity is defined by modulation amplitude x (gain x 10^{-3}); modulation amplitudes were 5-10 G; gains were variable, depending on signal strengths.

Methods

Model systems and controls (proteins or amino acids without added linoleate) were incubated over $CaSO_4$ at 37 C for up to ca. 20 days. Samples (100-200 mg) were withdrawn periodically and transferred to 3.5-mm inside diameter quartz tubes. A Varian E-9 spectrometer with 100 KHz modulation was used to conduct ESR analyses. First derivative spectra were recorded. The cavity resonance frequency was measured with a frequency meter connected in the waveguide between the microwave bridge and the cavity. A nuclear magnetic resonance (NMR) gaussmeter was used in conjunction with an electronic frequency counter to calibrate the magnetic field.

For comparison of radical production, sulfur amino acids were also exposed in air to 1 Mrad γ-radiation (Cobalt 60 source: Gammacell 60, Atomic Energy Commission of Canada, Ltd.) at a dose rate of 9.5 x 10^3 rads/min. Samples were analyzed by ESR immediately after irradiation.

RESULTS

Amino Acids

ESR signals were detected in only four of the nonsulfur amino acids studied: lysine, arginine, histidine, and tryptophan. Representative spectra are shown in Figure 1. Most of the

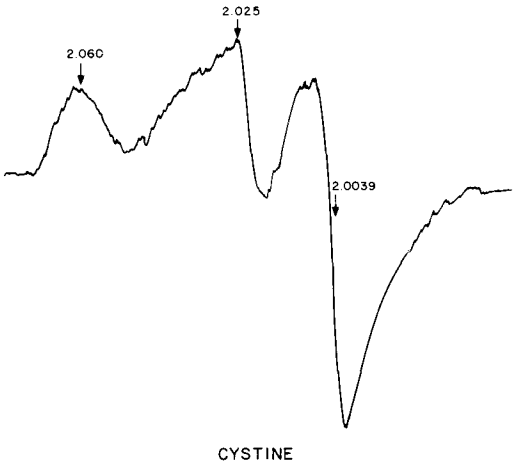

FIG. 2. Electron spin resonance spectrum of cystine reacted with oxidizing linoleate (6 days incubation; relative sensitivity 158, power 10 mW).

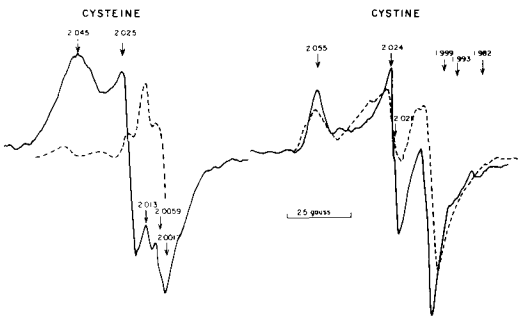

FIG. 3. Comparison of signals from cysteine and cystine exposed to oxidizing linoleate (broken line) and to γ-radiation (solid line). For irradiated cysteine: relative sensitivity 2.5, power 1 mW.

hyperfine structure apparent in the spectra of these amino acids after exposure to γ-radiation (10) is lost in oxidized samples—a not unexpected phenomenon considering the usual effects of oxygen on the hyperfine structure of most organic radicals (13). The spectra of all four amino acids indicate at least partial delocalization of spin density on the nitrogen atoms. However, only arginine had sufficient resolution to allow presumptive identification of a nitrogen-centered radical, in this case the guanidyl radical. The arginine, tryptophan, and histidine spectra were quite similar to those reported by Forbes and Sullivan (14) for UV-irradiated amino acids.

The absence of detectable ESR signals in the other amino acids indicated that steady-state radical concentrations, if any, were lower than ca. 10^{-10} M, the limit of detection of most ESR spectrometers. This phenomenon does not

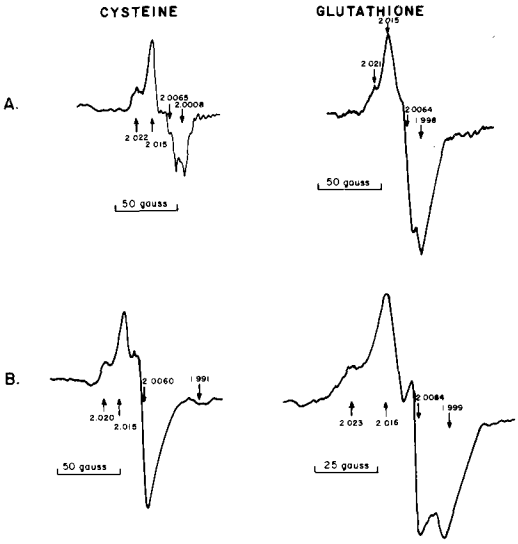

FIG. 4. Electron spin resonance spectra of cysteine and reduced glutathione reacted with methyl linoleate. **Cysteine:** (A) 2 days incubation (relative sensitivity 10, power 10 mW); (B) 8 days incubation (relative sensitivity 3.2, power 19 mW). **Glutathione:** (A) 1 day incubation (relative sensitivity 6.25, power 19 mW); (B) 11 days incubation (relative sensitivity 4, power 19 mW).

necessarily imply a lack of free radical interactions but may rather reflect greater instability of those radicals formed. Product analysis needs to be correlated with lipid oxidation parameters (e.g., peroxide and thiobarbituric acid values, fluorescence, and diene conjugation) to distinguish between these two possibilities.

Cystine reacted slowly with oxidizing methyl linoleate and gave weak initial spectra with single lines at g = 2.004 that indicated interactions at the α-carbon. After 6 days of incubation, downfield resonance typical of sulfur radicals (g = 2.025, 2.060) could be detected at high instrumental sensitivity (Fig. 2). The signals were quite similar to those generated by γ-radiation (Fig. 3) and presumably represented similar radical species.

Cysteine and glutathione exhibited both sulfur ($g \simeq 2.00$, g = 2.015, g = 2.022) and carbon (g = 2.0065) resonance after 1 day of incubation (Fig. 4A). Although quite similar, the spectra for the two compounds were not identical, reflecting different relative contributions from the various radical centers, which in glutathione include both glutamic acid and glycine as well as the component cysteine residue. Variations in line changes with incubation time (Fig. 4B) provide further evidence of additional radical centers and different relative contributions from the C· and S· centers in

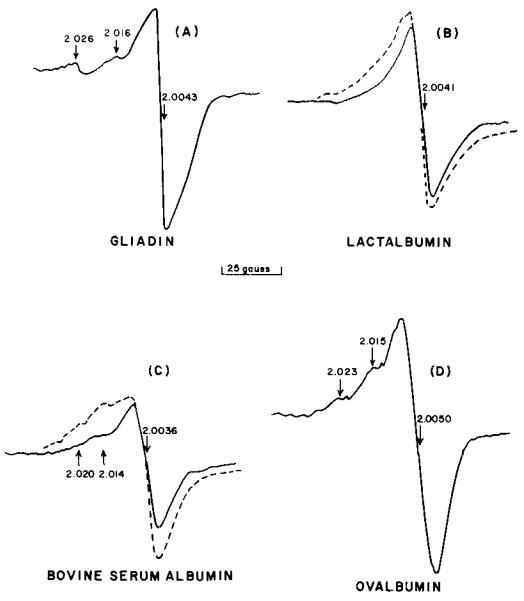

FIG. 5. Electron spin resonance spectra of proteins incubated with oxidizing methyl linoleate. (A) Gliadin (0/~8): 18 days incubation (relative sensitivity 5, power 20 mW). (B) Lactalbumin (0/4): 12 days incubation (relative sensitivity 4, power 2 mW [solid line] and 20 mW [broken line]). (C) Bovine serum albumin (0.7/17): 13 days incubation (relative sensitivity 4, power 2 mW [solid line] and 20 mW [broken line]). (D) Ovalbumin (4-5/1): 8 days incubation (relative sensitivity 5, power 10 mW).

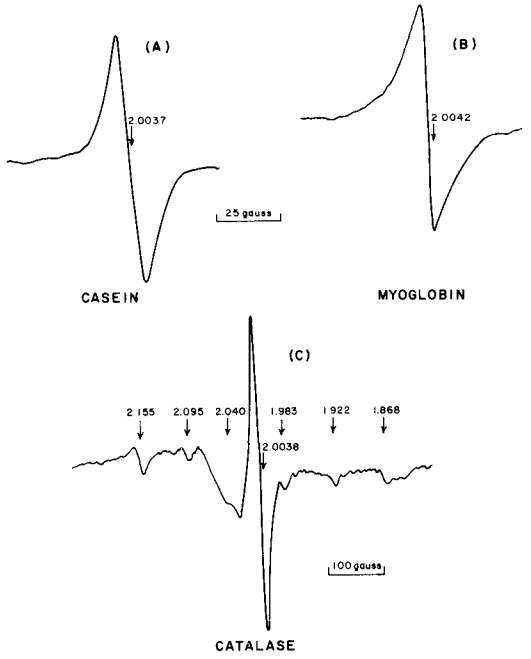

FIG. 6. Electron spin resonance spectra of proteins incubated with oxidizing methyl linoleate. (A) Casein (0-1/0): 10 days incubation (relative sensitivity 4, power 2 mW). (B) Myoglobin (0/0): 12 days incubation (relative sensitivity 2.56, power 20 mW). (C) Catalase (15/0-1): 5 days incubation (relative sensitivity 4, power 2 mW).

glutathione. No ESR signal was detectable at any time in methionine.

In contrast to cystine, the cysteine signals in the model systems differed from those generated by γ-radiation (Fig. 3A). The absence of a sulfur peak of $g \simeq 2.05$ to 2.06 and the stronger central carbon resonance in the oxidizing lipid systems are particularly interesting. These characteristics indicate differences in both sulfur free-radical species and in free electron distributions (e.g., greater localization on the α-carbons in the cysteine/lipid systems).

Proteins

No ESR signals were detected in control proteins incubated without lipid. Typical spectra for the proteins reacted with oxidizing ML are shown in Figures 5 and 6 (sulfhydryl/disulfide contents in residues/protein molecule are noted in parentheses in the figure legends).

Like spectra reported for irradiated proteins (15,16), these spectra exhibited two major components: a strong central singlet ($g = 2.004 \pm 0.001$) and weaker downfield shoulders ($g = 2.016$, 2.023). Peaks in catalase with g values differing greatly from those of free radicals ($g = 2$) arose from the heme iron.

The central lines of these spectra were all quite similar, but downfield shoulders generally appeared only when free sulfhydryl groups were present in the protein. Downfield resonance was absent in casein and myoglobin (no sulfur), and lactalbumin (disulfide).

The disulfide protein gliadin was an exception. The absence of the $g = 2.05$ signal seen in cystine apparently eliminates the possibility that some of the disulfide bonds near the molecular surface were cleaved by the lipid peroxides. Alternatively, gliadin is known to readily undergo disulfide interchange, and some reaction with lipids may occur during this process. However, the development of downfield resonance only after relatively long incubation periods (18 days) indicates low efficiency of whatever reaction is occurring. Furthermore, the high power levels required for clear detection of the downfield resonance reflected relatively low concentrations of sulfur radicals.

The development of weak downfield resonance in bovine serum albumin also required considerable incubation time (13 days). This protein, which binds lipids as part of its biological function, contains one free sulfhydryl that

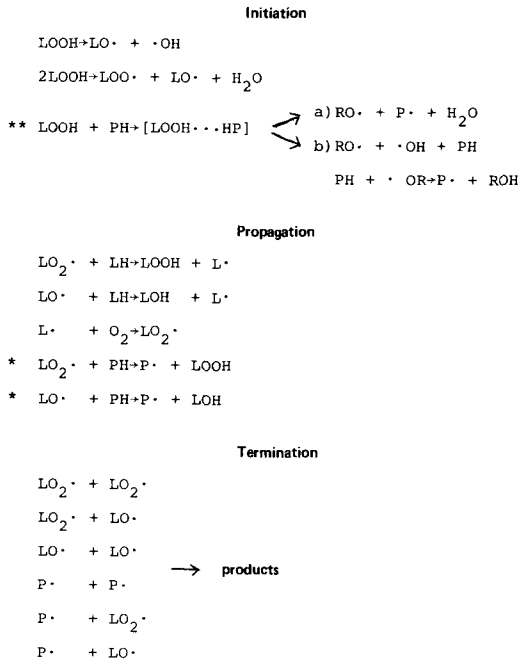

FIG. 7. Proposed overall reaction scheme for free radical interactions of proteins with peroxidizing lipids. *Reactions requiring direct contact between lipid radicals and proteins. **Reactions involving formation of complexes between lipid hydroperoxides and the N or S centers of the reactive amino acid residues. Reactive amino acids cysteine, lysine, arginine, histidine, and tryptophan form temporary complexes with lipid hydroperoxides via their corresponding sulfhydryl or positively charged nitrogen moieties. Radical transfer occurs either as the hydroperoxide and complex honolyze simultaneously (**-a), or as the hydroperoxide dismutates in the immediate vicinity of the amino acid (**-b). LH = lipid, PH = protein.

probably becomes exposed during the incubation period.

Catalase and myoglobin both contain heme iron, but only in catalase did the iron interact with lipid radicals. The lack of iron signals in myoglobin (compared with catalase) may be attributed both to differences in ligand binding in the two molecules and to steric protection of the heme group in myoglobin.

DISCUSSION

Free Radical Origins and Interaction Mechanism

ESR signals of free radicals generated in amino acids and proteins exposed to oxidizing methyl linoleate have been presented. We have shown previously (10-12) that these signals require the presence of oxidizing lipid and do, in fact, arise from nonlipid components of the systems. These findings thus substantiate the thesis advanced by Desai and Tappel (1) and Roubal (8), and supported by Zirlin and Karel (5), that free radicals produced in the lipid oxidation chain react with proteins.

No signals were evident in any of the samples before incubation, nor were they observed in lipid-free protein samples incubated in air for up to 40 days—in contrast to the suggestion of Munday et al. (17) that physically adsorbed oxygen is a source of ESR signals. ESR signals developed only in incubated systems containing at least partially oxidized lipid.

The signals persisted, although weaker, after extraction of the lipid. Furthermore, lipid radicals were undetectable at room temperature in either oxidized or γ-irradiated ML (1 and 2 Mrads, air and vacuum). ML oxidized on an inert microcrystalline cellulose matrix also gave no detectable ESR signals, even after 40 days of incubation. Similarly, peanut oil, safflower oil, and Wesson oil irradiated under identical conditions produced no signals detectable at room temperatures.

Others have reported similar difficulties in detecting free radicals in lipids. Lück et al. (18,19) were able to detect peroxy radicals in irradiated fats and crystalline fatty acids only after air incubation and low-temperature ESR measurements. Haydar and Hadziyev (20) noted a composite, nonsymmetrical ESR signal at g = 2.038 with a line width of 20 G—quite unlike the signals reported here or by Lück—in saturated fatty acid methyl esters exposed to γ-radiation at -196 C in a vacuum. When Haydar and Hadziyev (20) used UV radiation, the signals (g = 2.036, line width 2.5 G) were very weak and could be detected only at the limits of instrument sensitivity. Very high power levels and gain were also required to detect free radicals formed during the active ozonization of linoleic acid (21); when the ozone flow was stopped, the signal was lost. In addition to signals at g =2.004 and 1.998, a "peroxy" signal with a lifetime of ca. 20 min was noted at g = 2.014. However, in less energetic autoxidation systems, free radicals could not be detected in aqueous emulsions of hemoglobin and arachidonate peroxidizing at 10^{-5} M/sec (22). This failure was attributed to insufficient steady-state concentrations of free radicals.

These reports support our contention that lipid radicals in our peroxidizing solid systems are not detectable by ESR at room temperature. In addition, the nearly identical signals obtained from lysozyme exposed to oxidizing lipids, γ-radiation, high temperature, and t-butyl peroxy and alkoxy radicals (10,11)

provide presumptive evidence that the radicals observed in our model systems are, in fact, localized on the nonlipid components.

Alkoxy radicals seemed to be the most efficient initiators of protein radicals in a number of model systems containing lysozyme, casein, or ovalbumin incubated with butyl alkoxy and/or t-butyl peroxy radicals (10). By extrapolation, alkoxy radicals may be considered the major source of radical transfer in oxidized lipid-protein systems. Such reactivity is consistent with the observation that •OH radicals are the species most damaging to proteins irradiated in solution, and the •OOH radicals are somewhat less reactive (23).

If L•, LO•, and LOO• radicals are assumed to be too large to diffuse into the protein in a manner analogous to H• and •OH radicals produced during irradiation, then primary radicals can be expected to form only at sites on the protein surface. Effects of various environmental and systemic factors on radical concentrations (10,12) suggest that *contact* between lipid and protein components is critical to radical transfer. However, hydrogen abstraction by alkoxy or peroxy radicals in close proximity to amino acid residues (Fig. 7, * reaction) does not appear to account completely for our observations that (a) denaturation of the proteins, which one would expect to expose additional amino acid moieties for reaction, failed to increase radical concentrations per mg of protein (K.M. Schaich and M. Karel, unpublished data); (b) although ESR centers pre-formed by γ-irradiation of the various amino acids were almost equally stable under conditions comparable to our oxidized samples, of the 20 amino acids reacted with ML, only lysine, arginine, histidine, tryptophan, cysteine, and cystine exhibited detectable ESR signals; and (c) initial free radical production in proteins closely paralleled initial increases in lipid hydroperoxide values (10,12), suggesting that reactions between hydroperoxides and amino acid residues may also initiate the free radical chains (Fig. 7,** reaction).

We thus propose a mechanism in which, although contact is critical for direct hydrogen abstraction from protein sites by lipid peroxy and alkoxy radicals per se (Fig. 7,* reactions), radical transfer may also proceed via the formation of complexes between lipid hydroperoxides and the N or S centers of the reactive amino acid residues (Fig. 7, ** reaction).

There is considerable evidence that proteins and lipids interact through charge complexes. Luck (24), for example, found that fatty acids were found by positively charged amino acids via electrostatic interactions and van der Waals forces. More recently, lysine has been shown to form stable charge complexes with linoleic acid through the ε-amino group (25).

Under the reaction conditions of this study, lysine, arginine, and histidine all contained positively charged nitrogen moieties, and the indole nitrogen of tryptophan was resonance-stabilized and reactive. Thus, the possibility exists that these amino acids form temporary charge complexes with lipid hydroperoxides so that radical transfer occurs either as the hydroperoxide and complex homolyse (**-a), or as the hydroperoxide dismutates in the immediate vicinity of the amino acid (**-b), thus facilitating transfer (Fig. 7). Alternatively, the peroxide protein complex may have been formed via hydrogen bonding, e.g., between the amino acid nitrogen or sulfur atoms and an oxygen atom in the lipid hydroperoxide.

Nature of Observed Radicals

The typical triplet hyperfine structure of nitrogen-centered radicals was absent from the protein sites contributing to the observed signals. Considering the similarities of central singlet g values, line widths, and line shapes in these signals to those of analogous signals from irradiated proteins (10,15,16), the strong central resonance may be assigned to protein carbon radicals either on the backbone α-carbons or on the side chains.

The known characteristics of tryptophan as an electron sink in photolysis studies (26) and the spectral similarities of proteins and tryptophan (10,11; Figs. 1B, 5, and 6) make it quite tempting to suggest that tryptophan is the major contributor to the protein spectra. Preliminary studies correlating surface and total content of these four reactive amino acids with signal intensities in several proteins (K.M. Schaich and M. Karel, unpublished data) tended to support this role for tryptophan. However, since g values varied in different proteins, and saturation studies indicated the presence of several radical species, it is unlikely that any one amino acid is solely responsible for the central carbon resonance.

Since intramolecular radical migration is known to occur in irradiated proteins (27), a more plausible explanation is that the four reactive residues (whose long side chains protrude from the protein surface to provide greater accessibility to the lipid peroxides) serve as primary attack sites. Subsequent radical migration may allow electron localization on the α-carbons of a number of different residues along the protein backbone, resulting in the typical composite spectra.

Downfield sulfur resonance in signals of

both proteins and amino acids generally appeared only when sulfhydryl groups were present. The absence of sulfur resonance in proteins irradiated in air, or irradiated in vacuum and subsequently exposed to air, has been noted previously (10,16) and may be attributed to rapid disulfide recombination and/or oxidation of thiyl radicals to higher oxidation products (16). However, it is unlikely that such effects of oxygen were responsbile for the observed lack of sulfur resonance in the oxidizing systems reported here, since only very weak downfield resonance (possibly indicative of sulfur radicals) could be detected, even at high spectrometer gain and microwave power, when pre-formed lipid peroxides were reacted in vacuum with lysozyme.

Steric unavailability of disulfide bonds in the protein interior (e.g., of lysozyme for reaction with ML) was also apparently not solely responsible for the lack of sulfur signals, since denaturation of lysozyme with guanidine HCl before incubation with ML did not facilitate sulfur radical formation, except when the disulfide bonds were concurrently reduced to sulfhydryl groups with β-mercaptoethanol (10,11). Weak sulfur resonance in the disulfide proteins gliadin and bovine serum albumin, detectable after long incubations, may be attributed to specific functional structures in which sulfhydryl groups may become exposed during the incubation.

In free cystine, the central carbon resonance appeared first and remained predominant throughout the incubation period. Sulfur signals differing from those in sulfhydryl compounds were detectable after relatively long incubation periods and probably resulted from intramolecular electron migration to the disulfide bond or from increased steric susceptibility of the S-S bond due to conformational shifts after initial free radical attack.

This evidence seems to suggest that sulfur resonance in proteins and amino acids exposed to oxidizing lipids arises from radicals formed specifically on sulfhydryl groups and that disulfide bonds are relatively resistant to free radical attack from lipids. This conclusion is not surprising in light of the following mechanistic considerations: (a) oxidizing lipids presumably transfer free radicals to other compounds via hydrogen abstraction; and (b) sulfhydryl compounds form free radicals by losing labile hydrogens (28,29) and are thus compatible transfer sites for the lipids.

In contrast, free radical formation from a disulfide involves either removal of an electron from the disulfide bond (with the subsequent formation of a lower energy, three-electron bond S···S [30]) or S_{H2} displacement by other free radicals (e.g., on lipids [31]). The latter reaction requires an unhindered radical and is highly stereospecific and sensitive to the sizes of the molecules involved. Reaction of bulky lipid radicals with disulfides sterically protected in proteins is therefore highly unlikely. Reaction with free disulfides, like cystine, is more easily effected, although still kinetically unfavorable. Cystine's lack of reactivity toward lipid peroxides, relative to that of various thiols, is indirectly supported by numerous reports in the literature showing pro- or antioxidative effects of cysteine (32), gluthathione (33), and other small thiols (34), whereas none are known to show comparable effects for disulfides.

The sulfur resonance of ML-exposed cystine, when present, was essentially identical to that of irradiated cystine (Fig. 3B). However, the downfield resonance in sulfur proteins, cysteine, and reduced glutathione reacted with oxidizing ML appeared at $g \simeq 2.015$, 2.023. This configuration differs from the broad asymmetric envelopes with principal g values at 2.02-3 and 2.05-6 characteristic of sulfur-centered radicals in irradiated powders and crystals (Fig. 3A), indicating that the sulfur radicals formed by the two mechanisms are not identical.

Symons (35) has recently shown that spectra from irradiated thiols, and disulfides having anisotropic g values of 2.002, 2.025, and 2.060 arise from species in the isostructural family $RS \therefore SR_2$, $RS \therefore SR^+$, $RS \therefore SR^-$. Whereas such identification is appropriate for irradiated cysteine, and for cystine either irradiated or exposed to oxidizing ML, it cannot describe the radicals induced in thiols or sulfhydryl proteins by oxidizing lipids, where no $g \simeq 2.06$ signal component has been observed.

Doublet signals with g values in the 2.01-2.02 range have been reported for cysteine oxidized in flow systems by ceric ions (36) or hydroxyl radicals (37). However, molecules in solution may tumble freely, thus eliminating the anisotropy induced by fixed electron orbital orientations in solid samples. It seems unlikely that a situation simulating free tumbling could be achieved in the powder systems of this study, but the possibility of such effects cannot be eliminated.

A more plausible interpretation, however, is that the peaks at $g \simeq 2.016$, 2.023 may reflect delocalization of the free electron over other atoms besides sulfur, as would occur in sulfoxy or sulfur peroxy radicals. For example, when cysteine was oxidized in an aqueous flow system with ceric ions (36), two signals were

detected, one at g = 2.0106, identified as the radical $-CH_2-S\cdot$, and the second at g = 2.0186, attributed to -CH-SO· and/or $-CH_2-SOO\cdot$ radicals as most likely species. In frozen solutions of irradiated aminethiols, Copeland (38) subtracted the spectra obtained in nitrogen from those obtained when oxygen was present and demonstrated a doublet at g = 2.015, 2.023 that he suggests resulted from the formation of a sulfur-oxygen adduct, probably -SOO·.

It is not currently possible to identify the exact species of radical(s) responsible for the downfield resonance in the model systems reported here. Nevertheless, it *is* certain that the radicals arose from reaction at the cysteinyl residues, since (a) such resonance *only* occurred when free sulfhydryl groups were present, eliminating the possibility that the signals were alkyl peroxides, which also give g values of ca. 2.015, and (b) resonance in proteins was essentially identical to that in free cysteine and in reduced glutathione.

The signals detected by Roubal (8,9) in model systems of oxidizing lipid mixed directly with proteins and incubated under comparable conditions fit the same general pattern observed in the various proteins in this study. In Roubal's study, the downfield resonances of sulfhydryl proteins occurred at apparent g values in the 2.01-2.02 range, and the ESR spectral envelopes were structurally similar to the corresponding signals reported here. In proteins containing no sulfur amino acids (e.g., bovine hemoglobin) no downfield resonance was observed, as was also the case in our work. Thus, it may be presumed that the downfield resonance noted in Roubal's system was, in fact, caused by sulfur-type radicals, rather than by the matrix-stabilized lipid peroxy radicals.

Lipid peroxidation has been implicated both in vivo and in vitro in a variety of physiological and pathological conditions: in ozone toxicity (39,40); in increased membrane permeability of cells (41) and erythrocytes (42); in liver damage from ethanol (43), carbon tetrachloride (44), and aging (45); in formation of age pigments (46,47); and in vitamin E and antioxidant deficiencies (48,49). Recent evidence suggests that lipid oxidation begins concurrently with the induction of mixed-function oxidases involved in the metabolism of carcinogens (50,51). Lipid oxidation is also an important deteriorative reaction in metabolically static systems, e.g., it affects the storage stability (52) and nutritional quality (53) of food.

The free radical reactions of peroxidizing lipids may also have an important role in sensitizing cells or foods to oxidative damage. This sensitization occurs because oxidizing unsaturated lipids can propagate long chains of free radicals (Fig. 7) (an average chain length of 15-20 in cell membrane lipids [54]), which have the potential to transmit free radical damage from an initiating site, presumably a cellular or intracellular membrane, to a sensitive or "target" molecule some tens of Ångstroms away. In this way, lipid free radical intermediation may effectively increase the "target size" for damaging nonlipid molecules embedded in membranes or in contact with them.

The interaction mechanisms operative in the cooxidations of lipids and proteins in vivo or in the development of rancidity in foods during storage are not completely understood. Considerable study has been devoted to reactions involving lipid oxidation breakdown products. In contrast, although the participation of intermediate lipid free radicals has been hypothesized, such cooxidation reactions have not yet been demonstrated.

The evidence of free radical transfer from oxidizing ML to amino acids and proteins provides substantiation for the proposal that free radicals are a major mechanism for lipid peroxidation damage to proteins in general (3) and to sulfhydryl proteins in particular (55,56). Some preliminary attempts have been made to identify specific radical sites although, due to the nature of the dry solid model systems used, any precise identification is necessarily speculative at this time. Nevertheless, spectra were consistent and sufficiently characteristic to be useful for comparison with spectra obtained from γ-irradiated proteins and from tissues.

As noted previously in this text, spectra of proteins exposed to peroxidizing lipid or to γ-radiation are comparable. In addition, ESR signals essentially identical to those reported here for sulfhydryl proteins have been detected in frozen human blood (J. Paxton, Varian Associates, personal communication, 1974), in lyophilized human whole blood and erythrocytes (Schaich, Paxton, and Borg, unpublished data), and in frozen air-exposed rat blood (Schaich and Borg, unpublished data), indicating that radical transfers very similar to those demonstrated in dry model systems can also occur under physiological conditions. Similar ESR signals in rapidly frozen rabbit liver microsomes have, in fact, been attributed to protein-bound thio radicals induced by lipid autoxidation (57).

We have demonstrated the capacity of free radicals to transfer from oxidizing lipids to proteins and amino acids; other functional or structural molecules in contact with oxidizing lipids (e.g., vitamins or nucleic acids) may be similarly affected (Schaich and Borg, un-

published data). A more complete understanding of the mechanisms by which oxidizing lipids interact with other molecules will be needed, however, in order to use the knowledge for the prevention or inhibition of oxidative pathological damage in vivo or for the development of improved storage stability in food systems, particularly with respect to the efficacy and possible toxicity of antioxidants, both intrinsic dietary components and those added as preservatives, and of other food additives.

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Biosynthesis of Fatty Acids by the Carp, *Cyprinus carpio* L., in Relation to Environmental Temperature

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ABSTRACT

Incorporation in vivo of sodium 1-^{14}C-acetate into different lipid classes and fatty acids of total lipids and phospholipids of warm adapted and cold adapted carp livers was studied at 5 C and 22 C, respectively. The fatty acid composition of total lipids and phospholipids was also determined. The level of long chain polyunsaturated fatty acids in both total lipid and phospholipid fractions was higher in cold adapted fish than in warm adapted ones. The distribution of radioactivity among different lipid classes depended only on the actual incorporation temperature and was independent of the temperature history of the animals. Livers of fish incorporated a higher percentage of radioactivity into long chain polyunsaturated fatty acids of total lipids and phospholipids in 5 C than in 22 C. The distribution of radioactivity among different fatty acids was dependent on the experimental temperature rather than on the temperature to which the fish were adapted. The results suggest that fish are able to adjust the pattern of the biosynthesis of fatty acids very rapidly to the prevailing temperature and to assure by this way the proper physicochemical properties of their membranes. The possible mechanisms involved in this rapid response are discussed.

INTRODUCTION

Cholesterol content, the level of lysophospholipids, and the presence of unsaturated fatty acids in membranes are regarded as important in controlling membrane fluidity (1-5). Increasing the amount of these compounds or the degree of unsaturation of phospholipid fatty acids results in a shift of phase transition of membranes toward lower temperature with corresponding changes in permeability, activity, and allosteric behavior of several membrane-bound enzymes (6-19). It is very important, therefore, for all organisms to have these compounds in the right proportion in their membranes.

The phase transition point of membranes in warm blooded animals is well below the body temperature (20,21) and, except in a few cases (22), control of membrane fluidity is unnecessary. Poikilothermic animals, such as fish, do not have a constant body temperature. The adaptation of the physico-chemical properties of their membranes to ever-changing temperatures, therefore, has considerable survival value. This concept is supported by observations showing that prolonged cold exposure results in an accumulation of long chain polyunsaturated fatty acids in phospholipids of fish (23-27) and other aquatic animals (28-31). Increase in activity of some membrane-bound enzymes (32,33), including desaturation of fatty acids (34), was also noted. The present paper shows that the adjustment of long chain polyunsaturated fatty acids to changes in the environmental temperature is quite rapid in fish and does not depend on the temperature history of the animals.

MATERIALS AND METHODS

Animals

The fish, *Cyprinus carpio* L., weighing 150-200 g, were collected from their natural environment 2 days before the experiments. Warm adapted (WA) animals were collected in late summer (water temperature ≈ 22 C) of 1974 and cold adapted animals in mid-winter (CA-1) and late winter (CA-2) of 1974-1975 (water temperature ≈ 5 C). After collection, they were kept in aerated aquaria and transferred to the laboratory in nylon bags under oxygen on the day of the experiments. Sodium 1-^{14}C-acetate (sp act 1.18 mCi/mmol) or sodium 2-^{14}C-acetate (sp act 4.58 mCi/mmol) dissolved in 0.6% sodium chloride solution, was injected abdominally with the aid of a tuberculine syringe. After the injection (10 μCi/100 g body wt), the animals were placed in aquaria, set to 5 C and 22 C, respectively. They were stunned by a blow on the head 2 hr after the injection when incubated at 22 C and 6 hr after the injection when incubated at 5 C. These incubation times were established by incubating liver tissue slices at the above temperatures; the relatively long incubation at 5 C compensated

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for the decreased metabolic activity in cold exposed animals. Three animals were used in each group.

Extraction of Lipids

Livers were excised from freshly killed animals and pooled. After several rinses in ice cold physiological saline, they were blotted on filter paper and weighed. Lipid extraction was performed according to Folch et al. (35) by homogenizing the tissue in an all-glass Potter homogenizer in the presence of ice cold chloroform:methanol, 2:1. The homogenate was flushed with CO_2 and placed in a refrigerator overnight, then filtered and separated into two phases by adding 0.1 M KCl solution. The extract was washed free of radioactivity by Folch theoretical upper phase (35) containing 0.1 M cold sodium acetate. Finally, the volume of the extract was adjusted to 5 ml with chloroform. Aliquots of this solution were taken for total lipid determination, counting, and further analyses.

Separation of Lipids

Lipid class separation was performed by thin layer chromatography (TLC) on Silica Gel G plates using hexane:ethyl ether:acetic acid, 85:15:1, as solvent. In some cases, the separations were performed using the two-step developing techniques employed by Salle et al. (36). Several thousands of counts were applied to the plate by a micrometer syringe. The chromatograms were visualized by iodine vapor or by spraying with 0.1% Rhodamine B in ethanol and detecting under ultraviolet light. When the phospholipids were subjected to gas chromatography, no staining was employed at all; the origin was scraped directly into methylation ampoules.

Gas Liquid Chromatography (GLC)

Methyl esters were prepared by transesterification in absolute methanol containing 5% hydrochloric acid, in ampoules sealed under CO_2, at 80 C. The analyses were carried out on a JGC 1100 gas chromatograph equipped with flame ionization detector. The 6 ft long stainless steel column, 0.3 cm inside diameter, was packed with 15% diethylene glycol succinate on Gas Chrom P (Applied Science Lab., State College, PA), 100-200 mesh. The column temperature was 180 C when analytical runs were performed. For determination of radioactivity of fatty acids, a coiled glass column (0.6 cm inside diameter) was employed. The column was split before its outlet in such a way that only 25% of the eluate escaped to the detector while 75% was trapped on cotton wool. Preparative runs were programmed from 140 to 180 C at a rate of 1 C/min to ensure the complete separation of palmitoleic and oleic acids from palmitic and stearic acids, respectively. The samples were run in two or three replicates. Peaks were identified by using suitable standards (Applied Science Lab. N° K-108), by secondary standards (cod liver oil), and by plotting the logarithm of the relative retention times versus the number of carbon atoms in the molecule. Quantitation was performed by the triangulation techniques. The accuracy of the determination was $> 5\%$ in the case of major fatty acids and ca. 20% in the case of minor ones.

Determination of Radioactivity

Known aliquots of the chloroform extract were pipetted directly into counting vials and, after evaporation of the solvent, were dissolved in scintillation cocktail. Spots from the thin layer plates were scraped directly into counting vials. When the plates were visualized by iodine vapors, the iodine was allowed to evaporate from the spots before counting. The cotton on which the eluates were trapped, when the separation was performed by GLC, was placed without prior elution into counting vials. Toluene scintillation cocktail (4% PPO and 0.2% POPOP) was used throughout the experiments. Counting was performed by an Isocap 300 Nuclear Chicago or a Tri Carb Liquid Scintillation Spectrometer. Counts were corrected for quenching using sample channel ratio techniques and for counting efficiency.

RESULTS

Fatty Acid Composition of Warm and Cold Adapted Fish

Total fatty acids extracted from livers of cold adapted animals contained more arachidonic acid (20:4ω6) and docosahexaenoic acid (22:6ω3) than those obtained from warm adapted animals (Table I). The increase in the level of arachidonic acid was ca. ten-fold and, in the level of docosahexaenoic acid, ca. two-fold in CA-2 animals, as compared with WA fish. During cold adaptation, the level of palmitic acid (16:0) was reduced by ca. 50%. In contrast, there was an increase in the content of stearic acid. Oleic acid (18:1) did not exhibit any change during this period.

Phospholipids exhibited changes similar to the total fatty acids. However, they were richer in arachidonic and docosahexaenoic acids. These results are comparable to those obtained for *Gambusia affinis, Lebistes reticulatus, Salmo gairdneri,* and *Carassius aureatus* (23-27),

TABLE I

Fatty Acid Composition (% by wt) of Liver Total Fatty Acids and Phospholipids of Cold and Warm Adapted Carp[a]

Fatty acids	Total fatty acids			Phospholipids		
	WA	CA-1	CA-2	WA	CA-1	CA-2
14:0	1.7	0.8	0.8	0.5	0.7	0.5
16:0	20.3	32.2	11.6	32.0	18.1	14.6
16:1ω7	10.4	6.6	3.8	6.4	4.7	4.7
18:0	6.7	6.3	9.8	5.4	7.3	9.1
18:1ω9	35.7	22.0	34.0	10.4	10.5	15.7
18:2ω6	11.3	9.5	10.9	3.1	4.0	0.5
18:3ω6	0.7	0.6	0.4	0.7	1.0	0.2
18:3ω3	4.5	4.2	3.6	2.7	2.7	1.4
20:2ω6	0.6	0.1	0.1	1.1	1.2	0.3
20:3ω6	0.4	0.2	0.2	tr	1.3	0.3
20:3ω3	0.5	0.9	0.5	0.5	2.3	0.6
20:4ω6	1.1	8.9	10.8	16.8	23.0	25.4
20:4ω3	0.1	tr	tr	tr	0.3	0.1
20:5ω3	0.1	0.5	1.2	tr	0.3	0.1
22:4ω6	0.2	0.7	1.2	3.1	2.4	1.2
22:5ω6	0.6	0.8	3.8	3.9	2.9	2.1
22:6ω3	2.8	4.6	6.0	10.3	13.7	19.7

[a]WA = warm adapted fish; CA-1, CA-2 = cold adapted fish. WA animals were collected in late summer of 1974, CA-1 and CA-2 in mid and late winter of 1974-1975, respectively.

TABLE II

Incorporation of Radioacetate into Liver Lipids of Carp at Different Temperatures

Animals[a]	WA		CA-1	CA-2	CA-1	CA-2	CA-2[b]
Temperature (C)	22	5	5	5	22	22	22
nMol of acetate incorporated by 100 mg of liver	10.3	4.2	4.6	5.2	15.7	4.8	8.9
	Percent distribution of radioactivity						
Cholesterol esters	4.3	17.3	15.0	1.2	5.0	1.6	1.2
Triglycerides	2.5	1.8	35.0	24.5	30.0	15.2	14.0
Free fatty acids	28.0	29.6	0.6	6.1	0.2	5.8	3.8
Cholesterol	0.8	1.9	1.3	33.8	0.6	13.7	9.1
Diglycerides	2.2	4.4	5.9	2.1	1.9	1.2	0.5
Phospholipids	62.2	44.9	41.3	32.0	62.1	62.5	71.2

[a]WA = warm adapted fish; CA-1, CA-2 = cold adapted fish. WA and CA-1 animals received 1-^{14}C-sodium acetate; CA-2 animals 2-^{14}C-sodium acetate 2 hr (experiments at 22 C) or 6 hr (experiments at 5 C) before sacrifice.

[b]Animals received glucose before the injection of acetate.

with the exception that the changes evoked by the decrease in environmental temperature were more dramatic than with the above animals.

Incorporation of 1-^{14}C-Acetate into Liver Lipids

After injection of sodium 1-^{14}C-acetate, the liver lipids became highly labeled. Livers of WA fish incorporated ca. 60% less label at 5 C than at +22 C (4.2 nmol/100 mg liver versus 10.3 nmol). The rate of biosynthesis of lipids by CA-1 and CA-2 animals at +5 C was comparable to that obtained for WA fish at +5 C (Table II).

Incubation of CA-1 animals with acetate at 22 C resulted in a substantial increase in the incorporation of acetate. CA-2 animals failed to synthesize more lipid when treated with 2-^{14}C-acetate at 22 C unless glucose was injected abdominally before the experiment (Table II).

CA-1 and CA-2 animals had a lower proportion of radioactivity in their phospholipids than did their WA counterparts and more activity in cholesterol. Similarly, WA fish had a lower proportion of radioactivity in phospholipids when injected with acetate in the cold. The proportion of radioactivity in phospholipids after injection of CA animals with acetate in the warm was very similar to the values obtained with WA fish at 22 C. The free fatty acid

TABLE III

Distribution of Radioactivity among Total Fatty Acids of Livers of Warm and Cold Adapted Carp at Different Temperatures

Animals[a]	WA		CA-1	CA-2	CA-1	CA-2	CA-2[b]
Temperature (C)	22	5	5	5	22	22	22
nMol of acetate incorporated by 100 mg of liver[c]	9.8	3.4	3.9	3.4	14.9	4.1	8.0
Fatty acids	Percent distribution of radioactivity						
<16:0	3.9	6.3	5.7	4.2	4.6	4.8	1.7
16:0	49.6	15.2	14.7	11.4	45.2	32.0	35.9
16:1ω7	3.9	1.8	3.0	2.3	4.6	3.1	4.4
18:0	13.4	14.3	15.2	7.6	7.0	13.4	28.8
18:1ω9	7.9	11.1	4.8	5.4	9.2	4.1	2.6
18:2ω6	0.9	1.3	2.5	2.8	2.9	2.8	2.1
18:3ω6	1.6	5.9	2.1	2.1	1.4	2.7	2.2
18:3ω3	5.6	14.8	12.7	6.0	5.4	5.7	3.4
20:2 + 20:3	3.6	8.3	13.1	12.6	6.4	13.4	6.1
20:4ω6	2.3	5.4	5.9	7.2	2.5	3.8	3.0
20:4ω3	ND	ND	4.2	3.1	1.9	2.3	2.0
20:5ω3	0.9	1.3	2.9	5.2	0.9	3.1	1.6
22:4ω6	2.1	5.2	5.4	5.4	1.8	ND	ND
22:5ω6	1.0	2.4	1.9	3.8	2.0	2.9	2.2
22:5ω3	1.1	4.3	4.3	10.0	1.9	3.3	2.3
22:6ω3	2.0	2.2	3.6	10.6	2.0	2.2	1.7

[a]WA = warm adapted fish; CA-1, CA-2 = cold adapted fish. WA and CA-1 animals received 1-^{14}C-sodium acetate; CA-2 animals 2-^{14}C-sodium acetate 2 hr (experiments at 22 C) or 6 hr (experiments at 5 C) before sacrifice.

[b]Animals received glucose before the experiments.

[c]Values were corrected for activity of cholesterol.

[d]ND = not determined.

fraction of WA animals was highly labeled in contrast to the results with CA-1 and CA-2 fish. The sum of activity of triglycerides and free fatty acids was, however, similar in both WA and CA animals. A possible explanation is that the WA fish could have been in a fasting state and the α-glycerophosphate level of their livers might not have been sufficient to esterify the fatty acids formed to give triglycerides.

Apparently the distribution of radioactivity among the different lipid classes is dependent on the actual incorporation temperature rather than on the temperature history of the animals.

Distribution of Radioactivity among Liver Total Fatty Acids

Palmitic acid was the most heavily labeled fatty acid in WA fish when injected with acetate at 22 C (Table III); it represented about half of the radioactivity of total fatty acids. Stearic acid, containing 13% of the activity, was the second most heavily labeled compound. There was some radioactivity also in linoleic acid and a considerable activity in linolenic acid. This fact has also been observed by others (37) and can be explained by the presence of overlapping fatty acids (such as 20:1 in the case of linolenic acid) and/or by carboxy carbon exchange. Long chain polyunsaturated fatty acids were only poorly labeled. A similar pattern of labeling has been reported for the northern pike, *Esox lucius* L. (38), and goldfish, *Carassius auratus* L. (39).

When WA fish received acetate in the cold, there was a dramatic decrease in labeling of palmitic acid while a considerably greater proportion of label appeared in stearic, oleic, and arachidonic acids. The labeling of docosapolyenoic acids was also increased during this short cold exposure of WA fish. The only exception was docosahexaenoic acid, the labeling of which remained unchanged (Table III).

There was a further reduction in the incorporation of acetate into palmitic acid by CA-1 and CA-2 animals at 5 C. Labeling of long chain polyunsaturated fatty acids, including docosahexaenoic acid, increased from WA to CA-2 animals. In the latter fish, these unsaturated acids contained almost half of the radioactivity present in total fatty acids. This pattern of labeling could be completely reversed by injecting CA-1 and CA-2 animals with acetate in the warm. It is to be noted that, of the CA-2 fish, those receiving glucose before the experi-

TABLE IV

Distribution of Radioactivity among Liver Phospholipid Fatty Acids of Warm and Cold Adapted Carp at Different Temperatures

Animals[a]	WA		CA-1	CA-2	CA-1	CA-2	CA-2[b]
Temperature (C)	22	5	5	5	22	22	22
Fatty acid	Percent of total radioactivity						
<16:0	6.5	3.6	4.2	5.5	4.3	4.9	1.7
16:0	59.3	21.6	15.3	15.2	55.4	39.0	52.7
16:1ω7	1.4	1.0	1.4	3.1	1.6	2.0	2.5
18:0	11.8	16.5	16.4	11.2	16.2	15.1	19.9
18:1ω9	5.1	11.3	6.6	5.3	3.6	3.5	4.4
18:2ω6	0.4	0.8	7.8	3.3	1.8	2.1	2.9
18:3ω6	0.5	ND[c]	6.1	ND	1.1	2.3	1.9
18:3ω3	5.5	15.1	8.2	10.6	4.4	3.6	2.5
20:2 + 20:3	2.6	10.0	10.3	13.2	4.0	6.7	4.8
20:4ω6	1.4	5.8	4.0	6.7	1.6	3.4	1.4
20:5ω3	1.0	2.9	3.4	4.6	1.1	1.7	0.9
22:4ω6	1.7	5.0	4.2	7.2	0.2	2.9	1.3
22:5ω6	1.1	2.5	3.7	3.1	0.7	1.7	0.8
22:5ω3	0.7	0.6	5.0	6.1	0.9	2.2	1.1
22:6ω3	0.8	2.0	3.4	6.5	1.3	3.7	0.8

[a]WA = warm adapted fish; CA-1, CA-2 = cold adapted fish. WA and CA-1 animals received 1-^{14}C-sodium acetate; CA-2 animals 2-^{14}C-sodium acetate 2 hr (experiments at 22 C) or 6 hr (experiments at 5 C) before sacrifice.

[b]Animals received glucose before the injection of acetate.

[c]ND = not determined.

ment were more successful in reducing the labeling of long chain polyunsaturated fatty acids upon exposure to warm.

Labeling of Liver Phospholipid Fatty Acids

The pattern of labeling of phospholipid fatty acids was similar to that of total fatty acids (Table IV). However, an increase in labeling of docosahexaenoic acid in WA carp injected with acetate in the cold could be observed. We believe that higher levels of acyl transferase might result in this increase since there was no substantial difference in the labeling of docosahexaenoic acid in the total fatty acid fraction in WA animals at 22 C and 5 C (Table III). The labeling of arachidonic acid remained nearly constant during the whole period of incubation in the cold but that of docosahexaenoic increased gradually. When CA animals were injected with acetate in the warm, the distribution of radioactivity became very similar to that observed with WA fish at 22 C. As only 2 hr had passed between the injection and sacrifice of the animals, it is possible that fish possess a mechanism by which they adapt very precisely the biosynthesis of fatty acids and the fatty acid composition of phospholipids to the prevailing temperature.

DISCUSSION

The principal finding of this study is that fish, such as carp, can adjust their fatty acid metabolism to the prevailing temperature in such a manner that a decrease in the environmental temperature results in the accumulation of long chain polyunsaturated fatty acids. Conversely, increase in the temperature gives rise to the formation of saturated fatty acids. The response to changes in temperature is quite rapid and reversible, and does not seem to depend on the temperature history of the animals. Rapid disappearance of docosahexaenoic acid from the intestinal mucosa of cold adapted goldfish was observed after transferring the animals to warm (11). However, the data of Tables III and IV suggest that the nutritional state of the animals might have some effect on the intensity of this response.

Available evidence suggests that the membrane lipids of fish are in a fluid state at any temperature between 5 C and 25 C (18,40). The majority of these investigations were carried out on specimens adapted to 10 C. Investigating the fats of planktonic crustaceans, we have shown that the melting point of their fats was always a few degrees below the actual water temperature, and changes in unsaturation of their fats paralleled the melting point (28). Thus, the observed effect of temperature on the biosynthesis of fatty acids in carp may be partially connected with regulation of membrane fluidity.

However, the process allowing the animals to respond so sensitively to changes in their environment remains to be elucidated. We believe that temperature acts directly upon fat metabolism of the cells and, at least at the beginning of cold (or warm) adaptation, the endocrine system of the animals is not involved. In agreement with this are the results of in vitro experiments in progress in this laboratory. Liver slices of warm adapted fish incorporated a higher percentage of 1-^{14}C-acetate into arachidonic and docosahexaenoic acid when incubated at 5 C than at 25 C (unpublished results).

Saturated fatty acids are formed in the soluble fraction of the cells by the enzyme complex system, fatty acid synthetase (41), while long chain polyunsaturated fatty acids are formed in the endoplasmatic reticulum by chain elongation and subsequent desaturation of linoleic and linolenic acids (42). In the present study, no exogenous linoleic or linolenic acid was given to the animals. To undergo chain elongation and desaturation, these fatty acids must have been released from phospholipids or triglycerides, transferred to their CoA-esters, and, after completion of the reactions, the products had to be esterified to lysophospholipids or diacylglycerols to form complex lipids. Thus, labeling of long chain polyunsaturated fatty acids of phospholipids is a result of a precise cooperation of deacylation-reacylation reactions as well as the fatty acid biosynthetic processes.

The results obtained indicate that the fatty acid synthetase and the systems responsible for the formation of long chain polyunsaturated fatty acids respond divergently to changes in temperature. The drastic drop in the labeling of palmitic acid in the cold suggests that fatty acid synthetase has a high temperature coefficient while the systems producing polyunsaturated fatty acids might be less sensitive to the decreased temperature. Expression of the data on a tissue wt basis showed (data not included in this study) that a 20 C decrease in temperature results in ca. 95% loss in fatty acid synthetase activity but only 50-90% loss in producing various eicosa- and docosapolyenoic acids. The rate of production of saturated fatty acids remained at this low level in cold adapted fish but that of long chain polyunsaturated ones increased gradually (from 13% to 61% and from 27% to 33% in the case of docosahexaenoic and arachidonic acid, respectively.

The differences in the temperature sensitivities of the fatty acid synthesizing systems would facilitate the production of saturated and long chain polyunsaturated fatty acids in the right proportion at a particular temperature. This type of regulation is sensitive and rapid enough to assure the proper functioning of the membranes under changing temperature conditions. Data of Tables III and IV, on the other hand, point to the possibility that either the level or the activity of desaturases has been increased during prolonged exposure to cold. It is interesting in this connection that increase in the level of enzyme β-hydroxydecanoyl thioesterase has been found to be responsible for elevated production of cis vaccenic acid in cold exposed *Escherichia coli* (43). Fulco et al. (44) have demonstrated, on the other hand, that in certain bacilli a desaturase converting stearic to 5-octadecenoic and 5,9-octadecadienoic acids was induced by exposing the cultures to cold. Fatty acid synthetase, stearyl-CoA desaturase, and the enzyme system elongating and desaturating linoleic acid in mammalian cells can also be induced under certain conditions (45-47). However, the induction process, requiring several hours, is rather slow in comparison to the immediate demand for a higher proportion of polyunsaturated fatty acids when the temperature falls. Thus, it may be involved in controlling fatty acid composition only in later stages of cold adaption.

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Glycodihydrofusidate: Biliary Excretion and Its Effect on Biliary Secretion of the Rat

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ABSTRACT

Glycodihydrofusidate, which has the same detergent properties as bile salts, is excreted almost exclusively by the bile duct after intravenous injection in the rat. As with bile salts, it leads to a significant ($P \leq 0.05$) increase in excretion of lecithins and cholesterol (0.15 μmol lecithin and 0.026 μmol cholesterol per 1 μmol of glycodihydrofusidate excreted). In addition, this drug simultaneously inhibits excretion of both endogenous bile salts and bile pigments.

INTRODUCTION

Glycodihydrofusidate (GDHF), a structural analog of sodium cholate, is similar in its physico-chemical properties to bile salts (1); in particular, it is capable of dissolving amphiphilic molecules, which are water insoluble.

In the present work, we have studied the biliary excretion of GDHF and the modifications it provokes in the biliary secretion of the rat. The modifications observed involve, on the one hand, the secretion of bile salts and bile pigments and, on the other hand, the secretion of phospholipids and biliary cholesterol.

MATERIALS AND METHODS

Materials

The ethanolamine salt of glyco-24,25-dihydrofusidic acid (GDHF), $C_{37}H_{64}N_2O_9 \cdot 0.5H_2O$, was provided by Dr. Godtfredsen, Leo Laboratories, Copenhagen, Denmark. Radioactive GDHF was labeled with ^{14}C on the glycine moiety (sp act 1.158 μCi/mg; radiochemical purity 97%).

Methods

Male Wistar rats weighing 250 g were anesthetized with ether. A catheter of polyethylene No. 10 was placed in the proximal portion of the choledochus. Bile was collected during 12-14 hr. Once a plateau of basal secretion was attained, the GDHF was injected. Two groups of rats were studied.

First group: Six rats received 33 μmol of GDHF in 1 hr intravenously (intrafemoral catheter PE 10). Three rats received half of this dose (16 μmol).

Second group: Six rats received a single duodenal injection of 33 μmol of GDHF.

For the two groups, one μCi of ^{14}C-GDHF was added to the nonradioactive product. Bile was collected every hour during the 8 hr following the injection.

The biliary output, radioactivity excreted, total phosphorus (2), and cholesterol (3) were measured. Bile salts conjugated with taurine were analyzed by the amount of taurine liberated after acid hydrolysis (5.6N HCl, 18 hr at 110 C in vacuo) with amino acid analyzer JEOL model JLC 5AH.

The concentration of bile pigments was estimated by their absorption at 450 nm. Biliary metabolites of GDHF were extracted from a sample of bile with ethyl acetate and n-butanol and were purified by thin layer chromatography (TLC). Two solvent systems were employed:

System A: Isoamylacetate:propionic acid:n propanol:water (80:60:40:20, v/v).

System B: Chloroform:cyclohexane:methanol:acetic acid (80:10:2.5:10, v/v).

The metabolites fluoresce under ultraviolet light. After spraying with sulfuric acid, they turn violet. The amount of radioactivity contained by each compound was used to determine the relative percentage of each metabolite of GDHF.

RESULTS

Table I shows the average values (±SD) of the principal parameters studied in rats receiving 33 μmol of GDHF intravenously.

Biliary Excretion of Glycodihydrofusidate

The study of the first group of rats shows that the elimination of GDHF occurs principally by a biliary route. More than 81% of the input GDHF (intravenous) is found in the bile during the 8 hr following the injection. Its biliary excretion is maximal during the second hour following the onset of the perfusion, with an average discharge of 9 μmol/hr. The same maximum discharge is observed in the group of rats receiving 16 μmol/hr.

In the second group, 24% of the dose

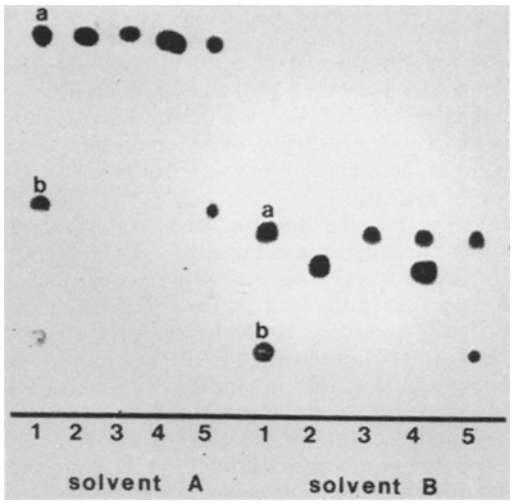

FIG. 1. Thin layer chromatogram of the biliary metabolites of glycodihydrofusidate (GDHF): 1 = rat bile, 2 = GDHF standard, 3 = ethyl acetate extract, 4 = ethyl acetate extract + GDHF standard, and 5 = butanol extract.

injected into the duodenum is excreted in the bile. Kinetics of excretion obtained with this group are very slow, and the hourly excretion is ca. 0.5 μmol.

Intrahepatic Metabolism

An analysis of the bile by TLC shows that, after injection of GDHF, this product is metabolized by the hepatocyte, giving two derivatives (Fig. 1):

Solvent system A: In this system, compound "a" has an R_f identical to the reference GDHF. Compound "b", more polar, migrates between taurochenodesoxycholic acid and glycocholic acid.

Solvent system B: Metabolite "a" and GDHF have different R_f's (Fig. 1). The residue of the ethyl acetate extract contains the less polar compound "a", while the residue of the butanolic extract contains a mixture of the two metabolites. Compound "b" represents between 10 and 27% of the totality of the fusidates excreted. These two metabolites have conserved their ^{14}C amino acid on their lateral chain.

Influence of Glyco-24,25-dihydrofusidate on Choleresis

Intravenous injection of GDHF induces a reduction of biliary flow (Table I). This cholestatic effect is maximal at the moment of maximal excretion of GDHF. In rats receiving the higher dose, biliary output is diminished 23%, on the average, in comparison to initial

TABLE I

Effect of Glycodihydrofusidate (GDHF) on Biliary Secretion[a]

Biliary components	Hour								
	-2	-1	1	2	3	4	5	6	7	8
Bile flow[b]	613 ± 55	588 ± 52	498 ± 48	443 ± 50	478 ± 68	460 ± 58	480 ± 78	505 ± 72	515 ± 67	515 ± 74
GDHF			4.34 ± 0.57	8.83 ± 0.76	7.16 ± 0.88	3.41 ± 0.55	1.21 ± 0.26	0.50 ± 0.14	0.28 ± 0.09	0.18 ± 0.06
Lecithins	0.57 ± 0.07	0.39 ± 0.06	0.73 ± 0.09	1.02 ± 0.23	1.07 ± 0.13	0.89 ± 0.07	0.56 ± 0.05	0.42 ± 0.05	0.30 ± 0.04	0.21 ± 0.03
Tauroconjugate bile salts	2.34 ± 0.48	2.29 ± 0.22	1.47 ± 0.19	0.88 ± 0.17	1.07 ± 0.18	1.14 ± 0.19	1.30 ± 0.23	1.57 ± 0.20	1.36 ± 0.17	1.33 ± 0.18
Cholesterol	0.141 ± 0.02	0.097 ± 0.01	0.188 ± 0.01	0.247 ± 0.04	0.323 ± 0.04	0.183 ± 0.03	0.124 ± 0.02	0.096 ± 0.02	0.079 ± 0.01	0.069 ± 0.01

[a]Lipid outputs are expressed in μmol/hr. Data are means (± SD) of six experiments in which rats received 33 μmol of GDHF intravenously.
[b]Bile flow is expressed as $\mu l \cdot hr^{-1}$.

secretion. Afterwards, the output regains its initial value.

Effect on the Secretion of Lipids

When injected intravenously, GDHF has the same action on biliary lipids in animals receiving 33 or 16 μmol/hr. Both phospholipid and biliary cholesterol excretion are significantly ($P \leq 0.05$) increased under the influence of GDHF. Phosphatidyl cholines are the only phospholipids excreted. One μmol of GDHF leads to the excretion of 0.15 μmol of lecithin, whereas 1.0 μmol of taurocholate under the same conditions leads to the excretion of 0.25 μmol of lecithin.

The secretion of cholesterol is similar to that of the lecithins and is parallel to the biliary secretion of GDHF, 1 μmol of GDHF causing the secretion of, on the average, 0.026 μmol of cholesterol.

Effect on Endogenous Bile Salts

The average secretion of tauroconjugated bile salts diminishes during the secretion of GDHF. The rate of secretion of these bile salts in the controls, before injection of GDHF, is 2.29 μmol/hr, and at the point of maximum excretion of GDHF is only 0.88 μmol/hr.

This reduction in the excretion of bile salts could explain, in part, the slight decrease in bile flow noted above.

Influence on the Excretion of Bile Pigments

GDHF leads to an inhibition of the biliary secretion of bile pigments. Before injection of GDHF, the OD_{450} of bile effluent is 0.145, and during the period of maximal excretion of GDHF (during the second hour following injection) $OD_{450} = 0.049$. Beginning at the third hour after injection, the OD increases markedly ($OD_{450} = 2.96$) and remains elevated until the sixth hour.

DISCUSSION

GDHF has micellar properties similar to those of other fusidates (1-4) and to those of bile salts (5). At physiological temperature, this compound aggregates in micelles at relatively low concentrations (critical micellar concentration $\cong$ 2 mM). It is capable of dissolving amphiphilic molecules which are water insoluble, such as lecithins and cholesterol, as efficiently as bile salts.

GDHF is excreted in the bile just as bile salts (6). Hepatocytic uptake of this compound is very efficient (7). Maximal biliary excretion of GDHF metabolites is nevertheless quite inferior to that of taurocholate in the same animal (8). The level of intestinal absorption of this derivative is quite low compared to that observed for 3-acetyl fusidate (9). Of the radioactivity injected into the duodenum, only 24% is excreted in the bile during 8 hr. It thus seems that the polarity of the molecule is an essential determinant in the intestinal absorption of fusidates.

In the rat, the secretion of lecithins and of biliary cholesterol is induced by GDHF, as it is also by 3-acetyl fusidate (9) and by taurocholate. The ratio lecithin:cholesterol (biliary) is slightly modified by perfusion with GDHF. Beaudoin (10) observed, however, an important decrease in this ratio in monkeys perfused with taurodihydrofusidate. It is reasonable to assume that the action of the product on biliary lipids is due to its detergent properties. It is agreed that the increased biliary secretion of lipids is produced only by bile salts capable of forming mixed micelles with lecithins. It is probable that the detergent properties of the drug are only slightly modified by its intrahepatic metabolism. Whereas only two metabolites of GDHF are recognized, nonconjugated forms of 3-acetyl fusidate and of fusidate undergo an intense hepatic metabolism resulting in numerous derivatives (9). Thus, the conjugation of the fusidate molecule with glycine reduces the number of metabolites.

The differences in this metabolism perhaps explain the variable influence of fusidates on bile flow. Contrary to observations on other species (10; S. Erlinger, personal communication), this product has no choleretic action in the rat. When the rate of perfusion with GDHF is 33 μmol/hr, a slight decrease in bile flow is observed. In spite of the decrease in excretion of endogenous bile salts, the excretion of organic anions is considerably increased under these conditions.

The excretion of endogenous bile salts is highly reduced. GDHF could act at the level of hepatic transport of bile salts. We have demonstrated a phenomenon of competition between GDHF and dehydrocholate, as much at the level of hepatocytic uptake as at the level of biliary excretion (11). Nevertheless, an action at the level of synthesis of bile salts from cholesterol is not excluded.

We are presently studying the influence of GDHF on bile pigments. This compound could act on the hepatic transport of bilirubin and/or its conjugation.

ACKNOWLEDGMENTS

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Quantitative Determination of Organic Solvent Soluble Lipofuscin Pigments in Tissues[1]

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ABSTRACT

Lipofuscin pigment determination in tissue extracts was quantitated by the use of its property of fluorescence. Chloroform:methanol tissue extracts were purified on Sephadex LH-20 columns before quantitative fluorescence measurements of the lipofuscin pigments. Interfering compounds separated by chromatography were retinol and a lower mol wt fluorescent compound. Irradiation of tissue extracts with ultraviolet light was not sufficient to eliminate the interference caused by retinol and the lower mol wt compound. Purified lipofuscin pigments from blood, lung, liver, spleen, brain, heart, and kidney tissues demonstrated distinct fluorescent emission maximum at 435 nm and excitation maxima between 345 and 350 nm.

INTRODUCTION

It has been proposed that lipofuscin pigments (LFP) accumulate in animal tissues due to lipid peroxidation in vivo and that these pigments are conjugated Schiff base products with the basic structure RN=CH-CH=CH-NH-R (1). This chromophore is reported to be derived from the reaction of malonaldehyde, a peroxidation product of polyunsaturated lipids, with primary amino groups in proteins and amino acids (2-6). Fluorescent lipofuscin pigments have been reported to occur as a result of lipid peroxidation in a wide variety of biological systems, including mitochondria, microsomes, and lysosomes (2,3,7-9). Lipofuscin pigments formed in peroxidizing systems have been reported to have fluorescent emission maxima from 420 to 470 nm and excitation maxima from 340 to 370 nm (6,7,10). Chemical assays for measurement of lipofuscin pigment are based on the fluorescence of the pigment, which is hypothesized to be a Schiff base chromophore. Interfering fluorescent compounds, such as retinol, have been previously removed by exposing the tissue extracts to ultraviolet (UV) light (6). This paper demonstrates the necessity of further purification of lipofuscin pigments before quantitative fluorescence measurements can be made.

EXPERIMENTAL PROCEDURES

Materials

All solvents used were spectral grade. Chemicals used were purchased from the following: Sephadex LH-20 from Pharmacia Fine Chemicals (Piscataway, NJ); *trans*-Retinol from Sigma Chemicals (St. Louis, MO); Quinine Sulfate from Fisher Scientific Co. (Chicago, IL). Tissue samples from Sprague-Dawley strain rats and Jackson C57BL/6J mice were used. *Octadon degus* tissues were obtained from the Department of Biochemistry, University of Minnesota.

Methods

Preparation of tissue extracts: Extraction of the pigment from tissues was carried out as follows: A portion of tissue (0.05-1.5 g) was accurately weighed and then homogenized in 20 vol (v/wet tissue wt) of 2:1 chloroform:methanol (v/v) at room temperature. For homogenization, a Polytron homogenizer with a PT20ST generator was used. The time of homogenization was 10-30 sec, at half speed, depending upon the type and toughness of tissue used. The homogenate was poured into a separatory funnel and washed twice with 50 ml of water. The two water washes were combined and washed 4 times successively with 25 ml of 2:1 chloroform:methanol. The combined organic layers were dried over anhydrous sodium sulfate. The solvent was removed on a rotary evaporator at 40 C to ca. 2 ml and then evaporated to dryness under nitrogen.

Column chromatography of tissue extracts: The dried chloroform:methanol extracts were made up to exact vol ranging from 0.125 to 5.0 ml, with 1:9 chloroform:methanol. Of these diluted tissue extracts, 0.1-0.5 ml aliquots were chromatographed. The dilution used depended upon the intensity of fluorescence in the tissue extract and upon the amount of tissue extracted. Chromatography was carried out on 1.5 x 35 cm Sephadex LH-20 columns with 1:9 chloroform:methanol as a mobile phase. Chloroform-methanol, 1:9, was found to

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give the best separation of the individual components of the tissue extracts. Sephadex LH-20 was allowed to swell overnight in 1:9 chloroform:methanol before pouring the column. Two-milliliter fractions of the column eluant were collected, and the relative fluorescence of each of the fractions was determined. It had to be determined that the fluorescent intensity of the most concentrated fractions had a linear relationship to successive dilutions.

Fluorescence measurements: Quantitative fluorescence measurements were made on an Aminco-Bowman Ratio spectrophotofluorometer (American Instrument Co., Silver Spring, MD) at an excitation wavelength of 365 nm and emission wavelength of 435 nm. The slit arrangements of the spectrophotofluorometer were 5, 5, 5, and 5 mm for slits 1, 3, 4, and 6, respectively. The excitation light source was a Hanovia Xenon lamp. The instrument was daily calibrated to read 100 relative fluorescence units against a Quinine Sulfate solution (1 μg/ml 0.1N H_2SO_4) at excitation wavelength of 365 nm and emission wavelength of 435 nm. In cases where the lipofuscin pigment extract was not purified by chromatography and the fluorescence was determined directly after the 2:1 chloroform:methanol extraction, the emission wavelength was set at 470 nm.

Exposure of tissue extracts to ultraviolet irradiation: Some of the unchromatographed chloroform:methanol extracts were exposed to high intensity UV irradiation for the destruction of retinol. Such exposures were carried out with the use of two 250 watt UV lamps, used simultaneously. The exposures were carried out for 2-3 min.

RESULTS

Exposure of Extracts to Ultraviolet Irradiation

Table I gives the intensity of fluorescence of extracts from six different tissues before and after exposure to high intensity UV light. Irradiation with UV light has been used for the destruction of the interfering retinol (6). The data show that UV irradiation had no effect on decreasing the measured fluorescence. In fact, in liver, kidney, and brain, fluorescence was actually significantly ($P<0.05$) increased as a result of the UV treatment; whereas in lung, heart, and spleen, no significant increase or decrease was measured.

Column Chromatography of Tissue Extracts on Sephadex LH-20

Typical Sephadex LH-20 elution profiles of rat blood, rat lung, and mouse kidney tissue extracts using 1:9 chloroform:methanol as the eluting solvent are shown in Figure 1. In rat blood and lung, three peaks were observed.

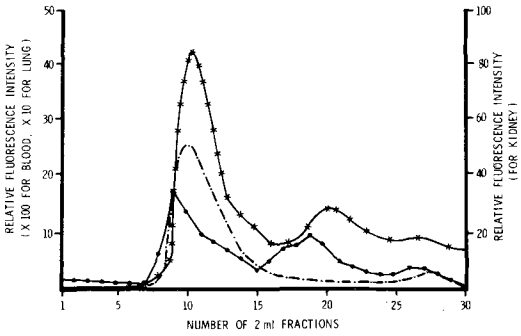

FIG. 1. Elution profiles for fluorescent compounds using Sephadex LH-20 chromatography with 1:9 $CHCl_3$-CH_3OH. •—• Extract from rat blood (left axis); ·×××·×××· extract from rat lung (left axis); •—•—• extract from mouse kidney (right axis).

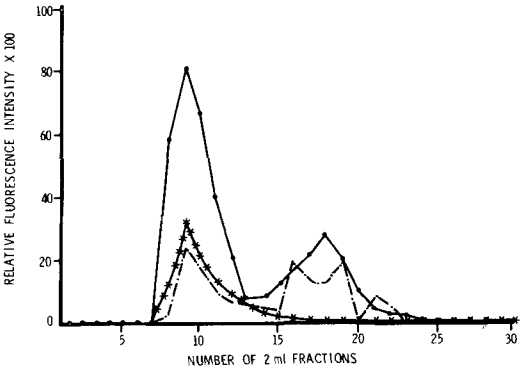

FIG. 2. Elution profile of Sephadex LH-20 chromatography of purified lipofuscin pigment (LFP) and retinol with 1:9 $CHCl_3$-CH_3OH. •—• Mixture of LFP (twice purified by LH-20 chromatography) and *trans*-retinol, chromatographed in the dark; x—x—x—x—x rat brain purified LFP; •—•—•—•—• mixture of rat brain purified LFP and *trans*-retinol, chromatographed in regular daylight.

The first peak eluted was identified, by fluorescent excitation and emission spectra, as lipofuscin pigment. Sharp emission (435 nm) and excitation (345-350 nm) maxima were observed in regions characteristic of the Schiff base chromophore. The second peak was later identified as retinol by fluorescent excitation and emission spectra and by co-chromatography with commercial, pure retinol. The third peak is an unidentified low mol wt fluorescent component. In mouse kidney, only two peaks were found. The first peak, which was identified as lipofuscin pigment, eluted similarly to the lipofuscin pigment from rat blood and lung. In kidney extract, no retinol was found, but a

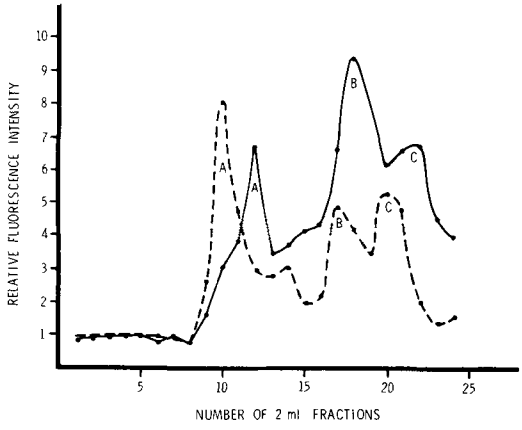

FIG. 3. Elution profiles of Sephadex LH-20 chromatography of crude liver extracts from *Rattus norvegicus* (——) and *Octadon degus* (— — — —). Peak A: lipofuscin pigments; Peak B: retinol; Peak C: unidentified material.

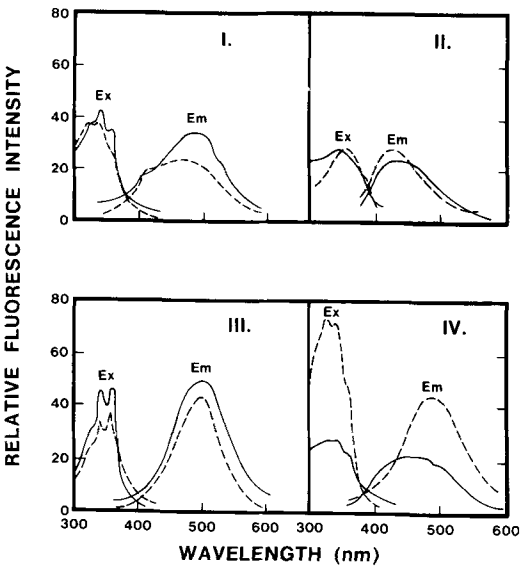

FIG. 4. Fluorescent emission (Em) and excitation (Ex) spectra of liver extracts from *Rattus norvegicus* (——) and *Octadon degus* (— — — —). I. Unpurified liver extracts. II. Peak A from Figure 3. III. Peak B from Figure 3. IV. Peak C from Figure 3.

second peak eluted in the same position as the unidentified third peak from rat blood and lung. Figure 2 shows the separation of purified lipofuscin pigments and pure *trans*-retinol in normal daylight and in the dark. Purified lipofuscin pigments were prepared by chromatography on Sephadex LH-20 columns. When purified lipofuscin pigments were re-chromatographed alone, no second peaks were observed by fluorescence measurement. Additional peaks were observed when *trans*-retinol was added to purified lipofuscin pigments. The number of additional peaks depended upon the light conditions during chromatography. In darkness, only one retinol peak was observed, whereas under normal laboratory lights and daylight, additional peaks appeared, possibly retinol degradation products. Comparison of Figure 2 to Figure 1 shows that the addition of pure *trans*-retinol to purified lipofuscin pigments gives an elution profile similar to that of the extract of rat blood and lung. Sephadex LH-20 elution profiles of tissue extracts with 1:9 chloroform:methanol from *Rattus norvegicus* and *Octadon degus* (two rodent species) were essentially identical (Fig. 3). The lipofuscin pigment and retinol elute from the column in the same regions in both species. An unidentified third peak was also observed in both tissues. Figure 4 shows the fluorescent emission and excitation spectra of peaks A, B, and C from Figure 3 as well as those of the unpurified liver extracts. These spectra further illustrate the similarities between purified lipofuscin pigments in each species.

The emission maxima of the unpurified extract (Fig. 4, I) from *R. norvegicus* and *Octadon degus* show some variation, being 480 and 470 nm, respectively. However, excitation maxima were similar. At 325, 340, and 360 nm, a shoulder or maximum was observed. The emission and excitation spectra of the purified lipofuscin pigment (Fig. 4, II) show emission maxima at a lower wavelength, 435 nm, and excitation maxima at 345-350 nm. The retinol (Fig. 4, III) shows emission maxima at 495 nm and excitation maxima at 345, 360 nm, and a shoulder at 325 nm. The spectra of the unidentified peak C from *Octadon degus* (Fig. 4, IV) resembles retinol). The broad emission spectrum of the unidentified peak C from *R. norvegicus*, which contains several shoulders, possibly represents a mixture of retinol and retinol degradation products. Sephadex LH-20 chromatography of extracts from a variety of tissues (lung, liver, heart, spleen, kidney, brain, and blood) all showed elution profiles similar to those in Figures 1 and 2. Fluorescent excitation and emission spectra for purified lipofuscin pigments from these various tissues were the same (Fig. 5). In all tissues, emission maxima of the pigment were determined to be at 435 nm and excitation maxima at 345-350 nm. Figure 6 demonstrates the change in the emission spectra of lipofuscin pigments due to the presence of retinol. Figure 6, I, shows a broad emission spectrum with maximum around 475 nm for unpurified liver extract of *Octadon degus*. Upon separation of the liver extract, by LH-20

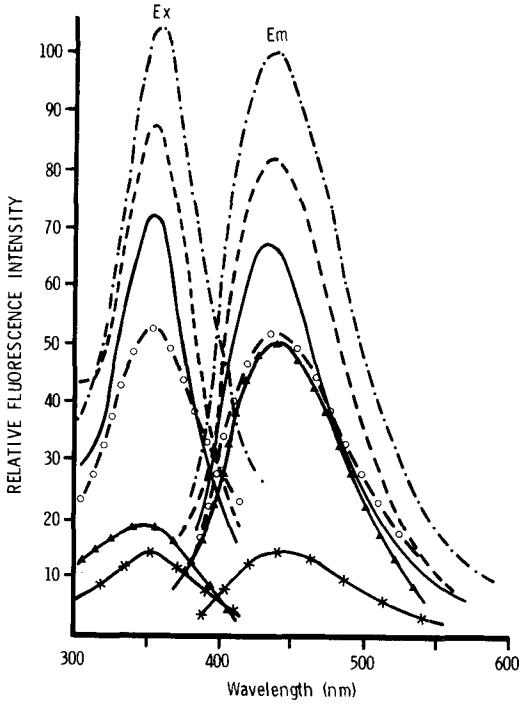

FIG. 5. Fluorescent emission (Em) and excitation (Ex) spectra of lipofuscin pigments purified by Sephadex LH-20 chromatography. Rat heart (—); rat liver (– – – –); rat lung (•–•–•–•); mouse lung (x–x–x); mouse kidney (o–o–o); and mouse spleen (▲–▲–▲–▲).

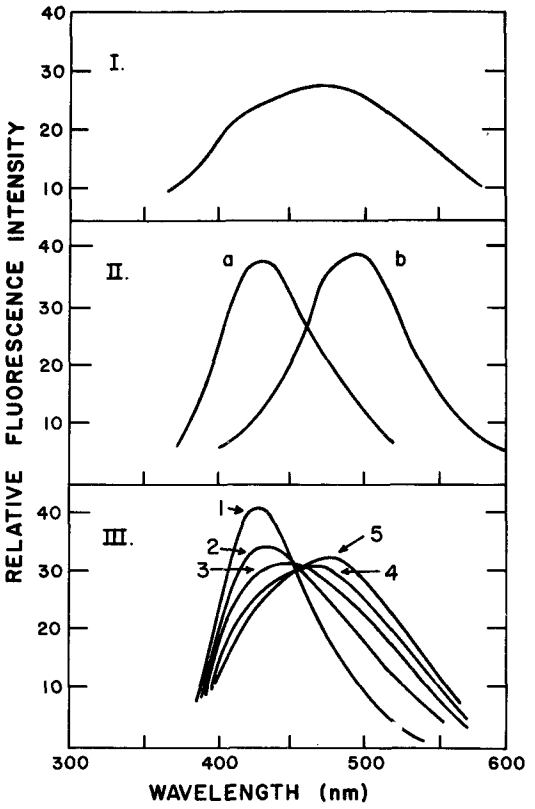

FIG. 6. Fluorescent emission spectra of crude and purified lipofuscin pigment and retinol from *Octadon degus* liver extracts (excitation at 365 nm). I. Unpurified 2:1 $CHCl_3$-CH_3OH liver extract. II. Purification of I (by Sephadex LH-20 chromatography) into components: (a) lipofuscin pigments and (b) retinol. III. Recombination of IIb with IIa (1 = purified lipofuscin pigments [IIa]; 2, 3, 4 = increasing stepwise recombination of IIb with IIa; and 5 = total recombination of IIb with IIa).

chromatography, into lipofuscin pigments and retinol components, sharp emission maxima appear for the purified lipofuscin pigments (IIa) at 435 nm, and for retinol (IIb) at 495 nm. Recombination of purified lipofuscin pigments with increasing amounts of retinol (Fig. 6, III) results in a successive broadening and shift of the emission spectra to higher wavelengths. In Figure 6, III, emission spectrum of 1 represents that of purified lipofuscin pigments (IIa), while spectra 2, 3, 4, and 5 show the effect of the addition of 25%, 50%, 75%, and 100%, respectively, of the separated retinol (IIb) to the original purified lipofuscin pigments. Increasing amount of retinol in the mixture caused the emission maximum to shift from 435 to 475 nm.

DISCUSSION

Although the purification and fluorescence measurements of lipofuscin pigments have been reported earlier (6,10-12), the interference caused by other fluorescent compounds has not been completely clarified. Organic solvent soluble lipofuscin pigments can be extracted with 2:1 chloroform:methanol; however, these extracts contain other interfering fluorescent compounds besides lipofuscin pigments. For this reason, purification of the tissue extracts is essential before quantitative fluorescence measurements can be made. Retinol, which has an emission maximum at 495 nm, is one of the primary interfering compounds in the fluorescence measurements. In unpurified 2:1 chloroform:methanol tissue extracts, retinol could be responsible for up to 80% of the measured fluorescence. Besides retinol and lipofuscin pigments, other lower mol wt fluorescent compounds have been found in the various tissue extracts. The concentrations of both retinol and the lower mol wt unidentified fluorescent compound exhibited great variations between tissue types (Figs. 1 and 3). Retinol concentrations in tissue extracts also

TABLE I

Relative Fluorescence of Tissue Extracts Before and After Exposure to High Intensity Ultraviolet Light for 2 Minutes in 2:1 Chloroform:Methanol

	Lung		Liver		Kidney	
	Before	After	Before	After	Before	After
	0.27	0.29	0.38	0.45	0.15	0.27
	0.14	0.16	0.42	0.50	0.20	0.37
	0.24	0.26	0.48	0.51	0.31	0.38
	0.27	0.32	0.12	0.52	0.28	0.30
	0.28	0.30	0.28	0.54	0.09	0.21
Mean ± SD	0.24 ± 0.06	0.27 ± 0.06	0.34 ± 0.14	0.50[a] ± 0.03	0.21 ± 0.09	0.31[a] ± 0.07
	Heart		Brain		Spleen	
	Before	After	Before	After	Before	After
	0.23	0.25	0.26	0.61	0.25	0.22
	0.31	0.36	0.15	0.56	0.29	0.27
	0.32	0.36	0.60	0.68	0.22	0.21
	0.31	0.32	0.34	0.68	0.16	0.22
	0.26	0.32	0.30	0.52	0.45	0.32
Mean ± SD	0.29 ± 0.04	0.32 ± 0.04	0.33 ± 0.17	0.61[a] ± 0.07	0.27 ± 0.11	0.25 ± 0.05

[a]Significantly higher by Student's *t*-test (at 95% level or greater).

depended upon the time elapsed between extraction and chromatography. As the time between extraction and chromatography increased, retinol concentrations decreased.

UV irradiation of tissue extracts as a means of purification from interfering fluorescent compounds has been shown to be unreliable. Exposure of tissue extracts to UV irradiation actually caused increased fluorescence in most cases (Table I). Similar increases in fluorescence after UV irradiation of tissue extracts have been reported to occur in the spleen and the adrenal glands (7). It seems possible that, while retinol was mostly decomposed in these tissue extracts by UV irradiation, its degradation products gave rise to fluorescence in similarly interfering regions of the spectrum.

Quantitative separation of retinol and the lower mol wt fluorescent compound, from lipofuscin pigments, was achieved by Sephadex LH-20 column chromatography. Fractionation by the use of 1:9 chloroform:methanol resulted not only in good separation of the compounds, but predictable elution profiles. There were practically no variations between types of tissue used or between tissues from different species. Fluorescent emission and excitation spectra of the first peak (lipofuscin pigment) eluted from Sephadex LH-20 show distinct excitation maxima at 345-350 nm and emission maximum at 435 nm (Fig. 4). It is of significant importance that the emission maximum was at 435 nm for purified lipofuscin pigments in all tissues assayed: lung, liver, heart, spleen, brain, kidney, and blood (Fig. 5). Earlier reports in the literature indicated a broad range of emission maxima from 420-470 nm to be representative of lipofuscin pigments (6,7). The reported variations in emission maxima are most probably due to the fact that the tissue extracts used had not been quantitatively purified from interfering fluorescent compounds. Retinol interference in pigment measurements caused the emission maximum to shift to higher wavelengths (Fig. 6). In an unpurified tissue extract, the emission maximum would be dependent upon the degree of contamination with retinol. The present method successfully separates the interfering fluorescent compounds from lipofuscin pigments. After purification by column chromatography, a quantitative estimation of the lipofuscin pigments can be achieved.

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SHORT COMMUNICATION

Effect of Lactation on Lipolysis in Rat Adipose Tissue

ABSTRACT

The concentrations of nonesterified fatty acids and glycerol in rat parametrial adipose tissue increased at peak lactation. Adipose tissue from lactating rats showed higher rates of release of nonesterified fatty acids and glycerol when incubated in vitro than did tissue from nonlactating rats, but there was a substantial increase in the esterification of fatty acids during involution. These results support earlier evidence that fat reserves were mobilized during lactation.

INTRODUCTION

Early studies based on carcass analysis demonstrated that female rats retained and stored a large amount of fat during pregnancy. These additional reserves disappeared during lactation, and it was assumed that they were utilized to meet the demands of milk production (1). Subsequent work showed that fat storage was confined to the first two-thirds of pregnancy (2) and revealed that the end of pregnancy was characterized by an increasing mobilization of these reserves (2,3). Recent studies in vitro in this laboratory (4) showed that fatty acid synthesis from glucose by rat adipose tissue virtually ceased during lactation but increased to very high levels after the young were removed. These results provided support for the concept that lipid stores were mobilized during lactation and replaced during involution. In this communication we report additional evidence in support of this conclusion.

TABLE I

Effect of Lactation on Contents of Nonesterified Fatty Acids (NEFA) and Glycerol in Rat Adipose Tissue[a]

Group	NEFA	Glycerol
Unmated	2.23 ± 0.29	1.03 ± 0.08
2 days lactation	3.38 ± 0.54[b]	1.33 ± 0.16
14 days lactation	4.19 ± 0.34[b]	1.70 ± 0.14[b]
3 days involution	1.69 ± 0.25	1.43 ± 0.10[b]

[a]Results are expressed as μmol/g of tissue and represent means ± SE for six rats.

[b]Values significantly ($P<0.05$) different from corresponding value for unmated rats.

MATERIALS AND METHODS

Hooded Norway rats from the Institute colony were used and were 3 months old at time of mating. Details of their management and feeding were as described previously (4). In addition to a group of unmated animals, groups of rats were studied on the 2nd and 14th days of lactation and the 3rd day after removing the young. In the last group, the young were removed on the 21st day after birth.

The rats were killed by breaking their necks and the parametrial fat bodies were quickly exposed. To obtain an estimate of the contents of nonesterified fatty acids (NEFA) and glycerol in vivo, portions of this tissue were removed, immediately frozen in liquid nitrogen, and analyzed for NEFA and glycerol as described below. Further portions of tissue (200-300 mg) were rapidly weighed and placed in small Erlenmeyer flasks with 3 ml of a modified Krebs Ringer bicarbonate medium (pH 7.4), which contained albumin (30 mg/ml) and one-half the recommended amount of calcium. Epinephrine, when used, was added to a final concentration of 1 μg/ml. Incubations were carried out at 37 C with continuous shaking for 60 min.

At the end of the incubation period, the flask contents were homogenized and a portion of the homogenate (1 ml) was used for the extraction of NEFA as described by Dole and Meinertz (5). The final heptane layer which contained the NEFA was removed, taken to dryness under nitrogen, and redissolved in chloroform. Nonesterified fatty acids were determined by a colorimetric method (6). A further portion of the homogenate (1.8 ml) was added to 0.2 ml of 1 M metaphosphoric acid and mixed. After centrifugation, the clear solution between the precipitated protein and supernatant fat was removed, neutralized with 1 M NaOH, and used for the determination of glycerol by an enzymatic method (7).

Bovine serum albumin (Fraction V) was obtained from BDH Chemicals Ltd. (Poole, England), and epinephrine was from Evans Medical Ltd. (Liverpool, England).

RESULTS AND DISCUSSION

The effects of lactation on the contents of

TABLE II

Effect of Lactation on Net Production of Nonesterified Fatty Acids (NEFA) and Glycerol by Rat Adipose Tissue In Vitro[a]

	NEFA		Glycerol	
	Basal	+Epinephrine	Basal	+Epinephrine
Unmated	1.09 ± 0.11	9.68 ± 1.40	0.80 ± 0.10	3.22 ± 0.38
2 days lactation	1.45 ± 0.50	12.39 ± 2.50	1.06 ± 0.10	4.84 ± 0.71[b]
14 days lactation	2.84 ± 0.53[b]	20.02 ± 1.16[b]	1.22 ± 0.13[b]	7.52 ± 0.23[b]
3 days involution	0.94 ± 0.34	10.52 ± 2.71	1.18 ± 0.12[b]	6.08 ± 0.29[b]

[a]Results are expressed as μmol/g of tissue per hr and represent means ± SE for six rats.
[b]Values are significantly ($P<0.05$) different from corresponding value for unmated rats.

NEFA and glycerol in rat adipose tissue in vivo are shown in Table I. There were substantial increases in the content of NEFA during lactation and somewhat smaller increases in glycerol content on the 14th day of lactation and during involution.

Results obtained in this way must be interpreted with some caution because the levels of NEFA in adipose tissue are sensitive to the method of killing the animal. However, other workers found that there was a significant ($P<0.02$) increase in the content of NEFA in rat adipose tissue during late pregnancy and ascribed this and other changes to enhanced lipolysis (3). Furthermore, in a recent study with cows, Metz et al. (8) analyzed samples of subcutaneous adipose tissue obtained by a biopsy technique and showed that there was an increase in the content of NEFA during early lactation. They considered that changes in the tissue content of NEFA were related to an alteration in the rate of lipolysis.

Table II shows the effects of lactation and involution on the release of NEFA and glycerol by rat adipose tissue in vitro. There was a significant ($P<0.05$) increase in the release of NEFA at peak lactation (14 days) but, on the 3rd day after removing the young, the value was similar to that for unmated animals. The release of glycerol was significantly ($P<0.05$) higher at peak lactation and during involution. There was a very similar pattern of change when basal lipolysis was stimulated by the addition of epinephrine (1 μg/ml) to the incubation medium (Table II).

The results for the basal rates of release of NEFA and glycerol (Table II) were used to derive estimates of the rates of lipolysis and of esterification as described by Vaughan (9). It seemed to be of interest to use this method to make a *comparison* between lactating and nonlactating rats, although the presence of low levels of glycerol kinase in adipose tissue may lead to re-utilization of some of the glycerol liberated by lipolysis (10,11). Estimates of lipolysis and esterification obtained by this method are, therefore, subject to error and cannot be regarded as absolute values. Nevertheless, these errors might not obscure a general change in the pattern of metabolism during lactation. As estimated by this method, the mean rate of lipolysis for unmated rats was 2.41 ± 0.29 μmol fatty acid/g adipose tissue per hr. On the 14th day of lactation and during involution, the values were significantly higher (3.65 ± 0.38 and 3.55 ± 0.36 μmol fatty acid/g adipose tissue per hr, respectively). The mean rates of esterification for unmated rats and for rats at peak lactation were 1.32 ± 0.28 and 0.84 ± 0.43 μmol fatty acid/g adipose tissue per hr, respectively. Although the value for lactating rats was somewhat lower, the difference was not statistically significant at $P = 0.05$. During involution, the mean rate of estification (2.61 ± 0.39 μmol fatty acid/g adipose tissue per hr) was significantly higher than the value for unmated rats and 3 times the value observed at peak lactation. At this stage, the extent to which these results may be influenced by changes in glycerol kinase activity has not been investigated.

The results presented in this paper, together with those of earlier studies (4,12), emphasize the changes in the metabolic activity of adipose tissue that occur during lactation in response to the demands of milk production. At the time when the metabolic activity of the mammary gland is at its highest, synthesis of long chain fatty acids by adipose tissue ceases (4) and dietary triglycerides are diverted from storage in fat depots to milk formation (12). In addition, these results suggest that triglycerides already stored in the fat depots are more readily mobilized at this time. These changes occur in spite of an increase in food intake during lactation (13) but are entirely consistent with the loss of fat that was noted by Spray at this time (1). The underlying mechanisms which regulate the changes are probably related to the hormonal changes that occur during

lactation, but their exact nature remains to be investigated.

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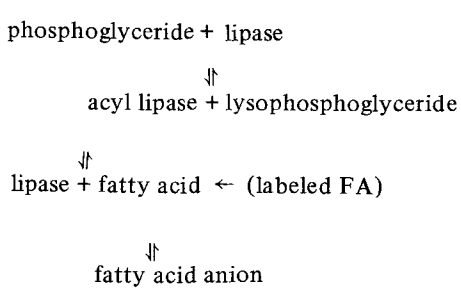
LETTER TO THE EDITOR

Introduction of Labeled Fatty Acid in Position 1 of Phosphoglycerides

Sir: Borgström (1) showed in 1954 that pancreatic lipase catalyzes the incorporation of free fatty acid into triglyceride. Since lipases also act on phosphoglycerides (2), it should be possible to introduce fatty acids into these lipids in the same manner. At present, most phospholipids can be labeled only by in vivo biosynthesis or by chemical syntheses that demand a heavy investment of funds, time, and expertise. A method that would consist of a simple incubation followed by thin layer (TLC) purification would make radioactive, mass-, spin-, or photolabeled phosphoglycerides much more easily available. In this letter, we want to demonstrate the possibility of such a method, without laying claim to having exhausted its potential or having found the optimal conditions for the reactions. Notwithstanding these reservations, the labeling of phosphatidylethanolamine (PE) as described here should be of considerable practical value.

The equilibrium for fatty acid exchange can be expressed as follows:



Since the substrate for lipase is the undissociated free fatty acid (3), the reaction should be carried out at low pH to suppress anion formation, and in the absence of agents complexing fatty acids, such as bile salts or calcium ion. The success of the exchange reaction is also jeopardized by the solubility in water of the lysophosphoglycerides, which may disengage themselves from the supersubstrate of the enzyme (4), i.e., the fatty acid-phospholipid micelle. Nevertheless, we have been able to introduce a labeled acid into phosphatidylethanolamine and, in low yield, into phosphatidylcholine (PC). We have not succeeded with the more acidic phospholipids.

Rhizopus delemar lipase (Miles Laboratories Inc., Elkhart, IN), 600 units/mg (manufacturer's information), was employed. Ten mg PE (egg, Applied Science Laboratories, Inc., State College, PA), 0.2 mg butylated hydroxytoluene (to prevent peroxidation), 2 x 10^6 cpm ^{14}C-oleic acid (ca. 0.01 mg), 0.4 ml 0.5 M Na-acetate (pH 3,4), and 1.4 ml H_2O were mixed and sonified for 20 sec; then, 2 mg lipase in 0.2 ml H_2O were added. After 4 hr shaking at 40 C under N_2, the lipids were extracted with a mixture of 2 ml chloroform and 1.6 ml methanol and separated by silicic acid TLC with chloroform:methanol:water:acetic acid, 64:32:2:2. Eighteen percent of the radioactivity was found in PE, the rest in the free fatty acid, none in lyso-PE. (Repeated experiments gave 14-23% yields). A phosphorus analysis of PE and lyso-PE showed a ratio of 2:8, and longer incubation times did not change the yield although the enzyme retained activity under these conditions. Thus, equilibrium had been attained. On hydrolysis of the PE with phospholipase 2 of snake venom (5), 100% of the activity was found in the lyso-PE. The exchange had taken place exclusively in position 1.

Higher sp acts of PE can be obtained by increasing the amount of labeled acid. Equimolar amounts of oleic acid added at the beginning of the incubation will result in PE of half the molar sp act of the acid. The reaction is not inhibited by such an excess of fatty acid; this was confirmed by experiments.

Although they were partly hydrolyzed by the lipase, phosphatidylserine and phosphatidylinositol did not incorporate label, nor did PC. Into this lipid, however, label could be introduced in the presence of PE. A mixture of 3.6 mg PE and 1.4 mg PC (egg), under the above conditions, had incorporated, after 3 hr, 2.4% of the label in PC and 7.3% in PE. In another experiment, 2.5 mg PC and 2.5 mg PE incorporated 3.4% and 6.1% label in 24 hr.

From the labeled PE, labeled PC might be accessible in better yield with the help of phospholipase 4, through exchange of the ethanolamine against choline (5). Phosphatidic acid, phosphatidyl-D,L-glycerol, and diphosphatidyl glycerol might be prepared in similar manner.

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A Guide for Authors is Located in Lipids 11(January):85(1976)

Structure of Some Intact Lipids of Petrel Stomach Oils

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ABSTRACT

The stomach or proventricular oils from 16 species of petrel have been analyzed and the carbon number distributions of the wax esters, triglycerides, and diacylglyceryl ethers are reported. The wax esters have been fractionated further into less and more polar species. To determine whether any intermolecular specificity existed, carbon number distributions for each lipid class were calculated, assuming random esterifications. The triglyceride and diacylglyceryl ether compositions observed were all found to agree closely with those calculated. The wax esters from three petrel species were found to have greater proportions of the middle range species with carbon numbers 34-38 than calculated. However, most of the lipids examined had random structures which have been found to be characteristic of marine sources. The results in general support the belief that the oils are derived directly from dietary sources rather than synthesis by the proventricular glands.

INTRODUCTION

Petrels, sea birds in the order Procellariiformes, are unique in being able to store large quantities of oil in the proventriculus of the alimentary tract. We have recently reported on the functions and the composition of the oils from 16 species, in terms of the classes of lipid present and the fatty acid, alcohol, and glyceryl ether composition of these oils (1). In the present work, the technique of high temperature gas chromatography (2,3) was used to separate the triglycerides, wax esters, and diacylglyceryl ethers of the oils according to their carbon number.

Histological studies (4) had indicated that the sources of the oils were the glandular cells surrounding the proventriculus, but recent work (1) has implicated dietary sources directly. Though it would appear (1) that in the proventriculus there is very rapid proteolysis and a relatively rapid hydrolysis and absorption of phospholipids, lipolysis of the fully acylated neutral lipids is very slow. Thus, the structure of the intact lipids here should almost be the same as at source. Some data are available on the intermolecular structures of the lipids from marine sources: the triglycerides of fish oil (5,6) and whale oil (2) have been analyzed and Nevenzel (7) has reviewed the structures of wax esters. Further data on wax esters have also been published (8-10). In all cases, the intact lipids had intermolecular distributions which could be accounted for by a random association of alcohols and/or acids. These were unlike the specific triglycerides synthesized by the mammary gland (11) or by mammalian gut or liver (12).

Therefore, if the proventriculus oils were of dietary origin and not glandular, a random intermolecular distribution would be predicted. A simple method for determining the specificity or randomness of the intermolecular structure of triglycerides has been described (11). In this method, the found molar proportion (F) of triglycerides having the same carbon number is compared with the molar proportion (R) calculated from random associations of fatty acids. Positive or negative F-R values can be summed to give the overall mol % specificity which is the degree of nonrandom association of fatty acids. In this paper, we present data for the wax esters and diacylglyceryl ethers as well as for the triglycerides.

MATERIALS AND METHODS

The detailed sources of the oils, the thin layer chromatographic (TLC) techniques used to separate the liid classes, and the quantitative analysis by gas-liquid chromatography (GLC) of fatty acids, alcohols, and glyceryl ethers have been described (1). The oils were obtained by inducing the birds to disgorge by "teasing." Specimens from *Fulmarus glacialis* and *Hydrobates pelagicus* were collected near Aberdeen, Scotland, while *Pagodroma nivea* and *Puffinus tenuirostris* oils were obtained at Cape Hallett, Antarctica, and Bass Strait, Australia, respectively. All other specimens were gotten from the New Zealand islands of Antipodes, Snares, and Campbell. Lipids were separated into their classes by TLC on 0.5 mm Silica Gel H (Merck, Darmstadt, W. Germany) using consecutive or separate developments with diethyl ether:light petroleum (bp 40-60 C) 5:95, 10:95, and 15:85, v/v. Only those lipid classes which comprised $>9\%$ of the total oil were analyzed

TABLE I

Fatty Acid and Alcohol Composition of the Less Polar Molecular Species of Wax Esters (mol %)

	Diomedea epomophora		*Diomedea melanophris*		*Daption capense*				*Pagodroma nivea*				*Pterodroma inexpectata*		*Pterodroma mollis*		*Puffinus tenuirostris*		*Puffinus griseus*	
					a[a]		b		a		b									
	FA	Alc	FA	Alc	FA	Alc	FA	Alc	FA	Alc	FA	Alc	FA	Alc	FA	Alc	FA	Alc	FA	Alc
14:0	7.5	1.4	2.7	5.0	31.7	-	19.2	-	2.6	8.6	3.1	8.0	1.6	6.0	0.9	6.1	1.9	6.0	1.3	5.1
16:0	10.7	20.9	3.2	57.4	11.0	-	11.8	-	2.6	39.8	3.2	39.3	2.8	41.7	3.4	36.0	1.9	43.7	1.7	52.1
16:1	12.0	-	16.4	4.0	3.1	-	4.8	-	19.0	4.1	20.6	2.8	19.2	3.0	20.1	-	19.1	5.8	21.2	5.0
18:0	1.2	7.1	-	5.5	0.3	-	1.4	-	-	3.2	-	1.9	-	2.1	1.3	8.4	-	5.8	-	3.3
18:1	46.2	29.8	61.1	10.8	7.0	2.7	12.7	2.0	57.5	25.5	53.2	27.9	57.0	16.7	44.8	21.8	53.0	7.4	59.4	8.4
18:2	0.5	-	1.0	-	0.4	-	1.0	-	1.9	-	1.7	-	1.0	-	1.3	-	1.5	-	2.2	-
20:0	-	-	-	0.2	-	-	-	-	-	-	-	-	-	0.4	-	1.6	-	0.6	-	0.6
20:1	15.5	26.7	8.1	8.3	25.1	70.5	27.8	72.5	9.6	11.5	10.1	13.9	9.0	14.4	18.4	17.3	10.4	15.0	5.7	11.4
20:2	-	-	-	-	-	-	-	-	0.3	-	0.3	-	-	-	0.5	-	0.2	-	0.2	-
20:4	0.2	-	0.3	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	tr	-
20:5	0.5	-	3.8	-	-	-	-	-	0.2	-	1.2	-	0.9	-	-	-	1.7	-	1.7	-
22:1	5.7	14.1	3.0	8.8	21.4	26.8	21.3	25.5	5.6	7.3	6.6	6.2	8.1	15.7	9.3	8.8	10.1	15.7	5.9	14.1
22:6	-	-	0.4		-	-	-	-	0.7	-	-	-	0.3	-	-	-	0.2	-	0.7	

[a]a and b refer to different specimens from the same colony.

TABLE II

Fatty Acid and Alcohol Composition of the More Polar Molecular Species of Wax Esters (mol %)

	Diomedea epomophora		*Diomedea melanophris*		*Daption capense*				*Pagodroma nivea*				*Pterodroma inexpectata*		*Pterodroma mollis*		*Puffinus tenuirostris*		*Puffinus griseus*	
					a[a]		b		a		b									
	FA	Alc	FA	Alc	FA	Alc	FA	Alc	FA	Alc	FA	Alc	FA	Alc	FA	Alc	FA	Alc	FA	Alc
14:0	-	1.1	-	5.5	29.9	-	26.4	-	-	6.3	-	8.4	tr	12.9	-	1.9	tr	8.5	tr	6.1
16:0	3.9	16.1	0.9	58.2	21.7	-	20.4	-	3.0	34.2	1.7	38.2	2.3	49.3	1.8	39.4	0.8	45.3	0.9	63.4
16:1	4.2	1.9	4.1	7.3	5.9	-	5.3	-	6.3	3.3	5.2	6.5	11.7	8.2	3.0	3.7	6.5	5.6	2.4	5.4
18:0	0.4	5.2	-	3.8	1.2	-	1.1	-	-	4.5	-	3.0	-	1.9	-	11.2	-	4.8	-	3.2
18:1	16.3	29.3	8.0	10.5	7.6	5.1	5.8	5.1	15.3	26.1	11.5	26.9	35.5	15.4	12.8	26.2	16.7	7.9	4.4	7.9
18:2	0.7	-	2.8	-	2.6	-	2.1	-	3.0	-	3.4	-	1.3	-	0.9	-	1.7	-	1.4	-
18:3	-	-	3.4	-	0.2	-	0.4	-	2.6	-	1.8	-	-	-	-	-	-	-	1.9	-
18:4	-	-	9.7	-	1.1	-	1.4	-	7.9	-	11.1	-	-	-	-	-	-	-	10.8	-
20:0	-	-	-	0.4	-	-	-	-	-	-	-	-	-	-	-	-	-	0.6	-	0.3
20:1	10.0	29.6	tr	10.0	13.1	72.4	14.5	70.8	4.3	18.2	0.9	12.3	7.2	8.3	4.5	13.7	7.7	14.9	tr	7.2
20:2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20:4	6.8	-	2.4	-	2.9	-	4.2	-	2.6	-	3.6	-	1.7	-	14.1	-	2.1	-	1.3	-
20:5	20.5	-	50.2	-	2.1	-	4.4	-	33.4	-	34.0	-	28.1	-	20.7	-	44.2	-	52.4	-
22:1	3.7	16.8	3.0	4.3	9.7	22.5	11.0	24.1	3.0	7.4	2.6	4.7	3.2	4.0	4.7	3.9	4.1	12.4	0.9	6.5
22:5	4.8	-	2.4	-	-	-	-	-	2.2	-	3.2	-	2.1	-	6.8	-	2.0	-	1.6	-
22:6	28.7	-	13.1	-	2.0	-	3.0	-	15.5	-	21.1	-	6.9	-	30.7	-	14.2	-	22.0	-

[a]a and b refer to different specimens from the same colony.

TABLE III

Wax Ester Composition (mol %)[a]

Total carbon number	*Diomedea epomophora*			*Diomedea melanophris*			*Daption capense* (a)			*Daption capense* (b)			*Pagodroma nivea* (a)		
	TWE[a]	LPWE	MPWE	TWE	LPWE	MPWE	TWE	LPWE	MPWE	TWE	LPWE	MPWE	TWE	LPWE	MPWE
30	1.9	2.0	0.4	2.7	2.3	0.6	-	-	-	-	-	-	2.5	2.8	0.7
32	6.8	7.6	1.7	14.0	17.4	5.1	1.6	0.4	1.8	1.7	1.4	2.7	14.8	16.0	4.7
34	18.8	20.6	4.6	31.2	40.9	17.6	17.9	22.9	14.4	12.3	16.6	8.1	32.3	33.1	15.7
36	25.8	27.6	13.8	25.2	16.3	39.5	23.9	21.8	37.2	19.5	17.3	32.8	24.4	24.2	28.7
38	22.1	21.9	27.0	15.5	11.4	24.6	15.2	10.3	22.2	18.2	11.6	23.5	14.5	13.8	26.6
40	16.0	14.1	30.1	7.3	8.1	8.8	18.1	18.5	12.4	22.3	25.2	19.1	8.9	7.9	17.4
42	6.8	5.1	16.7	3.5	3.2	2.8	17.3	19.0	9.1	20.1	21.7	11.8	2.6	2.2	6.2
44	1.8	1.1	5.7	0.6	0.4	1.0	6.0	7.1	2.9	5.9	6.2 1	2.0	-	-	-
Average carbon number	36.87	36.56	39.20	35.51	35.12	36.50	38.12	38.18	37.36	38.58	38.65	37.83	35.41	35.25	37.06
Overall mol % specificity	2.8	2.6	4.5	6.2	5.1	3.4	6.1	5.2	16.2	5.0	5.0	17.4	3.0	1.9	3.5
	Pagodroma nivea (b)			*Pterodroma inexpectata*			*Pterodroma mollis*			*Puffinus tenuirastris*			*Puffinus griseus*		
30	2.9	2.4	0.4	4.5	3.5	2.7	tr	-	-	2.1	2.3	0.4	1.9	2.9	0.4
32	16.0	16.3	4.0	13.8	15.4	13.7	3.0	3.8	1.4	12.2	16.2	3.9	13.0	17.7	4.1
34	33.4	34.4	17.2	22.6	27.7	26.2	24.9	32.2	8.6	24.4	33.0	13.6	30.1	34.8	17.0
36	26.1	26.9	30.2	21.7	18.8	27.7	31.2	34.9	23.1	21.1	16.5	31.8	20.5	14.4	35.6
38	12.4	12.3	29.4	16.3	15.0	18.3	23.9	18.4	36.2	19.0	16.1	26.2	17.3	13.9	25.6
40	7.0	6.4	13.6	12.9	11.4	7.9	13.4	9.0	13.1	12.6	10.7	12.8	10.7	11.0	9.8
42	2.2	1.3	5.2	5.9	6.4	2.9	3.6	1.7	7.6	6.2	3.7	8.5	4.7	4.1	5.4
44	-	-	-	2.3	1.8	0.6	-	-	-	2.4	1.5	2.8	1.8	1.2	2.1
Average carbon number	35.18	35.10	36.92	36.11	35.90	36.67	36.61	36.03	37.88	36.35	35.66	35.90	36.00	35.48	36.87
Overall mol % speciifcity	3.3	4.9	3.7	10.7	7.4	4.6	10.7	15.5	3.1	2.7	3.8	5.5	3.8	5.4	5.2

[a]TWE = total wax ester; LPWE = less polar wax ester; MPWE = more polar wax ester.

TABLE IV

Diacyl Glyceryl Ether Compositions (mol %)

Carbon number[a]	*Pterodroma mollis*		*Procellaria aequinoctialis*	
	F	F-R[b]	F	F-R
46	0.5	-0.1	0.5	-0.1
48	4.4	-1.0	3.9	0.8
50	12.7	0.0	11.7	1.6
52	19.4	-0.3	18.4	1.1
54	19.3	-3.9	20.2	-2.7
56	18.0	1.6	19.3	-3.0
58	14.9	1.6	14.8	-1.8
60	8.0	1.3	8.1	3.4
62	2.8	0.6	3.1	0.7
	54.45	5.3	54.60	7.6

[a]Carbon number is the total carbon number less the three carbons of glycerol. The figure at the bottom of the found composition (F) column is the average carbon number.

[b]F-R is the difference between F and the calculated composition assuming random esterification of glyceryl ethers and fatty acids. The figure at the bottom of the column is the overall degree of nonrandom associations of fatty acids and ethers.

by GLC. Wax esters readily separated into less polar and more polar fractions by TLC using ether:light petroleum, bp 40-60 C, 5:95 (v/v) as developing solvent. These fractions were, therefore, analyzed separately, and their fatty acid and alcohol compositions are reported in Tables I and II.

The intact lipids were fractionated by GLC using a 45 x 0.65 cm glass column of 3% (w/w) SE-30 (methyl polysiloxysilicone gum) on 100-120 mesh Universal support (Jones Chromatography Ltd., Llanbradach, Glamorgan, Wales). A temperature program of 240-330 C at 8 C/min was used with N_2 flow rate of 40 ml/min. A Pye model 204 dual FID Chromatograph was employed. The results were dealt with quantitatively using molar correction factors (3). Precision of measurement was $\leqslant 3\%$ for components comprising $> 5\%$ of the total sample, $\leqslant 5\%$ for components comprising 1-5% of the total sample, and $\leqslant 25\%$ for components comprising $< 1\%$ of the total sample.

The random structures of triglycerides (R mol %) were calculated by using the formula given by Kuksis et al. (13). The random composition of wax esters were calculated by

$$\sum (\text{acid mol \%} \times \text{alcohol mol \%}) \times 1/100$$

and, for the diacylglyceryl ethers, we used the formula:

$$\sum [\text{GE mol \%} \times (\text{A moles \%})^2 + 2\ \text{GE mol \%} \times \text{A mol \%} \times \text{B mol \%}] \times 1/10{,}000$$

where GE is a particular chain length glyceryl ether and A and B are particular chain length fatty acids. The formula was applied, in turn, to all possible pairs of fatty acids with each glyceryl ether.

Since intact lipids are separated by GLC according to their carbon number, fatty acids, etc., of differing degrees of unsaturation but of the same chain length were added together for the purpose of the above calculations.

RESULTS AND DISCUSSION

Tables I and II show the fatty acid and alcohol compositions of the less and more polar species of wax esters, respectively. Table III gives the compositions of the intact wax esters as the total or separated wax esters according to polarity. It is clear that the differences between the less and more polar species are reflected mainly in the fatty acid compositions; the more polar species contain no myristic acid but have high proportions of polyunsaturated fatty acids. The exception is *Daption capense*, where the total fatty acid composition (1) is unique in that there are very high proportions of both myristic and palmitic acids at the expense of the long chain polyunsaturates. Thus, in this instance, the contribution of the more polar species to the total is very small. The composition of the alcohols is very similar in all instances for both categories of wax esters. There is a very slight bias for longer chain alcohols to be found in the less polar group.

All the wax esters (Table III) fall into a carbon number range of 30-44, as in wax esters from marine invertebrates and fish (7). The average carbon numbers for the total wax esters are in the narrow range of 35.18 (*Pagadroma nivea*) to 38.58 (*D. capense*), while the less and more polar species have chain lengths slightly

Triglyceride Compositions (mol %)

Total acyl carbon number	*Diomedea epomophora*	*Diomedea melanophris*	*Diomedea chrysostoma*	*Diomedea bulleri*	*Phoebetria palpebrata*	*Macronectes halli*	*Fulmaris glacialis*	*Pterodroma inexpecta*	*Pterodroma mollis*	*Pterodroma lessoni*	*Procellaria aequinoctialis*	*Puffinus tenuirostris*	*Puffinus griseus*	*Hydrobates pelagicus*
44	-	0.2	0.3	0.3	-	-	0.2	-	-	0.7	-	-	-	-
46	0.9	1.4	1.2	1.7	1.0	0.9	1.2	1.0	0.3	1.4	0.6	1.6	1.5	0.6
48	4.4	5.5	4.3	6.7	3.6	3.4	4.7	3.4	2.0	4.8	3.6	5.2	4.2	2.9
50	12.6	13.3	10.6	15.2	9.9	10.9	12.5	9.0	6.7	11.2	9.1	10.6	11.7	8.2
52	21.2	19.8	16.4	21.5	17.6	20.6	20.0	16.2	12.6	17.5	15.6	16.2	18.1	14.6
54	20.3	20.7	20.0	20.4	21.6	23.1	20.1	20.2	18.8	22.5	18.9	19.4	21.6	19.0
56	19.9	18.0	19.7	16.5	22.0	19.0	19.0	21.8	21.2	20.1	21.9	18.6	20.2	21.3
58	13.4	12.1	15.8	11.7	15.3	13.1	14.8	15.4	20.2	13.6	18.6	14.2	14.4	17.0
60	6.8	7.2	9.4	5.0	7.0	7.5	5.9	8.8	12.4	7.7	11.2	9.1	6.8	11.3
62	0.5	1.8	2.3	1.0	2.0	1.5	1.6	4.2	5.8	0.5	0.5	5.1	1.6	5.1
C#[a]	18.04	18.01	18.21	17.86	18.20	18.12	18.05	18.32	18.59	18.06	18.32	18.24	18.13	18.45
Overall mol % specificity	5.7	3.2	3.4	3.9	3.7	2.5	9.9	2.1	4.3	6.7	6.1	1.7	5.1	5.4

[a]Average fatty acid chain length.

shorter or slightly longer, respectively. The agreement between the found compositions (F) and the calculated random compositions (R) is very close. The exceptions are *D. capense*, where the samples have an overall degree of nonrandom association of fatty acids and alcohols of 16.2 and 17.4% in the more polar fractions; *Pterodroma mollis*, with 10.7% and 15.5% in the total and less polar fractions; and *Pterodroma inexpectata*, with 10.7% and 7.4% in the total and less polar wax esters. Accumulated errors (11) would add to or subtract 3-5% from these results. Thus, in general, these wax esters represent a random esterification as found for wax esters from other marine sources (7,14,15). However, when some specificity of structure was observed, this was seen in the greater than calculated proportions of the species with carbon numbers 34-38.

Table IV shows the diacylglyceryl ether compositions for *Pterodroma mollis* and *Procellaria aequinoctialis*. Again the random compositions as calculated are close to those found experimentally.

The triglyceride compositions (Table V) show, without exception, random structures. Thus, almost all the lipids examined show an intermolecular structure which is akin to that found for the depot fats of land animals (11), some sea birds (16), and apparently fish oils (5,6,13) or whale fat (2). We have not found any intermolecular specificity of the kind shown by the milk triglycerides from many species (11) or by human serum triglycerides (12).

Histological studies (4) suggested that the stomach oils originate in secretions from the proventricular glands. However, the similarity of the structures of the stomach oils to those structures found in possible marine diets of the birds supports the belief that the stomach oils are of dietary origin.

These analyses do not rule out any underlying specificities of structure which arise from an intramolecular specificity in the cases of the diacylglyceryl ethers or triglycerides. The structures reported here could have arisen from the process in which an acyltransferase may selectively acylate a particular hydroxyl group of glycerol (the 2-position with polyunsaturated fatty acids, for example), but in which subsequent acylations are not influenced by the fatty acids already esterified (17). Slakey and Lands (17) have termed this "noncorrelative acylation."

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Effect of Alimentation on Human Serum Squalene Levels

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ABSTRACT

Five subjects consumed a meal containing ca. 1.5 mg squalene. Postprandial serum samples showed that in three of the subjects squalene and lipid levels reached maxima at 3 hr. Squalene levels in the low density lipoproteins of these subjects also increased. The results indicate that alimentation is a major factor in the variation in serum squalene levels.

INTRODUCTION

Squalene is well known as a precursor in the biosynthesis of sterols, but much less attention has been given to its distribution in nature. For example, there has been only one study of squalene in normal human plasma, which estimated the concentration as 29 μg/100 ml and reported that almost all of the squalene derived from labeled mevalonate occurred in the very low density (<1.019) lipoproteins (1). In a more recent study on patients with acute influenza, Gallin et al. (2) reported serum squalene concentrations of up to 190 μg/100 ml, whereas convalescent-phase patients had levels ranging from trace amounts to 47 μg/100 ml. To learn more of the normal levels in human serum, a study was undertaken in which samples of plasma from a blood bank were analyzed using a method previously employed on fish (3). plant tissues (4), and human atheromatous plaques (5). This gave an opportunity to test the method on plasma and to learn something of the normal range of variation, which in these samples proved to be between 1.9 and 34.0 μg/100 ml. As diet was regarded as a possible contributing factor in this variation, serum squalene levels were then measured in five subjects at hourly intervals following a "normal" breakfast. Finally, the squalene contents of the major components of the meal were determined to provide an estimate of the squalene intake.

MATERIALS AND METHODS

A preliminary series of analyses were done on nine samples of plasma obtained from the local blood bank. The collections of blood had been held at 4 C for up to 3 weeks for separation of the red cells and plasma. Six samples were nonlipemic, but three were selected for their distinct visible lipemia. Aliquots of 40 ml of plasma were used initially for analysis, but later this volume was reduced to 12 ml and still gave adequate amounts of squalene. Analyses have been done on as little as 9 ml of serum, but this seems to be approaching the limits of the present method.

The method for the analysis of squalene has been described previously (4) and consists essentially in the separation of the hydrocarbon fraction from a lipid extract by thin layer chromatography (TLC), the addition of a known amount of an internal standard, n-tetracosane, and the analysis of this fraction by gas-liquid chromatography (GLC). The method has given an average recovery of 86.7% and a reproducibility of within 10% (4). Exploratory analyses showed that the method needed several modifications for use with serum. The presence of a series of alkanes, presumably of dietary origin, interfered with the analysis and required the use of a different TLC solvent system. Tests showed that petroleum ether: benzene (97:3 v/v) allowed squalene (Rf 0.75) to be separated from all but three of these hydrocarbons (unidentified) which did not coincide with the squalene or internal standard peaks and thus did not interfere with the analysis. However, this solvent system is a rather poor solvent for some of the more polar lipids, and analysis of the customary 20-50 mg of lipid gave variable results stemming from an apparent occlusion of a portion of the squalene among the undissolved lipids at the origin. This problem was overcome by an initial predevelopment of the plate in chloroform for a distance of 2 cm to spread the origin. The TLC plate was then air dried for 2-3 min and given the full development in the petroleum ether:benzene solvent system. Side channels with a spot of squalene were used on each plate to locate the squalene band, which was often too faint to be detected after spraying with Rhodamine 6G. The area of the squalene band, which was well separated from other lipids, was scraped from the plate and eluted with diethyl ether. After addition of a known amount of n-tetracosane, usually 50 μg, and reduction of volume with nitrogen, the fraction was ready for analysis by GLC. Previously the GLC analysis was done isothermally at 260 C, but the column currently used (1% SE-30 at 60 ml N_2/min) was so much faster that two temperatures were used to

give peaks of greater width for more accurate measurement: 210 C until the tetracosane peak appeared and 240 C for the squalene peak. Using these modifications, repeated analyses of several samples of plasma gave coefficients of variance ranging between 7 and 15%.

The test meal given to five male subjects was chosen to represent a normal but substantial breakfast consisting of two fried sausages (36 g average wt), two large hen's eggs (63.7 g average wt) fried in butter, three slices of buttered toast, and coffee or tea. Squalene concentrations were determined only in those components having a considerable amount of lipid. This excluded egg whites, which contained no lipid, and bread, which had undergone prolonged exposure to higher temperatures. The samples taken for analysis included two lots of the two brands of sausages consumed, four lots of butter of the same brand, and three eggs from the same box. Lipids were extracted by the method of Folch et al. (6), after which the method was the same as that used for serum.

In the exploratory plasma analyses, comparisons were made between the amount of squalene found in the total plasma lipids extracted by the method of Sperry and Brand (7) and the amount found in the total lipoproteins obtained by dextran sulfate precipitation by the method of Burstein et al. (8). In the latter separation, the quantities of reagents were adjusted for the smaller volumes of plasma, and 5% dextran sulfate was used rather than 10% in the low density lipoprotein (LDL) precipitation to give larger and more accurately measured volumes. The quantities of squalene found by the two methods agreed within 10%, with neither method clearly exceeding the other. This indicated that the lipoproteins contained nearly all of the serum squalene. For this reason, it was decided that in the postprandial analyses, the low density lipoproteins (chylomicrons, very low density lipoproteins [VLDL], and LDL) would be analyzed separately from the high density lipoproteins (HDL) to provide further information on squalene distribution.

Postprandial blood samples were taken from five male subjects at five hourly intervals starting with the end of the meal as time zero. A venous catheter was inserted in the forearm in time for the first 25 ml sample at 1 hr. The blood was held at 4 C until centrifuged at 1800 G for 15 min. The clot was compressed with a spatula to expel serum, and the centrifugation was repeated. The supernatant serum was taken by pipette, and its volume (12-13 ml) was noted. The lipoproteins were precipitated by dextran sulfate as discussed above, and their lipids were extracted by the method of Folch et al. (6) and taken to dryness under nitrogen and high vacuum for weighing. Limitations on the amount of blood that might be reasonably requested from the donors precluded an examination of the individual lipoprotein fractions and necessitated a compromise sampling schedule that omitted the zero hour sample in favor of the 5 hr sample.

TABLE I

Squalene Content of Dietary Components

Component	Wt (g)	Lipid (%)	Squalene (μg/g)
Sausage A - 1	35	27.6	8.9
Sausage A - 2	31	25.5	9.3
Sausage B - 1	33	17.6	4.9
Sausage B - 2	37	18.3	5.2
Egg yolk - 1	20.0	26.2	12.3
Egg yolk - 2	20.5	27.9	13.5
Egg yolk - 3	19.8	30.8	11.0
Butter[a] Lot 1	-	-	55.0
Butter Lot 2	-	-	27.0
Butter Lot 3	-	-	51.0
Butter Lot 4	-	-	25.0

[a]Assumed to be entirely lipid.

RESULTS

The nine samples of plasma from the blood bank had squalene concentrations ranging between 1.9 and 34.0 μg/100 ml. Squalene reached the highest levels of 11, 13.3, and 34.0 μg/100 ml in the three lipemic samples, whereas the nonlipemic samples did not exceed 9.7 μg/100 ml. There was no correlation with lipid content, for the lipemic samples did not always have the highest lipid contents. These findings are consistent with the varied dietary histories and hormonal balances to be expected in random blood donors.

The squalene contents of the dietary components (Table I) reveal that, of the three major sources of lipid in the test meal, butter contained an average of 3 times more squalene than egg yolk, the next lowest (39.5 μg/g and 12.3 μg/g, respectively). The sausages gave two groupings of squalene and lipid levels that corresponded to the two brands used in the test meal. The average squalene content for the group was 7.1 μg/g. Butter showed a similar 2-fold range of values, but the four lots that were analyzed all came from the same brand.

The postprandial serum analyses (Fig. 1) show that in three of the subjects the total squalene levels rose through the first 3 hr and declined thereafter. This was not seen in No. 4,

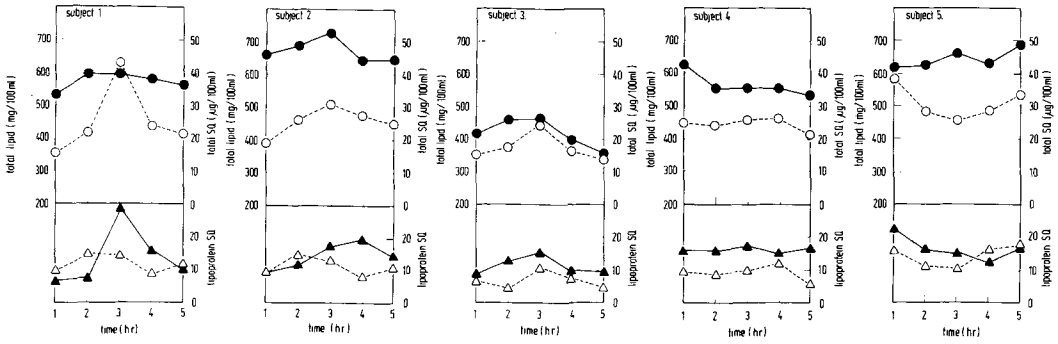

FIG. 1. Postprandial serum squalene and lipid concentrations in five subjects. ● - total serum lipids, ○ - total serum squalene, ▲ - squalene in low density lipoproteins (chylomicrons, very low density and low density lipoprotein), △ - squalene in high density lipoproteins.

where the squalene level diminished through the middle of the sampling period. In contrast to the plasma samples, the serum lipid levels tended to correlate with squalene levels, having maxima at the same time as squalene in three of the subjects and showing the same lack of change as squalene in Subject No. 4. Number 5 was again unique in that lipid levels tended to rise while squalene levels fell.

Further individual differences are seen in the relative amounts of squalene in the two major lipoprotein fractions. In Nos. 1 and 2, the squalene in the HDL fraction exceeded that of the LDL fraction in the first 2 hr, whereas, in Nos. 3 and 4 of the LDL, squalene was always greater than the HDL. No. 5 was again different in that the HDL squalene exceeded the LDL squalene at 4 and 5 hr rather than at 1 and 2 hr as in Nos. 1 and 2.

DISCUSSION

The information on the squalene concentration in normal human plasma has been limited to one report of an estimated 29 µg/100 ml (1). The present study was undertaken to test the feasibility of determining the squalene concentrations in relatively small amounts of serum and to learn something of the normal range of variation and its possible causes. The preliminary analysis of nine samples of plasma from a blood bank showed that a reproducibility of within 15% could be maintained on 12 ml aliquots and that the range of concentrations extended from 1.9 g to 34 µg/100 ml. These findings indicated that squalene levels vary considerably in normal blood donors as well as in influenza patients.

The results of the postprandial serum analyses from five subjects show that diet can have a marked effect. The test meal was selected to represent a normal breakfast, and in three of the subjects the total squalene levels rose by 28, 12, and 9 µg/100 ml. These increments appear to be valid indications of elevations in serum levels, for they far exceed the experimental error of the analytical method. That these elevations were an effect of alimentation is indicated by the corresponding rise in serum lipid levels in these subjects of 64, 68, and 40 mg/100 ml that would be expected with an intake of dietary lipid. Moreover, the squalene and lipid levels reached their maxima after approximately the same interval of 3 hr. The previously reported concentration of 29 µg/100 ml (1) is within the postprandial range found in this study, but, as this was from a fasting blood sample, the reason for this rather high concentration is not clear.

The analysis of the test meal was restricted to the three components—sausages, egg yolks, and butter—having considerable amounts of lipid. The results permit a rough estimate to be made of the squalene available in the test meal. Calculating on the basis of two yolks averaging 20 g each, two sausages of 36 g each, and 21 g of butter on three slices of toast plus 5 g for frying, gave a total of 1769 µg of squalene. Allowing for a 15% loss during cooking would reduce this to ca. 1500 µg or 1.5 mg of squalene.

It is rather surprising that such a small amount of dietary squalene could be responsible for the changes in serum squalene levels. To have this effect, the exogenous squalene would have to be almost entirely absorbed, but the studies of Channon and Tristam (9) have shown that squalene is largely absorbed when administered to rats in small percentages (<0.25%) of the total diet. Assuming that the 1.5 mg of squalene in the meal was completely absorbed, this would give a concentration of 32 µg/100 ml in the 5.4/liter blood volume of an average 70/kg adult human. It is of interest

that this calculated value is in the general range of the average concentrations (17.1-31.0 μg/100 ml) found in the subjects of this study.

In the earlier report on human plasma, squalene derived from labeled mevalonate occurred almost entirely in the very low density (<1.019) or the chylomicron and VLDL fractions (1). In contrast, the present study shows that squalene occurs in both the low density (chylomicron, VLDL, LDL) and high density (HDL) fractions, with the concentrations in the latter occasionally exceeding those of the low density lipoproteins. Three of the subjects showed an increase in the squalene content of the low density fraction reaching maxima at 3-4 hr after alimentation. This agrees with the generally accepted concept that exogenous lipid, and hence squalene, appears first in the chylomicrons and secondly in the VLDL after lipolysis of the former in the liver. This pattern was not found in Subjects No. 3 and 4. The exogenous squalene may be transferred to the HDL fraction at some later stage, but the limited data of this study do not indicate when this would occur.

The variation in individual serum lipid levels among subjects who have eaten similar, if not identical, meals is rather unexpected. The average lipid levels over the 5 hr period ranged from 417 to 674 mg/100 ml, giving a range of 257 mg. As it is unlikely that lipid intake among the subjects would have varied through the amount of butter used and the lipid content of the sausages by > 10-15%, these differences probably represent individual responses to dietary lipid. Although intake of dietary lipid has long been known to elevate serum lipid levels, no studies of individual differences appear to have been published.

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Tirucalla-7,24-dienol: A New Triterpene Alcohol from Tea Seed Oil

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ABSTRACT

A new triterpene alcohol is isolated from tea (*Thea sinensis*, Theaceae) seed oil, and its structure is shown to be 5α-tirucalla-7,24-dien-3β-ol. This triterpene alcohol is considered to be a possible biogenetic precursor to the meliane and meliacan series of oxygenated triterpenes. Gas liquid chromatographic and proton magnetic resonance spectroscopic correlations between euphane and tirucallane series triterpenes also are discussed.

INTRODUCTION

In the previous study on the unsaponifiable fraction of tea (*Thea sinensis*) seed oil, the presence of several unidentified triterpene alcohols besides β-amyrin, butyrospermol, and lupeol was indicated in the triterpene alcohol fraction of this oil (1).

This paper describes a further study of one of these unidentified triterpenes leading to the conclusion that it is 5α-tirucalla-7,24-dien-3β-ol (Fig. 1, IIc), a new triterpene alcohol from natural sources.

EXPERIMENTAL PROCEDURES

Authentic specimens of 5α-eupha-8,24-dien-3β-ol (Fig. 1, Ia, euphol) and 5α-tirucalla-8,24-dien-3β-ol (Ic, tirucallol) were obtained as gifts, and 5α-eupha-7,24-dien-3β-ol(IIa, butyrospermol) was isolated from tea seed oil (1). Three triterpenes of 5α-lanostane series (2)–(24ξ)-24-methyl-5α-lanost-9(11)-en-3β-ol (IIIe), 5α-lanosta-8,24-dien-3β-ol (IVa, lanosterol), and 5α-lanost-7-en-3β-ol (Vb)—also are used as reference specimens.

Melting points were determined with a micro melting point apparatus (Yanagimoto Seisakusho Ltd., Kyoto) and are uncorrected. All recrystallizations were performed in acetone-methanol. Preparative argentation thin layer chromatography ($AgNO_3$-TLC) for the fractionation of triterpene acetates was carried out on 20 x 20 cm plates coated with 0.5 mm of silica gel (Wakogel B-10, Wako Pure Chemical Industries Ltd., Osaka) impregnated with 10%

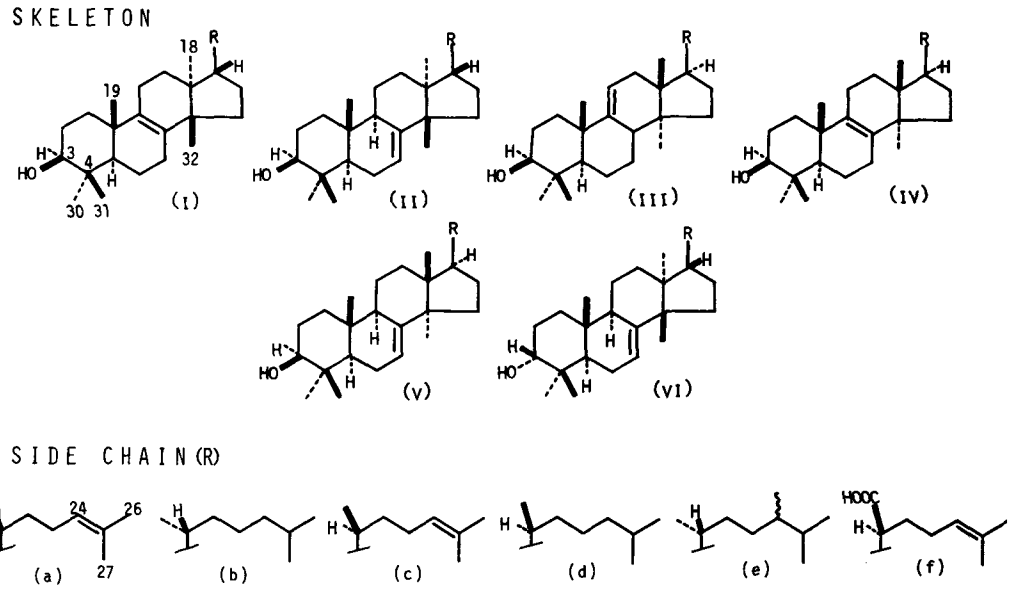

FIG. 1. Diagram of the skeletons (I-VI) and side chains (a-f) of euphane, tirucallane, and lanostane series of triterpenes.

or 20% silver nitrate. Methylene chloride was used as the eluting solvent. After development (17 cm), the separated zones were visualized and recovered as described previously (1). Analytical TLC of free triterpene alcohols was carried out on the 20 x 20 cm plate spread with 0.25 mm of silica gel. A mixture of hexane: ether:acetic acid (80:20:1) was used as the eluting solvent. Gas liquid chromatography (GLC) was performed on a Shimadzu GC-5A gas chromatograph (Shimadzu Seisakusho Ltd., Kyoto) equipped with a flame ionization detector and a 2 m x 3 mm inside diameter glass column packed with 3% OV-17 on Gas Chrom-Z, 80-100 mesh, prepared by Nihon Chromato Works Ltd. (Tokyo). The chromatograph was operated at a column temperature of 255 C. The carrier gas was nitrogen with a flow rate of 50 ml/min. Detector and injection port temperatures were 280 C. Relative retention time (RRT) was expressed by the ratio of the retention time to that of sitosterol (β-sitosterol, [24*R*]-24-ethylcholest-5-en-3β-ol). The figures indicated were the mean values determined on several runs.

Infrared (IR) spectra were recorded in KBr pellets on a Type IRA-2, IR spectrophotometer (Japan Spectroscopic Co., Tokyo). Optical rotations were measured in $CHCl_3$ using a Carl Zeiss Polarimeter 0.01° (Carl Zeiss, Oberkochen, West Germany). Concentrations used were indicated in parentheses as g/100 ml.

Proton magnetic resonance (PMR) spectra were recorded on a JNM-C-60-HL (60 MHz) or on a JNM-MH-100 (100 MHz) instrument (Japan Electron Optics Laboratory Co., Tokyo) for the solutions (0.2 M) of 80 μmol of triterpenes in 0.4 ml of deuteriochloroform ($CDCl_3$) at 28 C. The chemical shifts (δ) and the lanthanide-induced shifts (LIS) (δ[LSR]) were expressed in ppm downfield from internal tetramethylisilane. The LIS were recorded at 60 MHz with the solutions of triterpenes in the presence of a molar equivalent of tris (dipivalomethanato) europium (Eu[DPM]$_3$), a lanthanide shift reagent (LSR). For the convenience of comparison, the LIS were normalized as proposed by Buckley et al. (3). In this study, the δ(LSR) 11.00 was given to the lowest field methyl signal, 31 (4β)-methyl signal. The experimental details of this PMR work with the LSR were described in the previous paper (4).

Mass spectra were taken with a Hitachi RMU-7M mass spectrometer (Hitachi Ltd., Tokyo), electron energy 70 eV, target current 42 or 58 μA, ion source temperature 180 C, sample temperature 110-120 C, and accelerating high voltage 4.5 KV. The samples were introduced directly into the ion source through a vacuum-lock.

Other procedures such as hydrogenation (platinum oxide catalyst, ether solution), acetylation, and hydrolysis of triterpenes were carried out in a similar manner as described previously (1).

Isolation of an Unknown Triterpene Alcohol from the Unsaponifiable Material of Tea Seed Oil

A solution of unsaponifiable material (43.7 g) separated from tea seed oil (9.3 kg) in hexane was poured onto a column containing 1 kg of alumina (Wako). The following eluting solvents were then passed in succession: (i) 2.4 L (liters) of hexane (H), fraction 1, 10.3 g; (ii) 6.0 L of H-ether (E) (95:5), fraction 2, 9.2 g; (iii) 4.8 L of H-E (9:1) followed by 7.0 L of H-E (4:1), fraction 3, 1.2 g; (iv) 1.8 L of H-E (7:3), fraction 4, 2.8 g; (v) 7.0 L of H-E (7:3), fraction 5, 7.1 g; and (vi) 13.6 L of H-E (1:1), fraction 6, 1.7 g. The fractions 2-6 consisted exclusively of triterpene alcohols.

The fraction 5 (7.1 g) was acetylated and the product was recrystallized. The crystalline material consisting almost exclusively of the acetates of lupeol and butyrospermol was left aside from the further investigation. On the other hand, the filtrate was recovered to give a yellow, pasty solid (3.1 g), which was then roughly fractionated into five fractions by preparative $AgNO_3$-TLC. The fraction (1,600 mg) from the second zone from the starting line contained an unknown triterpene acetate and also the acetates of lupeol and butyrospermol. This fraction was further fractionated by preparative $AgNO_3$-TLC into seven fractions, of which the fraction (637 mg) from the third zone from the starting line eventually gave a uniform triterpene acetate (110 mg, >99% pure by GLC, RRT 1.59) after repeated recrystallization. The melting point was 123-124 C (needles), $[\alpha]_D^{21}$ -39° (c 0.82). The IR spectrum showed bands at 842 and 822 (trisubstituted double bond), 1730 and 1242 (acetoxyl), and 1394, 1380, and 1370 (geminal dimethyl) cm^{-1}. The overall patterns of the spectrum are closely similar to those recorded for eupha-7,24-dienyl (Fig. 1, IIa) acetate. The acetate on hydrolysis gave free alcohol (RRT 1.32), mp 76-79 C (fine needles). This unknown triterpene alcohol is shown by high resolution mass spectrometry to have a molecular formula $C_{30}H_{50}O$ (calculated mol wt 426.3859); the molecular ion (M^+) was given at m/e 426.3819 (relative intensity 20%), with other principal ions at m/e 411.3595 (100%), 393.3492 (22%), 313.2574 (3%), 271.2403 (3%), 259.2088 (6%), 255.2117 (5%), 241.1910 (2%), and

TABLE I

Relative Retention Times and ΔR_{Ac}-Values of 5α-Euphane and 5α-Tirucallane Series Triterpene Alcohols Determined on 3% OV-17 Column

Compound	RRT[a] 3β-OH	RRT[a] 3β-OAc	ΔR_{Ac}[b]
5α-Euphane series			
Ia	0.89	1.07	1.20
Ib	0.74	0.89	1.20
IIa	1.17	1.40	1.20
IIb	0.96	1.16	1.21
5α-Tirucallane series			
Ic	1.01	1.22	1.21
Id	0.84	1.01	1.20
IIc	1.32	1.59	1.20
IId	1.08	1.31	1.21

[a]Retention time for sitosterol (30 min) is taken as 1.00.

[b]ΔR_{Ac}-value is expressed by the ratio of RRT of the acetate to RRT of the free alcohol.

229.1944 (4%). The triterpene alcohol showed on analytical TLC a mobility identical with that of eupha-7,24-dienol(IIa).

Hydrogenation of the Unknown Triterpene Acetate

Hydrogenation of the triterpene acetate afforded the dihydro acetate (RRT 1.31), mp 131-133 C (fine needles). IR of the dihydro acetate still showed the absorptions at 840 and 821 cm^{-1} related to a trisubstituted double bond; however, the intensity of these bands was found weaker than that observed for the acetate mentioned above. Hydrolysis of the dihydro acetate gave free alcohol (RRT 1.08), mp 95.5-97 C (plates). MS = m/e 428 (M^+, 19%), 413 (100%), 395 (77%), 315 (3%), 299 (3%), 297 (3%), 273 (9%), 259 (7%), 255 (4%), and 241 (5%).

Isomerization of the Dihydro Derivative of the Unknown Triterpene Acetate by HCl

A solution of the dihydro acetate (43 mg; RRT 1.31) in $CHCl_3$ (20 ml) was treated with a stream of dry HCl at 0 C for 3 hr in a similar manner as described by Dawson et al. (5). GLC of the isomerized product (40 mg) gave a major component peak at RRT 1.01 (relative abundance 76%), with three other minor component peaks at RRT 0.83 (17%), 1.07 (5%), and 1.31 (2%). Crystallization of this isomerized product afforded flat needles (22 mg) with mp 146-148 C (GLC: RRT 1.01, 97%; RRT 1.07, 3%). IR of the acetate showed no absorptions related to trisubstituted double bonds. MS = m/e 470 (M^+, 15%), 455 (75%), 410 (2%), 395 (100%), 357 (1%), 315 (2%), 313 (2%), 301 (5%), 299 (5%), 297 (2%), 255 (6%), and 241 (13%).

Tirucalla-8,24-dienol (Fig. 1, Ic) and Its Dihydro Derivative (Id)

An authentic specimen of tirucalla-8,24-dienol showed M^+ at m/e 426 (34%), with other ions at m/e 411 (100%), 393 (52%), 313 (3%), 297 (4%), 273 (7%), 271 (6%), 259 (17%), 255 (8%), and 241 (13%) in the mass spectrum. Hydrogenation of its acetate (mp 161-163 C, fine needles) (lit. mp 163.5 C [6]) gave tirucall-8-enyl (Id) acetate, mp 146-148 C (fine needles) (lit. mp 147-149 C [6]). IR, MS, and PMR spectra, as well as RRT in GLC and melting point of this acetate, were identical with those observed for the isomerized dihydro acetate derived from the unknown alcohol described above.

Eupha-7,24-dienol (IIa) and Its Dihydro Derivative (IIb)

Eupha-7,24-dienyl (IIa) acetate isolated from tea seed oil (1) showed mp 146-147 C (fine needles), $[\alpha]_D^{21}$ +10° (c 0.81) (lit. mp 146.5-147.5 C, $[\alpha]_D$ +11° [7]). IR showed the presence of trisubstituted double bonds (841, 832, and 816 cm^{-1}). Hydrolysis of the acetate, gave free alcohol, mp 107-110 C (fine needles) (lit. mp 111-113 C [7]). MS = m/e 426 (M^+, 22%), 411 (100%), 393 (48%), 313 (13%), 297 (4%), 273 (5%), 271 (9%), 259 (12%), 255 (8%), and 241 (6%). Hydrogenation of the IIa acetate gave euph-7-enyl (IIb) acetate, mp 138-140 C (fine needles) (lit. mp 137-139 C [7], 134-135 C [8]), which on hydrolysis gave free alcohol IIb. The IR absorptions attributable to the trisubstituted double bond (838, 821, and 812 cm^{-1}) were found weaker than those observed for the IIa acetate described above. MS = m/e 428 (M^+, 8%), 413 (100%), 395 (70%), 315 (2%), 299 (7%), 273 (20%), 259 (14%), 255 (6%), and 241 (10%).

Isomerization of Euph-7-enyl (IIb) Acetate by HCl

Euph-7-enyl (IIb) acetate (100 mg, >99% pure by GLC, RRT 1.16) in $CHCl_3$ (20 ml) was treated with dry HCl as described above. The isomerized product (98 mg) was shown in direct GLC to consist of a component with RRT 0.89 (relative abundance 86%) and two minor components with RRT 0.78 (10%) and 0.98 (4%). No detectable amount of the starting material (IIb-acetate, RRT 1.16) was found in the GLC. The product on recrystallization gave fine needles (70 mg) with mp 126-127 C (GLC: RRT 0.89, 96%; RRT 0.98, 4%). The product is regarded as a Δ^8-isomer of euph-7-enyl (IIb) acetate, i.e., euph-8-enyl (Ib) acetate

(lit. mp 124-127 C [5], 124-126 C [9]). IR of the Ib acetate showed no absorptions correlated to trisubstituted double bonds. MS = m/e 470 (M^+, 22%), 455 (86%), 410 (2%), 395 (100%), 357 (2%), 315 (2%), 313 (1%), 301 (4%), 299 (4%), 297 (4%), 255 (5%), and 241 (11%).

RESULTS AND DISCUSSION

The mobility in TLC and ΔR_{Ac}-value (Table I) for the unknown alcohol isolated from tea seed oil are indicative of the presence of the usual 30 (4α), 31 (4β)-dimethyl-5α-stan-3β-ol grouping in the ring A (10,11). The high resolution mass spectrum indicates a molecular formula $C_{30}H_{50}O$ for the alcohol. The presence of the ions at m/e 313.2574 ($M^+ - C_8H_{15}$ [side chain] - 2H, requires 313.2530) and m/e 259.2088 ($M^+ - C_8H_{15} - C_3H_6$ [part of ring D] - CH_2, requires 259.2061) shows that this triterpene alcohol possesses a monounsaturated side chain (12) and also a monounsaturated skeleton with the additional C-32 methyl group (13). Both the double bonds were found as trisubstituted since the IR absorptions related to trisubstituted double bonds and observed for the unknown alcohol at 840 and 821 cm^{-1} were still observed as weakened bands for its dihydro derivative. The PMR spectrum of the unknown alcohol showed two olefinic protons (δ 5.12 and 5.16), of which the one (δ 5.12) must be on the side chain isopropylidene group since it disappeared in the spectrum of the dihydro alcohol; signals due to two vinylic methyls (26,27-methyls) (14) were also observed at δ 1.62 and 1.70 in the spectrum of the unknown alcohol. The presence of an axial proton at C-3 with a broad multiplet (δ 3.22) is also in support of the 3β-configuration of the hydroxy group (15). The spectrum of the alcohol also showed five tertiary methyl groups which must be located in the ring system with the singlets at δ 0.79 (3H), 0.82 (3H), 0.87 (3H), and 0.99 (6H). The chemical shifts of these methyl signals are found nearly identical with those observed for 5α-eupha-7,24-dien-3β-ol (Fig. 1, IIa). These facts may be rightly interpreted by regarding the ring system of the unknown alcohol as identical with that of IIa, the ring system of euphane-tirucallane series with Δ^7-bond (II).

The five tertiary methyl groups of the unknown alcohol showed the signals at δ(LSR) 11.00 (3H), 10.14 (3H), 4.49 (3H), 2.09 (3H), and 1.58 (3H) after the normalization of the LIS in the spectrum recorded in the presence of a molar equivalent of $Eu(DPM)_3$. Careful preliminary experiments in which the spectra were measured with every amount of $Eu(DPM)_3$ added have revealed that these methyl signals are associated with the signals at δ 0.87, 0.99, 0.79, 0.99, and 0.82, respectively, in the spectrum measured in the absence of the LSR. The signals at δ(LSR) 11.00 and 10.14 are correlated with 31 (4β)-methyl, the methyl group nearest to the 3β-OH, and 30 (4α)-methyl groups, respectively (3,4,16). The third methyl signal from the lower field at δ(LSR) 4.49 is attributable to the 19-methyl group. The Δδ-value ($\Delta\delta = \delta[LSR] - \delta$) of 19-methyl signal (Δδ 3.70) gives strong support to admit that the position of the trisubstituted double bond of the unknown alcohol is Δ^7 in the ring system because 5α-lanost-7-en-3β-ol (Fig. 1, Vb; Δδ 3.70), 5α-eupha-7,24-dien-3β-ol (IIa,Δδ 3.75), and 5α-euph-7-en-3β-ol(IIb,Δδ 3.76) also show similar Δδ-values for the corresponding methyl signal and there is no difference in the rings A and B chemistry between the triterpenes of lanostane and euphane-tirucallane series. Furthermore, the triterpenes with $\Delta^{9(11)}$- or Δ^8-bond indicate somewhat larger Δδ-values for the methyl signal: 24-methyl-5α-lanost-9(11)-en-3β-ol (IIIe, Δδ 3.87), 5α-lanosta-8,24-dien-3β-ol (Id, Δδ 3.91), 5α-euph-8-en-3β-ol (Ib,Δδ 3.95), and 5α-tirucall-8-en-3β-ol (Id,Δδ 3.90). The possibility of $\Delta^{9(11)}$-bond for the skeletal double bond of the unknown alcohol is, therefore, excluded.

The remaining two methyl signals with δ(LSR) 1.58 (Δδ 0.76) and 2.09 (Δδ 1.10) are attributable to 18- and 32-methyl groups, respectively, because it is seen from the Δδ-values listed in Table II that, among 18- and 32-methyl groups, the β-oriented one shows a larger Δδ-value than the other α-oriented one; the Δδ-values for the triterpenes are 18-methyl (β-oriented) > 32-methyl (α-oriented) on the lanostane series and, on the other hand, 18-methyl (α-oriented) < 32-methyl (β-oriented) on the euphane-tirucallane series. The δ(LSR)-values of all five methyl signals in the ring system of the unknown alcohol are identical within the experimental error with those measured for IIa. This is fully explicable by the ring system of II (Fig. 1) for the unknown alcohol. When the C-9 proton of the unknown alcohol is β-oriented, it causes some difference in the PMR data described above between the unknown alcohol and the reference eupha-7,24-dienol (IIa), of which the α-orientation of C-9 proton has already been ascertained (5,8).

The unknown alcohol, thus shown to possess the ring system II (Fig. 1), and its dihydro derivative indicate that the MS, IR, and PMR (without the LSR) spectral patterns are nearly identical with those of eupha-7,24-dienol (IIa) and its dihydro derivative (IIb), respecitvely,

TABLE II

Chemical Shifts (δ, ppm)[a] and Normalized Lanthanide-induced Shifts (δ[LSR], ppm)[b] of Proton Signals of 5α-Euphane, 5α-Tirucallane, and 5α-Lanostane Series of Triterpene Alcohols

Compound	C-3 substituent	Methyl groups									
		18	19	30	31	32	21	26,27	3β-OAc	3α-CH	Others
5α-Euphane series											
Eupha-8,24-dienol(Ia)	3β-OAc	0.76	0.98	0.88	0.88	0.88	0.85[e]	1.62,1.69	2.05	4.50*m*	5.08*t*(24-CH)
	3β-OH	0.76	0.97	1.00	0.81	0.88	f	1.62,1.69		3.20*m*	5.08*t*(24-CH)
Euph-8-enol (Ib)	3β-OAc	0.76	0.99	0.88	0.88	0.88	0.85[e]	0.87*d*	2.05	4.56*m*	
	3βOH[b]	1.48	4.92	10.32	11.00	1.96	1.09*d*	0.89*d*			
	3βOH	0.77	0.97	1.02	0.83	0.89	f	0.88*d*			
	$\Delta\delta$[c]	0.71	3.95	9.30	10.13	1.07		0.01			
Eupha-7,24-dienol(IIa)	3β-OAc[d]	0.82	0.78	0.96	0.85	1.00	f	1.62,1.70	2.05	4.56*m*	5.10*m*(24-CH),5.21*m*(7-CH)
	3β-OH[b]	1.58	4.51	10.18	11.00	2.10	1.16*d*	1.62,1.68			5.21*m*(24-CH),6.89*m*(7-CH)
	3β-OH	0.81	0.76	0.98	0.87	0.98	0.89[e]	1.62,1.70		3.25*m*	5.10*m*(24-CH),5.23*m*(7-CH)
	$\Delta\delta$	0.77	3.75	9.20	10.13	1.12		0 -0.02			0.11(24-CH) 1.66(7-CH)
Euph-7-enol(IIb)	3β-OH[b]	1.61	4.52	10.19	11.00	2.13	1.16*d*	0.91*d*			6.91*m*(7-CH)
	3β-OH	0.83	0.76	0.99	0.88	0.99	0.89[e]	0.88*d*		3.22*m*	5.24*m*(7-CH)
	$\Delta\delta$	0.78	3.76	9.20	10.12	1.14		0.03			1.67(7-CH)
5α-Tirucallane series											
Tirucalla-8,24-dienol(Ic)	3β-OAc[d]	0.76	0.99	0.89	0.89	0.89	0.96[e]	1.62,1.69	2.05	4.48*m*	5.11*m*(24-CH)
Tirucall-8-enol(Id)	3β-OAc	0.77	0.99	0.89	0.89	0.89	0.96[e]	0.87*d*	2.06	4.53*m*	
	3β-OH[b]	1.50	4.87	10.37	11.00	1.94	1.16*d*	0.95*d*			
	3β-OH	0.77	0.97	1.02	0.82	0.88	f	0.87*d*			
	$\Delta\delta$	0.73	3.90	9.35	10.12	1.06		0.08			
Tirucalla-7,24-dienol(IIc)	3β-OAc[d]	0.82	0.79	0.94	0.87	0.99	0.93[e]	1.62,1.70	2.05	4.54*m*	5.10*m*(24-CH),5.24*m*(7-CH)
	3β-OH[b]	1.58	4.49	10.14	11.00	2.09	1.19*d*	1.74,1.80			5.31*m*(24-CH),6.90*m*(7-CH)
	3β-OH	0.82	0.79	0.99	0.87	0.99	0.94[e]	1.62,1.70		3.22*m*	5.12*m*(24-CH),5.26*m*(7-CH)
	$\Delta\delta$	0.76	3.70	9.15	10.13	1.10		0.12,0.10			0.19(24-CH) 1.64(7-CH)
Tirucall-7-enol(IId)	3β-OH	0.82	0.76	0.98	0.87	0.98	f	0.87*d*		3.22*m*	5.24*m*(7-CH)
5α-Lanostane series											
24-Methyllanost-9(11)-enol (IIIe)	3β-OH[b]	1.70	4.92	10.26	11.00	1.72	1.30*d*	1.04*d*[g]			0.99*d*(28-CH_3)[g],6.96*m*(11-CH)
	3β-OH	0.66	1.05	1.00	0.82	0.75	f	0.87*d*		3.25*m*	5.24*m*(11-CH)
	$\Delta\delta$	1.04	3.87	9.26	10.18	0.97		0.17			1.72(11-CH)
Lanosta-8,24-dienol(IVa)	3β-OH[b]	1.79	4.92	10.24	11.00	1.79	1.31*d*	1.75,1.84			5.38*m*(24-CH)
	3β-OH	0.70	1.01	1.01	0.82	0.88	f	1.62,1.69		3.26*m*	5.11*m*(24-CH)
	$\Delta\delta$	1.09	3.91	9.23	10.18	0.91		0.13,0.15			0.27
Lanost-7-enol(Vb)	3β-OH[b]	1.74	4.59	10.04	11.00	1.85	1.32*d*	1.06*d*			6.92*m*(7-CH)
	3β-OH	0.65	0.89	1.00	0.90	1.00	f	0.88*d*		3.26*m*	5.21*m*(7-CH)
	$\Delta\delta$	1.09	3.70	9.04	10.10	0.85		0.18			1.71(7-CH)

though between these two isomers apparent differences are recognized in GLC, melting point, and $[\alpha]_D$. These facts may be explained by giving the side chain c (Fig. 1), C-20 epimer (20αH. 20*S*) of a (20βH, 20*R*), to the unknown alcohol since between 5α-eupha-8,24-dien-3β-ol (Ia) and 5α-tirucalla-8,24-dien-3β-ol (Ic), a known couple of C-20 epimers, similar correlations in the physical characteristics can be observed. That the unknown alcohol has the side chain c is justified also by the PMR spectroscopy with the LSR. Three triterpenes of euphane series, Ib, IIa, and IIb (Fig. 1), afforded Δδ -0.02 - 0.03 associated with 26,27-dimethyl signal(s), whereas the Δδ 0.08 was observed for the dimethyl signal of tirucall-8-enol (Id). The difference in Δδ-values between these two series is thought to have arisen from the inverted configuration at C-20. Then, the unknown alcohol exhibiting the Δδ 0.10 and 0.12 for the dimethyl signals is considered to carry the side chain of tirucallane series, i.e., c. Accordingly, the unknown alcohol isolated from tea seed oil may be concluded to have the structure of IIc (Fig. 1), 5α-tirucalla-7,24-dien-3β-ol, a new triterpene alcohol from natural sources.

The structure IIc given for the new alcohol is in full accord with the result of HCl isomerization. As euph-7-enol gives its Δ^8-isomer by treatment with HCl in $CHCl_3$ (5), so isomerization of euph-7-enyl (IIb) acetate in this study also indicates the formation of the acetate of the Δ^8-isomer (Ib). On the other hand, the dihydro acetate of the new alcohol on HCl treatment afforded a compound identical with the authentic specimen of tirucall-8-enyl (Id) acetate, and therefore the new alcohol must be a Δ^7-isomer (IIc) of 5α-tirucalla-8,24-dien-3β-ol (Ic).

It should be noted here that acid-catalyzed isomerization is reversible for lanost-8-enol (IVb) (2,17,18) and 32 (14α)-methylcholest-7-enol (18). These compounds yield an equilibrium mixture of Δ^7- and Δ^8-isomers by HCl treatment. On the other hand, as has already been shown (5) and also as described in the experimental section, the Δ^7-compounds of euphane and tirucallane series by HCl treatment under a very mild condition yield principally the Δ^8-isomers with several minor products. Attempted HCl treatment of euph-8-enol (Ib) under mild conditions gave no evidence for the formation of the Δ^7-isomer by GLC. Under the more violent conditions of acid isomerization, euph-8-enyl acetate is known to yield isoeuph-13(17)-enyl acetate rather than the Δ^7-isomer (19).

Gas chromatographic correlations of the tri-

[a]60 MHz, 80 μmol of triterpene in 0.4 ml of $CDCl_3$, 28 C, internal tetramethylsilane = 0 ppm; each signal was a singlet unless otherwise stated, in which case the multiplicity is given after the chemical shift: *d* = doublet (*J* 6.0 Hz), *t* = triplet (*J* 6.0-7.2 Hz), and *m* = multiplet.

[b]Normalized lanthanide-induced shift: 60 MHz, 80 μmol of triterpene alcohol in 0.4 ml of $CDCl_3$, molar ratio of $Eu(DPM)_3$ to alcohol is 1, 28 C, internal tetramethylsilane = 0 ppm; *d* = 21-CH_3, *J* 3.6-4.8 Hz; 26,27-methyls, *J* 5.4-6.6 Hz.

[c]Δδ-Value = δ [LSR] - δ, correlated to the 3β-OH data.

[d]Measured on a 100 MHz instrument under the condition described in footnote "a".

[e]Shoulder peak, part of a doublet signal.

[f]The presence of the shoulder peak is not clear.

[g]These doublets could not be sharply separated from one another, presumably due to the presence of C-24 epimers.

TABLE III

C-20 Epimeric (5α-Tirucallane/5α-Euphane) and Skeletal Isomeric (Δ^7/Δ^8) Separation Factors of Triterpene Alcohols on 3% OV-17 Column

C-20 Epimeric (5α-Tirucallane/5α-Euphane) Separation Factor

Compound compared		Separation factor 3β-OH	Separation factor 3β-OAc
Δ^8	Ic/Ia	1.13	1.14
	Id/Ib	1.14	1.13
Δ^7	IIc/IIa	1.13	1.14
	IId/IIb	1.13	1.13

Skeletal Isomeric (Δ^7/Δ^8) Separation Factor

Compound compared	Separation factor 3β-OH	Separation factor 3β-OAc
5α-Euphane series		
IIa/Ia	1.31	1.31
IIb/Ib	1.30	1.30
5α-Tirucallane series		
IIc/Ic	1.31	1.30
IId/Id	1.29	1.30

terpenes of euphane and tirucallane series furnish strong support for structure IIc of the new alcohol. Table I shows RRT determined on 3% OV-17 column and ΔR_{Ac}-values (10,11), the ratio of the RRT of the acetate to the RRT of the corresponding free alcohol, of the two series of triterpenes. The ΔR_{Ac}-values of these triterpenes are essentially similar to those observed on 5α-lanostane series compounds (10,11), as expected inasmuch as there is no difference in the spacial configurations of rings A and B between these series. Table III shows C-20 epimeric (5α-tirucallane/5α-euphane) and skeletal isomeric (Δ^7/Δ^8) separation factors of the triterpenes on 3% OV-17 column calculated from the retention data listed in Table I. Triterpenes of tirucallane series are more strongly retained than those of euphane series on OV-17 stationary phase. Ikan and Gottlieb (20) also observed a similar tendency between the two series of triterpenes as their trimethylsilyl ether derivatives on XE-60 column.

Many meliane (the group of compounds having a tirucallane type skeleton with an oxygenated side chain) and meliacan (the group of compounds possessing an apo-tirucallane type skeleton) series of oxygenated triterpenes have recently been ascertained to be present in the Meliaceae and the related Rutaceae and Simaroubaceae species (21-27). The new alcohol (IIc) isolated from tea seed oil in this study is just a compound which has been considered as a possible biogenetic precursor of these oxygenated tetracyclic triterpenes (21,23,26); nevertheless, its occurrence in natural sources has not yet been known, though the previous existence of the tirucalla-7,24-diene structure in the form bearing a carboxylic group at C-20 is known as one of the elemolic acids (3α-hydroxy-5α-tirucalla-7,24-dien-21-oic acid, Fig. 1, VIf) (22). The presence of the new triterpene alcohol (IIc) is ascertained also in the seed oils of two other theaceous plants, *Camellia japonica* and *Camellia Sasanqua* (unpublished data).

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Enzymatic Hydrolysis of 1-Monoacyl-SN-Glycerol-3-Phosphoryl-choline (1-Lysolecithin) by Phospholipases from Peanut Seeds

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ABSTRACT

Hydrolysis of 1-lysolecithin (1-acyl glycerophosphorylcholine [1-acyl GPC]) by preparations of phospholipase D from peanut seeds was investigated. 1-Lysolecithin was hydrolyzed at a much slower rate than phosphatidylcholine (lecithin). Although Ca^{+2} ions are required for the cleavage of lecithin by the enzyme, their effect on the hydrolysis of lysolecithin depended upon the concentrations of the substrate: at 0.2 mM 1-lysolecithin, Ca^{+2} ions increased the reaction rates, whereas at concentrations of the substrate lower than 0.1 mM, Ca^{+2} ions were inhibitory. A broad pH activity curve between 5 and 8 was obtained with higher rates in the alkaline range, both in the absence and presence of Ca^{+2} ions. The increased hydrolysis of lysolecithin due to Ca^{+2} was noticed over the entire pH range. Upon storage of the enzyme solutions at 4 C, decreased rates of hydrolysis of lecithin were observed, with t 1/2 values of ca. 50 and 100 days depending on the purity of the preparation. During the same period, no reduction occurred in the activity of these preparations on lysolecithin as substrate. The effects of Ca^{+2} ions and the analysis of the products of 1-acyl GPC cleavage by the enzyme preparations revealed the presence of more than one enzyme and the formation of the following compounds: lysophosphatidic acids (1 acyl glycerophosphoric acids), free fatty acids, glycerophosphorylcholine, and choline. The possible pathways leading to the degradation of lysolecithin and the formation of these products include reactions catalyzed by lysophospholipase A_1 (lysophosphatidylcholine 1-acyl hydrolase, E.C. 3.1.1.5) and a phosphodiesterase (L-3-glycerylphosphorylcholine glycerophosphohydrolase, E.C. 3.1.4.2), in addition to phospholipase D (phosphatidylcholine phosphatidohydrolase, E.C. 3.1.4.4).

INTRODUCTION

Preparations of phospholipase D (phosphatidylcholine phosphatidohydrolase, E.C. 3.1.4.4) from peanut seeds have been shown to act on various phospholipids (1). With the exception of cardiolipin as substrate, ether, organic solvents, or detergents were required for the hydrolytic activity (2). Ca^{+2} ions were also needed for the catalytic reaction (2,3). In similar studies, Long and his associates have investigated the properties of a soluble phospholipase D. Their partially purified enzyme from cabbage leaves, which catalyzed the cleavage of lecithin and other glycerophosphatides, hydrolyzed lysolecithin at a rate ca. 0.15 that of lecithin (4,5). Ether and detergents were not required and even decreased the rates of lysolecithin hydrolysis. Ca^{+2} ions at a concentration of 5 mM were essential for the reaction. They also reported on the absence of deacylation of this substrate during the enzymatic degradation (5). In our previous studies, we failed to detect other lipolytic activities, e.g., phospholipase A (phosphatide acyl-hydrolase, E.C. 3.1.1.4) activity in homogenates of peanut seeds, using ^{14}C-fatty acid labeled as substrate (2).

In the present study, we intended to clarify the mode of hydrolysis of lysolecithin by preparations of phospholipase D. We could demonstrate that lysolecithin was indeed cleaved by phospholipase D under conditions not requiring the presence of either organic solvents or detergents. However, more detailed results on the possible pathways leading to a complete hydrolysis of lysolecithin are presented. This indicates clearly the presence of additional enzymes in dry peanut seeds participating in a metabolic pathway hitherto not described in plants. A preliminary account has been published (6).

MATERIALS

Enzymes

Phospholipase D from dry peanut seeds was purified according to Heller et al. (1,7) briefly as follows. *Step 1:* soluble, crude enzyme of the seed homogenate after centrifugation at 100,000 x g for 60 min. *Step 2:* precipitation with 35% $(NH_4)_2SO_4$ (w/v). *Step 3:* DEAE-cellulose column chromatography, using a KCl gradient. *Step 4:* eluate obtained from a sepha-

rose 6B column.

Substrates

^{3}H-choline labeled lecithin was extracted from tissue culture cells grown in the presence of ^{3}H-choline (2). Alternatively, it was prepared by chemical synthesis from phosphatidyl-N,N-dimethylethanolamine and ^{3}H-methyl iodide according to Stoffel (8). ^{3}H-palmitate labeled lecithin was extracted from livers and kidneys of rats injected with potassium 9,10 (n) ^{3}H-palmitate. The labeled lecithin preparations were purified on columns of silicic acid (Unisil, Clarkson Chemical Co. Inc., Williamsport, PA) using increasing concentrations of methanol in chloroform for elution. Both chemical and radiochemical purity was determined. The labeled lecithins were diluted with ovolecithin to any desired specific radioactivity.

^{3}H-choline or ^{3}H-palmitate labeled 1-lysolecithins (1-monoacyl-SN-glycerol-3-phosphorylcholine) were prepared from their respective lecithins following hydrolysis with phospholipase A_2 from *Crotalus adamanteus* in the presence of ether (9). The products were extracted and chromatographed on a column of silicic acid (Unisil) using gradients of methanol in chloroform. The 1-lysolecithin was eluted from the column with methanol. Both chemical and radiochemical purity of these substances were determined (see below) and they were diluted as required, with unlabeled 1-lysolecithin prepared in a similar manner. ^{3}H-choline labeled glycerophosphorylcholine was prepared from ^{3}H-choline labeled lecithin following alkaline hydrolysis and purification according to Brockerhoff and Yurkowsky (10). 1-Lysophosphatidic acid was prepared from phosphatidic acid following hydrolysis with phospholipase A_2 and purified by SiO_2 column chromatography. Phospholipase A_2 was a Sigma product (Sigma, Ramat Gan, Israel), and the radioactive materials were purchased from Amersham (Buckinghamshire, England).

METHODS

The purity of the substrates and the products was determined by the following procedures: (a) determination of acyl ester bonds according to Stern and Shapiro (11); (b) analysis of phosphorous by the method of Bartlett (12); and (c) development of thin layer chromatograms of lipids in either (i) chloroform:methanol:water (65:35:4, v/v/v), (ii) chloroform:pyridine:88% formic acid (50:20:7, v/v/v), or (iii) ether:hexane:glacial acetic acid (60:40:1, v/v/v). Thin layer chromatograms of water soluble substances, e.g., choline or glycerophosphorylcholine, were developed in chloroform:methanol:3M trichloracetic acid: water (80:120:40:25, v/v/v/v). The lipid spots were made visible by iodine vapors and by using a spray reagent for phosphorous (13). Water-soluble spots were visualized according to Doizaki and Zieve (14).

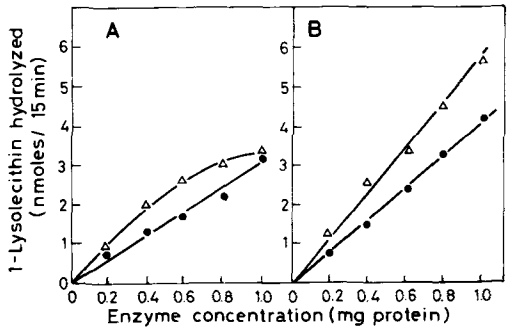

FIG. 1. Dependence of lysolecithin hydrolysis on protein concentrations. A final volume of 0.2 ml contained 100 mM acetate, pH 5.6, enzyme at step 4 with protein concentrations as indicated, in the presence (△—△) or absence (●—●) of 30 mM $CaCl_2$. Incubation was done at 37 C for 15 min, and the reaction was terminated with isobutanol and was processed as described in Methods. Substrate: 1-lysolecithin-^{3}H-choline (1.5 μCi/μmol)–(A) 0.1 mM; (B) 0.2 mM.

Radioactive labeled compounds chromatographed on thin layer plates were scanned with a radioactive scanner (Berthold, Wilbad, West Germany). Quantitative evaluation of the labeled compound on the plates was done by scraping the areas into counting vials. Two milliliters of Triton x-100:ethanol (1:1, v/v) were added, the vials were mixed and left on the bench at room temperature, then 10 ml of a scintillation fluid (made of 130 mg POPOP and 5 g PPO in 1 liter toluene) were added, mixed, and counted. Correction for quenching was done by the channel ratio method. Protein was determined according to Lowry et al., using bovine serum albumin as standard (15).

Assay

Phospholipase D activity was assayed using ^{3}H-choline labeled lecithin as substrate (1). With 1-lysolecithin as substrate, the following assay conditions were employed. The incubation mixture contained 0.15 mM 1-lysolecithin (1.5 μCi/μmol or 7.5 μCi/μmol); 0.1 M acetate, pH 5.6; 30-50 mM $CaCl_2$; and 0.1-0.9 mg protein of enzyme preparations at steps 2 or 4 of the purification scheme (1) in a total volume of 0.2 ml. The reaction proceeded for 15 min at 37 C and was then terminated by the addition

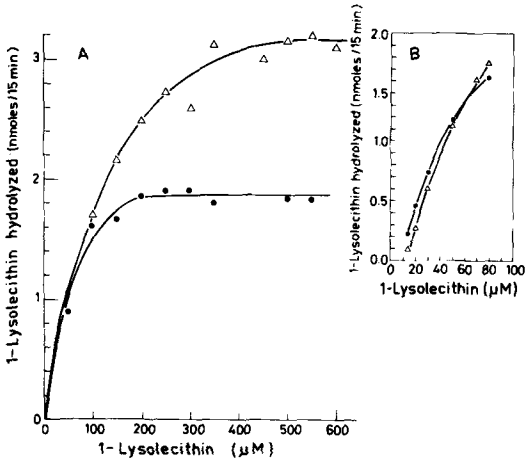

FIG. 2. Rates of lysolecithin hydrolysis as a function of substrate concentrations. The conditions were similar to those given in Figure 1, except that the amount of enzymatic protein was 0.37 mg and the reaction was measured in presence (△—△) or absence (●—●) of 30 mM $CaCl_2$. The range of lysolecithin concentrations was (A) 50-600 μM and (B) 14-18 μM.

of 1 ml isobutanol. After mixing, 1 ml of H_2O was added; the tubes were vortexed and centrifuged in a desk centrifuge. The upper organic phase was removed and replaced by 1 ml of isobutanol; the tubes were mixed again, centrifuged, and the upper phase removed. Chloroform (1 ml) was added, and an aliquot of 0.5 ml of the now aqueous upper phase was withdrawn for determination of the water-soluble radioactive products. Counting was done in ini glass vials (flat bottom tube closers, Hospital Laboratory Supplies, London, England) following the addition of 3.5 ml Insta-gel (Packard Instrument Co., Downers Grove, IL) in a Scintillation spectrometer (Packard). The results were corrected for quenching. The recovery of ^{3}H-choline in the aqueous phase of the above described procedure was almost 100%, with negligible contamination by 1-lysolecithin (M. Deutsch-Gonen and S. Gatt, personal communication, 1975). In a few instances the reaction was terminated by adding chloroform and methanol according to Folch et al. (16) or Bligh and Dyer (17).

RESULTS

Effects of Protein and Calcium Concentrations

The hydrolysis of ^{3}H-choline labeled 1-lysolecithin by partially purified preparations of peanut phospholipase D was followed by measuring the release of water-soluble products, employing the biphasic system of isobutanol and water (M. Deutsch-Gonen and S. Gatt, personal communication, 1975).

This experiment was performed under conditions which were found to be suitable for the hydrolysis of lecithin, e.g., pH 5.6 and the presence of Ca^{+2} ions (1). Similarly, the optimal concentration of Ca^{+2} ions for the degradation of 1-lysolecithin was 30-50 mM. Increasing amounts of enzyme were incubated with either 0.1 mM or 0.2 mM 1-lysolecithin (Fig. 1). It should be noted that lysolecithin was hydrolyzed even in the absence of Ca^{+2} ions, although at a rate lower than in its presence. The effects of Ca^{+2} ions on the rate of lysolecithin hydrolysis depended on the substrate concentration. The velocity of the cleavage of lysolecithin depended linearly on the amount of protein up to 1 mg of enzyme in the presence or absence of Ca^{+2} ions with 0.2 mM lysolecithin (Fig. 1B). At a concentration of 0.1 mM lysolecithin, in the presence of Ca^{+2} ions, the activity deviated from linearity (Fig. 1A).

Effects of Substrate Concentrations

To clarify the previously obtained results, the rate of hydrolysis as a function of 1-lysolecithin concentrations, in the presence or absence of Ca^{+2}, was examined. At lysolecithin concentrations below 14 μM, 30 mM of Ca^{+2} ions had no effect on the rate of hydrolysis of the substrate. However, Ca^{+2} ions caused a considerable decrease in the rate of degradation up to 65 μM 1-lysolecithin (Fig. 2B). At higher substrate concentrations, up to 600 μM, a marked increase in the maximal rates of reaction, by $> 50\%$, was observed (Fig. 2A).

Effects of pH

The rate of hydrolysis of lysolecithin increased progressively toward the alkaline range and was more than twice as high as in the presence of Ca^{+2} over the entire range of pH (Fig. 3). The curve obtained in the absence of Ca^{+2} was discontinuous and exhibited a few breaking points: one at ca. 5.8 and the other at ca. 6.8. When Ca^{+2} ions were present, a smooth curve was obtained up to pH 7.5, which then leveled off. This is in contrast to the hydrolysis of lecithin, which has a pH optimum at 5.6 (1,2).

Stability of the Enzymatic Preparations

In all fresh preparations, lecithin was hydrolyzed at rates 200-300 times faster than 1-lysolecithin.

The effect of storage of phospholipase D preparations on the rates of hydrolysis of lecithin and lysolecithin was compared. Figure

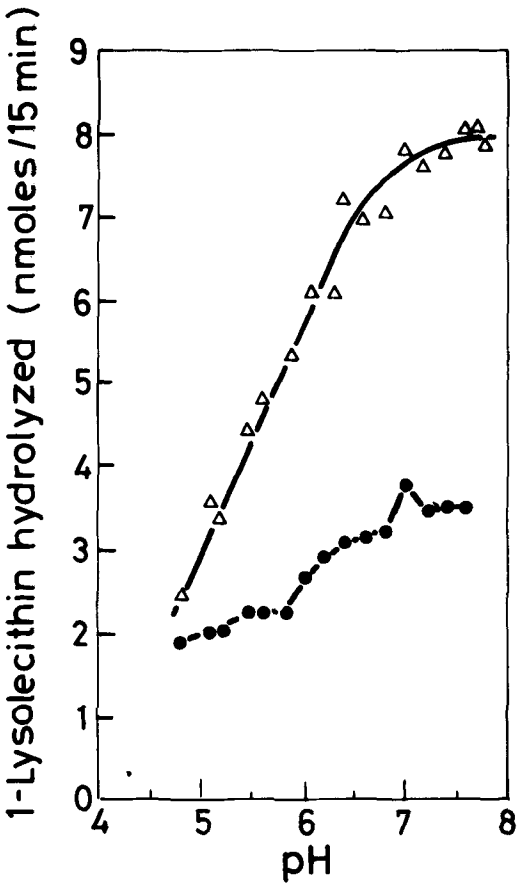

FIG. 3. Dependence of lysolecithin hydrolysis on pH. 0.3 mM ^{3}H-choline labeled 1-lysolecithin (1.5 μCi/μmol) was incubated with 0.37 mg protein of the enzyme at step 4 for 15 min at 37 C in presence (△—△) or absence (●—●) of 50 mM $CaCl_2$. The buffers used were acetate, dimethyl-glutarate, collidine, and imidazole at 200 or 300 mM concentrations.

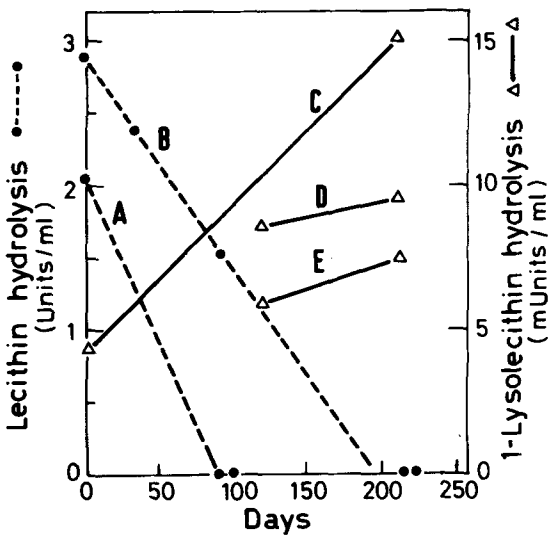

FIG. 4. Stability of phospholipase D preparations to storage. Solutions of enzyme preparations in 50 mM tris-HCl, pH 7.5-8.0, were stored at 4 C. The activity with ^{3}H-choline labeled lecithin or 1-lysolecithin was determined on aliquots of the preparations at steps 2 or 4 (cf. Materials): A. With lecithin (5 mM) as substrate, pH 5.6, with enzyme step 2. B. With lecithin (5 mM) as substrate, pH 5.6, with enzyme step 4. C. With lysolecithin (0.15 mM) as substrate, pH 7.8, enzyme at step 2. D. With lysolecithin (0.20 mM) as substrate, pH 7.8, enzyme at step 4. E. With lysolecithin (0.10 mM) as substrate, pH 7.8, enzyme at step 4.

4 indicates that the catalytic activity towards lecithin of buffered solutions of phospholipase D stored at 4 C diminished and disappeared after ca. 100-200 days for enzymatic preparations at steps 2 and 4, respectively. The activity of the same preparations with 1-lysolecithin as substrate increased upon storage.

Analysis of the Products

The results of the experiments shown in Figures 2 and 3, i.e., rate of lysolecithin hydrolysis as a function of pH or substrate concentrations, suggested that this substrate might also be attacked by enzymes present in these preparations other than phospholipase D.

Two enzyme preparations were employed: one, a crude preparation (at step 2), which hydrolyzed both lecithin and 1-lysolecithin; and the other, at a higher degree of purification (step 4), which had already lost all its phospholipase D activity with lecithin as substrate. Table I shows the results obtained when ^{3}H-palmitate labeled 1-lysolecithin was incubated with the two enzyme preparations. The controls, without enzyme, indicate that only negligible amounts of the substrate were deacylated or otherwise degraded (18). The crude enzyme liberated ca. 20% of the radioactivity of lysolecithin as free fatty acids (26.5% at pH 5.6 and 17.9% at pH 7.8). Lysophosphatidic acids were formed only at pH 5.6. The more purified enzyme preparation, on the other hand, at both pH values (5.6 and 7.8) converted $>70\%$ of the labeled substrate into labeled fatty acids and only ca. 17-18% into lysophosphatidic acids, i.e., in the open or cyclic forms (cf. Ref. 19). We were unable to identify other neutral lipids, e.g., glycerol-1-palmitate. These results are consistent with the presence of a lysophospholipase A_1 acting on either 1-lysolecithin or on 1-lysophosphatidic acids or on both.

To identify the water-soluble products, we have incubated both enzymatic preparations under almost the same conditions as above, with ^{3}H-choline labeled 1-lysolecithin and the

TABLE I

Isobutanol-soluble Degradation Products of ^{3}H-Palmitate Labeled 1-Lysolecithin[a]

Tube	1	2	3	4	5	6
Enzyme	-	I	I	II	II	-
Final pH	5.6	5.6	7.8	5.6	7.8	7.8
Fatty acids and neutral lipids[b]	6.5	26.5	17.9	82.3	71.2	8.3
Unidentified phospholipid	2.8	0.5	1.08	0	1.3	0.78
Cyclic lysophosphatidic acid	0	5.5	0	17.0	15.0	0
Lysolecithin	89.0	45.7	79.8	0	9.4	90.4
(Origin) Lysophosphatidic acid	0.9	21.4	1.1	0.4	3.0	0.4

[a]The incubation mixture in a final volume of 0.2 ml contained 0.15 mM ^{3}H-palmitate labeled lysolecithin (1.5 μCi/μmol); 30 mM $CaCl_2$; 200 mM acetate, pH 5.6, or 200 mM imidazole, pH 7.8; and either enzyme I (purified past step 2, 1 mg protein) or enzyme II (purified past step 4, 0.37 mg protein). The tubes were incubated for 210 min at 37 C, and the reaction was terminated with isobutanol. Subsequent operations were done as described in Methods.

[b]The isobutanol-soluble compounds were chromatographed on thin layer plates covered with Silica Gel G and developed in $CHCl_3$:CH_3OH:H_2O (65:25:4, v/v/v). In this solvent system, these lipids appeared at the solvent front. The radioactive recovery was >90%. The results are expressed in percent distribution of total radioactivity in each tube (lane).

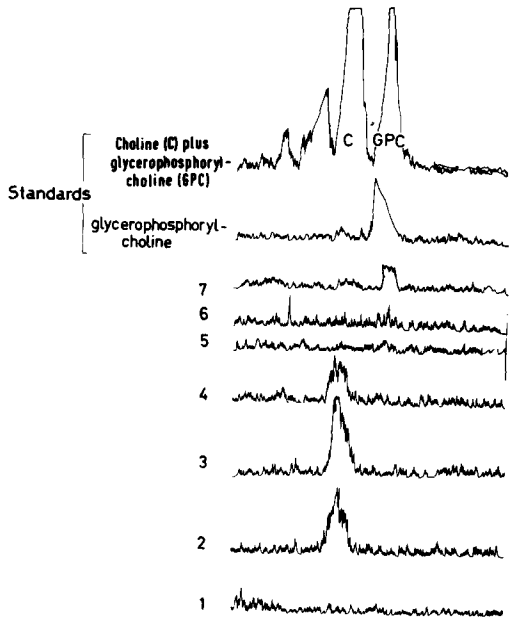

FIG. 5. Radioactive scan of the water-soluble degradation products of ^{3}H-choline labeled lysolecithin. Incubation mixture of 0.2 ml contained 0.2 mM ^{3}H-choline labeled 1-lysolecithin (12.2 μCi/μmol); 30 mM $CaCl_2$; 100 mM acetate, pH 5.6, or 100 mM imidazole, pH 7.8; and either 0.5 mg protein of the crude enzyme (step 2) or 0.9 mg protein of the purer enzyme (step 4). Incubation lasted 300 min at 37 C, and the reaction was terminated with 0.8 ml of $CHCl_3$:CH_3OH (2:1, v/v) (16). The aqueous phase was chromatographed on thin layer plates using $CHCl_3$:CH_3OH:H_2O:CCl_3COOH (3M) (80:120:25:40, v/v/v/v) as described in Methods. The composition of each tube is given as follows:

Tube	1	2	3	4	5	6	7
$CaCl_2$	+	+	+	+	+	+	–
Enzyme (step)	–	4	4	2	2	–	2
pH	5.6	5.6	7.8	5.6	7.8	7.8	7.8

water-soluble products chromatographed and traced on a radioactive scan (Fig. 5). In agreement with the results obtained previously and shown in Table I, the crude enzyme catalyzed the liberation of ^{3}H-choline at pH 5.6 only; ^{3}H-glycerophosphorylcholine was formed at pH 7.8 in the absence of Ca^{+2} ions. On the other hand, the enzyme preparation at a more advanced stage of purification released only ^{3}H-choline at the two pH values in the presence of Ca^{+2}. Therefore, at a cruder stage of purification, the enzyme catalyzed the formation of products of the phospholipase D reaction at pH 5.6 but not at 7.6 and most probably also catalyzed the reaction of a lysophospholipase A_1. At a more advanced stage of purification, there is evidence for the existence of both phospholipase D and lysophospholipase A_1. The stoichiometric relationship between the water-soluble and lipid-soluble products apparently does not fit because, in the presence of Ca^{+2} ions, the only water-soluble product detected was choline, whereas the major lipid product was free fatty acids. These results raised the possibility of the existence of another enzyme in the preparations of phospholipase D, i.e., a phosphodiesterase responsible for the hydrolysis of water-soluble diesters such as glycerophosphorylcholine to yield free ^{3}H-choline. We have, therefore, incubated ^{3}H-choline labeled glycerophosphorylcholine (5 μM; 20 μCi/μmol) with the same enzyme preparations, under conditions similar to those given in Figure 5. From the chromatographic analysis and radioactive scanning of the results (not shown), the crude enzyme at pH 5.6 did not hydrolyze glycerophosphorylcholine, either in the presence or absence of Ca^{+2} ions at both pH values. The purer enzyme (step 4) catalyzed

the hydrolysis of glycerophosphorylcholine at pH 5.6 only when Ca^{+2} ions were present.

Failure to detect phosphodiesterase activity in the crude preparations of the enzyme is most probably due to its low activity. In the more advanced stage of purification (step 4), its activity was detected because it was probably purified together with phospholipase D.

DISCUSSION

Long et al. have shown that a partially purified phospholipase D from cabbage leaves catalyzes the hydrolysis of 1-lysolecithin to form two degradation products in addition to choline. These products were identified as lysophosphatidic acid (1-monoacyl-SN-3-glycerophosphoric acid) and a cyclic form of this acid (19). The optimal conditions for the enzyme's activity required the presence of Ca^{+2} ions and a pH ranging from 5.4 to 6.4. The requirements for Ca^{+2} were quite strict (5). These authors were unable to detect any deacylase activity in their enzyme preparations (5) but could show a phosphodiesterase acting on diphenyl phosphate at ca. 2% of the rate found with ovolecithin, and on glycerophosphorylcholine at ca. 0.2% of the rate with ovolecithin (4). The phospholipase D and phosphodiesterase activities were separated by treatment with calcium phosphate gel (4). For its action on lysolecithin, the amount of the cabbage enzyme had to be increased, since Long et al. found that the phospholipase D was only 15% as active with this substrate as with ovolecithin as substrate (4,5).

Analysis of the protein pattern comprising the partially purified preparations of phospholipase D from peanut seeds has been done previously (Fig. 3 in Ref. 7). A number of protein bands were observed on the gels obtained after disc-gel electrophoresis of the enzyme even at an advanced stage of purification, e.g., following gel filtration on sepharose 6B, step 4 (7). At the beginning of our studies, we found a similar ratio of activities using lecithin or 1-lysolecithin as substrates whether crude (step 2) or purer (step 4) preparations of phospholipase D were employed. When using a fresh preparation of the crude enzyme (step 2) which retained its activity towards lecithin, we identified among the products lysophosphatidic acid, choline, and fatty acids. Since no phosphodiesterase activity could be measured under the conditions used, the choline obtained is probably the product of phospholipase D action on 1-lysolecithin (Fig. 6, pathway C). The fatty acids formed by this preparation are probably the products of a lysophospholipase

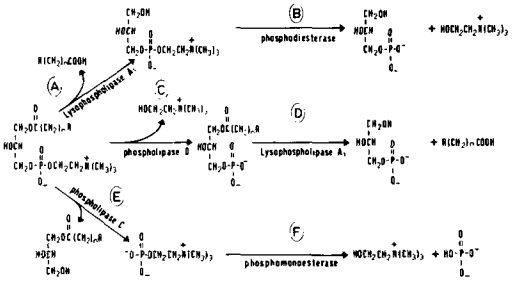

FIG. 6. Possible pathways of lysolecithin catabolism.

A_1 activity, acting on lysophosphatidic acid (Fig. 6, pathway D). These activities were obtained at pH 5.6 (but not at 7.8), which is the optimum value for phospholipase D action on lecithin. It is, therefore, suggested that fresh, crude preparations of phospholipase D hydrolyze 1-lysolecithin by pathways C and D (Fig. 6). The activity toward lecithin decayed with time, wheras the catalysis of lysolecithin hydrolysis remained unchanged. Despite the loss of activity toward lecithin, choline was identified among the products of the enzymatic reaction as well as lysophosphatidic acids. Since a phosphodiesterase activity was detected in this preparation, the formation of fatty acids and choline, instead of glycerophosphorylcholine, can be explained by assuming the catabolism of lysolecithin according to pathways A and B in Fig. 6. Lysolecithin is hydrolyzed by a lysophospholipase A_1 (pathway A) to form fatty acids and glycerophosphorylcholine, which is then degraded by a phosphodiesterase to choline and glycerophosphate (Fig. 6, pathway B).

Since lysophosphatidic acid was also formed, pathways C and D are probably also operating, and the respective enzymatic activities were detected both at pH 5.6 and 7.8. Choline could also have been formed by the combined action of phospholipase C and a phosphomonoesterase (pathways E and F). We could not, however, detect glycerol-1-palmitate, the product of phospholipase C action on 1-lysolecithin, which seems to exclude this route. Despite the fact that phospholipase D activity on lecithin as substrate is expected to be very low under the assay conditions, choline plus lysophosphatidic acids could be formed by the enzyme inasmuch as large quantities of protein were employed in these studies. The phosphodiesterase and lysophospholipase A_1 activities copurified with the phospholipase D but were stable upon storage. Both the phospholipase D and the phosphodiesterase of plant origin were shown to be activated by Ca^{+2} (4), although the mammalian

phosphodiesterase has been reported to be inhibited by this cation (20).

In our experiments, Ca^{+2} ions had different effects, depending on substrate concentrations (Fig. 2). Since more than one enzymatic activity is concerned, we cannot draw conclusions on the nature of the effect of this cation.

Very little published work is found on the occurrence and properties of lysophospholipase A_1 from plant sources (21,22). From the hitherto known properties of this enzyme from various other sources, it does not seem to require Ca^{+2} for activity (21,22).

All these enzymatic activities, using lysolecithin as substrate, were detected in freshly prepared solutions of the enzyme at a crude stage of purification (step 2). Using lecithin as substrate, only phospholipase D activity was detected in these preparations (1,2).

From the data presented in these studies, it is difficult to conclude whether the phospholipase D which acts on lecithin is the same protein acting on 1-lysolecithin. Further studies are under way to clarify this and other problems.

ACKNOWLEDGMENT

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Vitamin E, Cholesterol, and Lipids during Atherogenesis in Rabbits

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ABSTRACT

Rabbits were fed diets including cholesterol and 10% butterfat to determine whether polyunsaturated butter (9% 18:2) would be less atherogenic than normal saturated butter (3% 18:2) when fed for 12 weeks. The cholesterol diets alone, 0.5% or 2%, produced aortic plaque development, and plasma cholesterol increased 20 times, lipids increased 10 times, and vitamin E increased 5 times. The inclusion of both fat and cholesterol in the diet produced a synergistic effect, doubling these values to 40 times for cholesterol, 20 times for lipids, and 10 times for vitamin E. The higher circulating levels of cholesterol caused increased tissue levels of cholesterol. With 2% cholesterol and fat, liver and aorta cholesterol increased 10 times, heart 4 times, and muscle cholesterol 2 times. The lower 0.5% dietary cholesterol load was successful in limiting the amount of tissue cholesterol increase. Liver, aorta, heart, and muscle levels of cholesterol were only about half the concentration attained when 2% cholesterol was fed. It was concluded that there were no differences in plasma or tissue cholesterol, vitamin E, or atherosclerosis attributable to the polyunsaturated nature of the diet. The 10% butterfat diets alone, whether saturated or unsaturated, did not induce aortic plaques and did not increase blood or tissue cholesterol, lipids, or vitamin E. Our results suggest that the lipid mobilizing effect is mediated by cholesterol, probably by conversion to bile acids and a stimulation in intestinal absorption.

INTRODUCTION

Rabbits readily and rapidly develop atherosclerosis when fed a high lipid diet containing cholesterol (1-3). There are some discrepancies in the literature concerning the influence of the nature of the lipid upon the atherogenesis. Thus, Kritchevsky et al. (4,5) found a lower severity of experimental atherosclerosis in rabbits fed polyunsaturated fat. In a later study in which rabbits were fed cholesterol diets high and low in triglycerides containing linoleic acid (corn oil vs. olive oil), Swell et al. (6) observed no significant differences in the degree of atherosclerosis.

Our laboratory has been producing cow's milk containing higher than normal levels of polyunsaturated fats for possible use by the cardiac-concerned consumer (7). Recently, we initiated experiments to determine whether butter made from this polyunsaturated milk would be less atherogenic than normal butter when fed to rabbits. The influence of an unsaturated and saturated butter upon a number of serum and tissue lipid components was also of interest. Accordingly cholesterol, total lipids, and vitamin E were measured during the development of atherosclerosis.

TABLE I

Diets Used in Experiments

Expt.	Group	N	Cholesterol	Added fat[a]	Treatment
1	1	8	0	0	Fed for 12 weeks, killed
	2	8	+ 2%	0	
	3	10	+ 2%	+ 10% Herd butter	
	4	9	+ 2%	+ 10% SOC butter	
	5	10	+ 2%	+ 10% SOC-F butter	
	6	9	0	+ 10% Herd butter	
2	7	12	0	0	Killed at 4, 8, 12 weeks
	8	12	+ 0.5%	0	
	9	16	+ 0.5%	+ 10% Herd butter	
	10	14	+ 0.5%	+ 10% SOC-F butter	

[a]HERD denotes butter from cows fed grain and hay; SOC denotes butter from cows fed safflower oil-casein, grain, and hay; and SOC-F denotes butter from cows fed safflower oil-casein-formaldehyde, grain, and hay.

TABLE II

Fatty Acid Composition of Diets (% by wt)[a]

Fatty acid	Chow	Herd butter	SOC butter	SOC-F butter	Chow + 10% herd butter	Chow + 10% SOC-F butter
C8:0	-	1.2	0.6	0.8		
C10:0	0.01	3.0	1.5	2.1	1.8	1.1
C12:0	0.06	3.7	1.8	2.6	3.0	1.7
C14:0	0.4	11.4	7.0	9.0	9.2	5.6
C16:0	13.9	30.1	19.1	20.9	27.1	21.7
C16:1	0.6	2.7	2.5	1.2	1.5	1.1
C18:0	3.3	11.0	15.1	16.8	8.6	12.8
C18:1	21.9	25.5	35.6	31.2	25.3	28.7
C18:2	45.5	2.0	4.3	8.9	14.5	18.4
C18:3	13.9	0.8	2.2	2.3	5.0	5.4
C20:0	0.2	0.1	0.1	0.2	0.1	0.2
Others	0.2	8.5	10.2	4.0	2.9	2.3
Iodine value	134.6	30.0	46.2	49.4	61.4	71.7

[a]HERD denotes butter from cows fed grain and hay; SOC denotes butter from cows fed safflower oil-casein, grain, and hay; and SOC-F denotes butter from cows fed safflower oil-casein-formaldehyde, grain, and hay.

MATERIALS AND METHODS

Experimental Animals

New Zealand White male rabbits, 4-5 months old, weighing 2.9-3.4 kg, individually caged, were used in two atherogenic experiments. In experiment 1, 2.0% cholesterol was fed; in experiment 2, the diet contained 0.5% cholesterol.

Diets

The rabbits were fed at 8:00 a.m. daily 125 g of a powdered commercial chow containing the added dietary ingredients, and the feed cups were removed from the cages at 4:30 p.m. Details of the diet and treatments are given in Table I. Cholesterol (USP-Nutritional Biochemicals, Cleveland, OH) was added in a powdered form, and butter, melted on a steam bath, was mixed with the chow in a Hobart food mixer. Butters were prepared from milk of cows fed (a) hay and grain (Herd butter), (b) hay and grain containing 6.5% added safflower oil and 3.5% added sodium caseinate (SOC butter), or (c) hay and grain containing 10% formaldehyde treated safflower oil-caseinate (SOC-F butter). After pasteurization, 30 min at 145 C, 40% cream was prepared from the milk in a De Laval cream separator. After holding at 5 C overnight, the cream was churned into butter with a household electric hand mixer. Batches of ca. 2 kg butter were prepared at biweekly intervals during the course of the experiments or, if prepared previously, were stored at -20 C. The fat content of the butter averaged 82.2%; moisture was 17.1%. Analysis of the commercial chow indicated a fat content of 4.1%; the chow diet containing 2% cholesterol and 10% butter of experiment 1 averaged ca. 14% fat, and the chow diet containing 0.5% cholesterol and 10% butter of experiment 2 averaged 12.6% fat, since the butter only contained 82% fat.

The milk from which the butters were prepared varied only slightly in cholesterol content (7). Consequently, cholesterol content of the butters was quite similar, being 2.76-3.28 mg/g butterfat. In experiment 1, in which the diets contained 2% added cholesterol (2,000 mg/100 g diet), the butter contributed an additional 24 mg/100 g diet, making the total cholesterol content 2.024%. In experiment 2, in which the diet contained 0.5% added cholesterol (500 mg/100 g diet), the cholesterol content of the butter made the total cholesterol concentration 0.524%.

The fatty acid composition of the rabbit chow, the saturated and unsaturated butters, and diets containing 10% of these butters is given in Table II. The 4% vegetable fat of the rabbit chow is primarily polyunsaturated in contrast to the animal fat, which is primarily saturated. The herd butter contained only 2% C18:2 in contrast to the SOC-F butter, which contained 8.9% C18:2 and 2.3% C18:3.

The vitamin E content of the rabbit chow was 58.3 μg/g. The butters which were substituted for chow contained 17.9 μg tocopherol per gram of fat. Accordingly, the tocopherol content of the diets varied slightly: group 1, 58.3 μg/g; group 2, 57.1 μg/g; groups 3, 4, 5, 53.1 μg/g; group 6, 54.3 μg/g.

Bleeding and Sampling of Tissues

Every 2 weeks the rabbits were weighed and bled from the marginal ear veins into a 125 ml

TABLE III

Body Weight and Feed Consumption of Rabbits Fed an Atherogenic Diet Containing 2% Cholesterol

Group[a]	Diet[b]	Body weight (kg)		Gain[c]		Mean daily feed consumption (g/day)
		0 wk	12 wk	kg	%	
1	N	2.94	3.20	0.26	8.8 ± 2.0^{A}	92.2 ± 5.4^{A}
2	N+C	2.96	3.18	0.22	7.4 ± 1.4^{AB}	84.1 ± 4.2^{A}
3	N+C+H	3.06	3.37	0.31	10.1 ± 2.4^{BCA}	83.0 ± 6.5^{A}
4	N+C+SOC	3.23	3.70	0.47	14.6 ± 1.5^{BDC}	82.5 ± 4.3^{A}
5	N+C+SOC-F	2.99	3.35	0.36	12.0 ± 0.4^{ABCD}	82.2 ± 2.4^{A}
6	N+H	3.09	3.62	0.53	17.2 ± 0.3^{D}	91.0 ± 6.8^{A}

[a]8-10 rabbits in each group.

[b]N = chow; C = 2% cholesterol; H = 10% herd butter (from cows fed grain and hay); SOC = 10% SOC butter (from cows fed safflower oil-casein, grain, and hay); SOC-F = 10% SOC-F butter (from cows fed safflower oil-casein-formaldehyde, grain, and hay).

[c]Values bearing different superscripts differ significantly.

TABLE IV

Degree of Atheroma and Heart Weights of Rabbits on Atherogenic Diets Containing 2% Cholesterol

Group[a]	Diet[b]	Aortic surface with plaque (%)[c]	Heart weight		Mortality
			g	g/kg body weight[c]	
1	N	0^{A}	5.86 ± 0.40	1.83 ± 0.10^{A}	1/9
2	N+C	23.4 ± 5.4^{B}	5.79 ± 0.30	1.89 ± 0.07^{AB}	1/10
3	N+C+H	23.7 ± 9.0^{B}	6.40 ± 0.44	2.28 ± 0.19^{AB}	2/10
4	N+C+SOC	21.4 ± 2.1^{B}	7.47 ± 0.43	2.08 ± 0.06^{AB}	2/10
5	N+C+SOC-F	27.3 ± 6.3^{B}	7.01 ± 0.39	2.42 ± 0.10^{B}	1/10
6	N+H	0.3 ± 0.3^{A}	7.21 ± 0.29	1.99 ± 0.04^{AB}	0/10

[a]8-10 rabbits per group.

[b]N = chow; C = 2% cholesterol; H = 10% herd butter (from cows fed grain and hay); SOC = 10% SOC butter (from cows fed safflower oil-casein, grain, and hay); SOC-F = 10% SOC-F butter (from cows fed safflower oil-casein-formaldehyde, grain, and hay).

[c]Values bearing different superscripts differ significantly.

heparinized Erlenmeyer flask. Plasma was separated from the chilled blood by centrifugation. In experiment 1, the rabbits received the diet for 12 weeks and were then fasted overnight and killed under ether anesthesia by exsanguination. In experiment 2, rabbits were killed after 4, 8, and 12 weeks on the diets after overnight fasting.

The aorta, heart, liver, and samples of biceps femoris muscle and abdominal fat were quickly removed and weighed. The aorta was carefully cleared of adherent fat, sliced longitudinally, and pinned to a cork board. The area covered by a visible atherosclerotic plaque was drawn on a template grid and the extent of coverage of the aorta estimated. Grading of atherosclerosis was therefore based upon the percentage of the aortic surface covered by plaque.

Chemical Determinations

Weighed samples of tissues were immediately homogenized in chloroform-methanol and extracted by the technique of Bligh and Dyer (8). The resulting lipid extract in chloroform was stored at -20 C after addition of butylated hydroxytoluene (0.1% of lipid weight) as recommended by Johnson (9).

The fat content of tissues and diets was determined gravimetrically after ether extraction using the Goldfisch extraction apparatus.

Vitamin E was determined in plasma by the α,α'-dipyridyl reaction by the method of Quaife et al. (10). Milk vitamin E content was analyzed after chromatographic separation on alumina as described by Low and Dunkley (11). Tissue vitamin E was determined on hexane extracts of tissue after saponification in the presence of pyrogallol by the method of Bieri et al. (12).

Plasma cholesterol was determined on acetone-ethanol extracts (13) by the method of Pearson et al. (14). Free and total cholesterol in tissues were determined by the ferric chloride procedure of Zlatkis et al. (15) after digitonin isolation of the cholesterol (16).

Total plasma lipids were determined by a colorimetric method based on the sulfophosphovanillin reaction (17,18). Lipids for detailed fatty acid analysis were extracted using the

TABLE V

Liver Weights and Composition of Rabbits Fed Atherogenic Diets Containing 2% Cholesterol

Group[a]	Diet[b]	Weight		H_2O (%)[c]	Lipid (%)[c]
		g	g/kg body weight[c]		
1	N	72.0 ± 5.3	22.35 ± 1.37^{A}	72.9 ± 1.0^{A}	3.39 ± 0.13^{A}
2	N+C	97.8 ± 7.0	31.54 ± 1.20^{B}	70.1 ± 0.6^{B}	8.22 ± 0.47^{B}
3	N+C+H	112.1 ± 9.2	37.45 ± 2.54^{BC}	70.0 ± 1.6^{AB}	10.75 ± 1.14^{BCD}
4	N+C+SOC	123.0 ± 4.6	36.45 ± 1.75^{C}	67.6 ± 1.4^{B}	11.80 ± 0.77^{CD}
5	N+C+SOC-F	108.3 ± 9.0	36.59 ± 1.62^{C}	66.9 ± 0.9^{B}	11.60 ± 1.04^{D}
6	N+H	82.7 ± 3.0	22.88 ± 0.72^{A}	70.8 ± 0.6^{A}	4.54 ± 0.40^{E}

[a]8-10 rabbits per group.

[b]N = chow; C = 2% cholesterol; H = 10% herd butter (from cows fed grain and hay); SOC = 10% SOC butter (from cows fed safflower oil-casein, grain, and hay); SOC-F = 10% SOC-F butter (from cows fed safflower oil-casein-formaldehyde, grain, and hay).

[c]Values bearing different superscripts differ significantly.

Goldfisch extraction apparatus. Methyl esters were prepared by the method of Christopherson and Glass (19) and their fatty acid composition determined by programmed gas-liquid chromatography, using a column packed with 15% EGSS-X on Gas-Chrom P (100/120 mesh).

Statistical comparisons were made by using either Student's *t*-test with correction for unequal group size or by analysis of variance with mean separation by Student-Newman-Keuls test.

RESULTS

Experiment 1: Atherogenic Diet Containing 2% Cholesterol

Body weight and feed consumption (Table III): The rabbits fed the saturated herd butter without cholesterol exhibited the greatest body weight gain. There appeared to be a trend for greater weight gains in all dietary groups containing 10% added fat. The average daily feed intake of the groups fed cholesterol appeared to be less than those not receiving the sterol (groups 2-5 vs. 1 or 6), but these were not statistically significant differences.

Aortic atheroma and heart weight (Table IV): The degree of atheroma is given in Table IV. Rabbits fed chow alone (group 1) exhibited no aortic plaque, and the feeding of 10% fat alone (group 6) induced only slight plaque deposition. Feeding cholesterol with or without fat led to severe plaque deposition covering ca. 25% of the aortic surface (groups 2-5). There were no apparent differences in the severity of the lesions in rabbits receiving either the saturated or unsaturated butters. Only one rabbit in groups 2-5 had no atherosclerotic lesion, and in one rabbit almost 96% of the aortic surface was covered with plaque. Whitish lipid areas and streaks were commonly seen, and, in the more severe cases, thick, rough, nodular lipid plaques were present.

As the feeding progressed, some of the rabbits receiving both cholesterol and fat exhibited clear signs of cholesterol poisoning (20). After ca. 8 weeks, several rabbits in groups 2-5 had decreased feed consumption, cold and yellowish ears, and were lethargic.

Heart weights of the rabbits fed cholesterol and fat were larger than those of controls, but the difference was statistically significant only in group 5.

Changes in liver size and composition: Liver weights were much greater in all groups fed cholesterol and lipid (Table V). The liver enlargement ranged from 40 to 70%. Rabbits receiving only the 10% added fat (group 6) did not exhibit any liver enlargement. Many of the enlarged livers exhibited abnormal pathology. Common observations on gross examination indicated yellowish or mottled livers with granular necrotic areas.

Water and lipid changes accompanied the liver enlargement. Livers from rabbits fed cholesterol and fat showed a decrease in their water content and a concomitant increase in percentage lipid. Control rabbit livers had a lipid content of only 3%, which increased to ca. 12% in the livers from rabbits fed cholesterol and fat. On a dry weight basis, lipid comprised ca. 12% of the liver dry weight in group 1 but increased to 36% in livers from fat and cholesterol fed rabbits (groups 3, 4, and 5).

Time course of changes in plasma cholesterol, total lipids, and vitamin E: The addition of cholesterol to the diet increased plasma cholesterol ca. 10 times after 5 weeks and reached a plateau at ca. 20 times after 9 weeks on the diet (Fig. 1). Although plasma cholesterol was not increased at all by the sole addition of 10% fat to the diet, the inclusion of

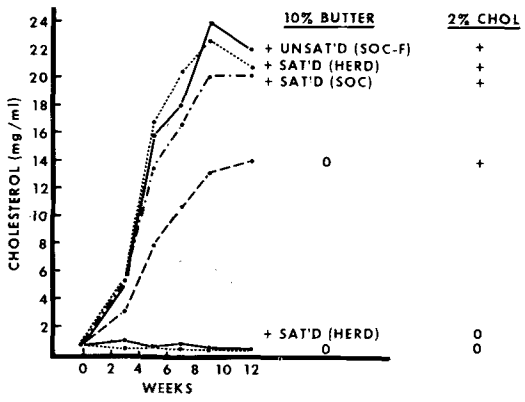

FIG. 1. Changes in plasma cholesterol of rabbits fed diets containing 2% cholesterol and/or 10% butter. HERD denotes butter from cows fed grain and hay; SOC denotes butter from cows fed safflower oil-casein, grain, and hay; and SOC-F denotes butter from cows fed safflower oil-casein-formaldehyde, grain, and hay.

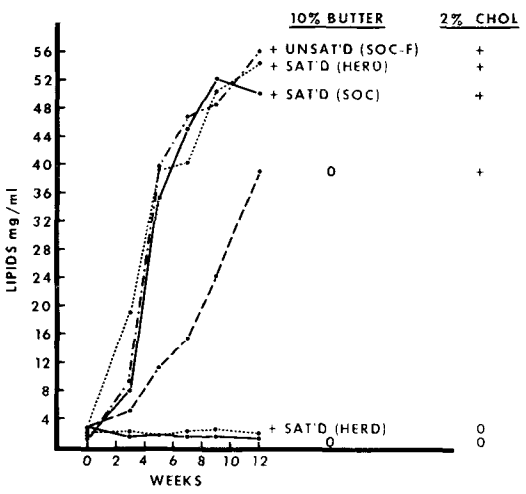

FIG. 2. Changes in plasma total lipids of rabbits fed diets containing 2% cholesterol and/or 10% butter. HERD denotes butter from cows fed grain and hay; SOC denotes butter from cows fed safflower oil-casein, grain, and hay; and SOC-F denotes butter from cows fed safflower oil-casein-formaldehyde, grain, and hay.

both fat and cholesterol produced a synergistic effect, inducing a 40 times increase in plasma cholesterol level after 9 weeks feeding. Maximal cholesterol levels were achieved after ca. 5-7 weeks on the diets. The degree of saturation or unsaturation of the butters did not influence plasma cholesterol changes.

A very similar pattern was observed in plasma lipids (Fig. 2). The addition of cholesterol to the diet induced a steady increase, but the addition of only 10% fat to the diet did not increase plasma lipids. After about 5 weeks on the diet, rabbits receiving both cholesterol and 10% fat had plasma lipids which were ca. 20 times control levels.

Plasma vitamin E also exhibited similar changes (Fig. 3). Vitamin E levels of the rabbits receiving 10% added fat increased from ca. 2 μg/ml to 3-4 μg/ml. Cholesterol in the diet increased plasma vitamin E to ca. 10 μg/ml, and the synergism of cholesterol and fat resulted in vitamin E levels of ca. 18 μg/ml after 5 weeks feeding.

The parallel increases in these three constituents suggested a strong interrelationship among these lipids. When all values for each constituent were plotted against each other, very high correlations were observed. Total lipid and plasma cholesterol gave the best correlation (r = 0.97). Vitamin E was well correlated with plasma cholesterol (r = 0.90) and plasma lipids (r = 0.81).

Tissue cholesterol (Table VI): Rabbits receiving 10% added fat with no added cholesterol (group 6) did not exhibit increased tissue cholesterol levels when compared to those on the control chow diet (group 1). As atherogenesis proceeded, the rabbits which developed aortic plaques (groups 2-5) had greatly elevated serum, aortic, heart, muscle, and liver cholesterol levels. After 12 weeks on the atherogenic diet, serum cholesterol concentrations were elevated ca. 30 times by the presence of cholesterol in the diet (group 2), and the presence of lipid (groups 3, 4, 5) acted synergistically to increase serum cholesterol still more, to ca. 40 times control levels.

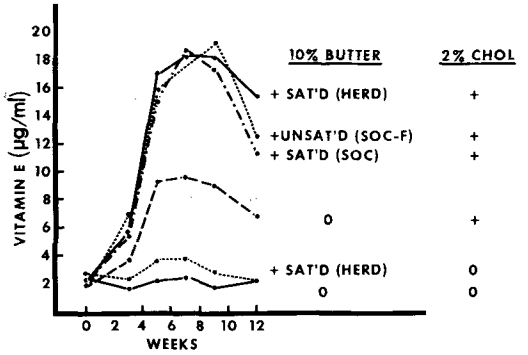

FIG. 3. Changes in plasma vitamin E of rabbits fed diets containing 2% cholesterol and/or 10% butter. HERD denotes butter from cows fed grain and hay; SOC denotes butter from cows fed safflower oil-casein, grain, and hay; and SOC-F denotes butter from cows fed safflower oil-casein-formaldehyde, grain, and hay.

The proportion of the cholesterol in the serum that is transported as ester declined

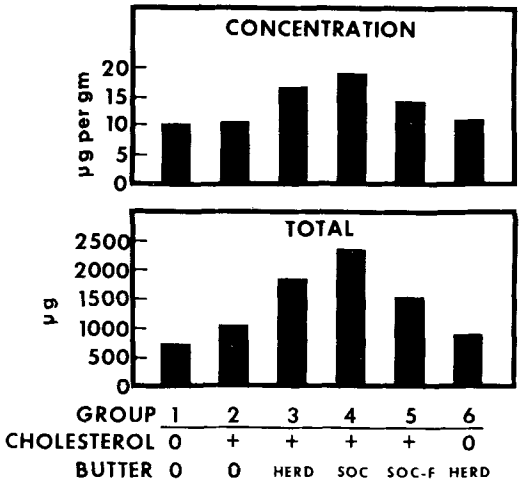

FIG. 4. Liver vitamin E concentration and content of rabbits fed diets containing 2% cholesterol and/or 10% butter. HERD denotes butter from cows fed grain and hay; SOC denotes butter from cows fed safflower oil-casein, grain, and hay; and SOC-F denotes butter from cows fed safflower oil-casein-formaldehyde, grain, and hay.

during atherogenesis. In controls (group 1) and in the rabbits receiving 10% added fat (group 6), ester cholesterol was about three-fourths of total cholesterol. The groups receiving both cholesterol and fat had serum cholesterol ester levels ranging from 57 to 66% (groups 3, 4, 5). The percentage of ester cholesterol in the rabbits receiving only cholesterol (group 2) was not significantly different from controls.

Aortic and liver cholesterol levels increased ca. 10 times, heart cholesterol increased 3-4 times, and muscle cholesterol doubled (groups 2-5). The percentage of total cholesterol ester increased markedly in rabbits with these high cholesterol levels. Thus, in control rabbits (group 1) almost all of the cholesterol in aorta, heart, and muscle is free cholesterol. In atherosclerotic rabbits (groups 2-5), ester cholesterol comprises 60, 40, and 25% of the total cholesterol of aorta, heart, and muscle, respectively. Cholesterol ester in the liver increases from ca. 20% in controls to ca. 60% in atherosclerotic rabbits (groups 2-5).

Liver vitamin E (Fig. 4): Rabbits fed cholesterol and added fat exhibited 40-90% increases in their liver tocopherol concentrations. Taking into account the liver enlargement that occurred, total liver vitamin E increased two- to threefold. The liver increase approximately paralleled the increase in liver lipids (Table V).

Experiment 2: Atherogenic Diet Containing 0.5% Cholesterol

Body weight and feed consumption (Table

TABLE VI

Cholesterol Levels in Tissues (mg/100 g fresh tissue) of Rabbits Fed Atherogenic Diets Containing 2% Cholesterol

Tissue[a]	Group: Diet:[b] 1 N	2 N+C	3 N+C+H	4 N+C+SOC	5 N+C+SOC-F	6 N+H
Serum	48 ± 6^{A}	1,484 ± 131^{B}	1,818 ± 187^{BCD}	2,161 ± 243^{C}	2,080 ± 143^{D}	63 ± 10^{A}
% Ester	73 ± 2^{AE}	71 ± 2^{ACE}	57 ± 2^{B}	66 ± 2^{CD}	60 ± 6^{ABD}	76 ± 2^{E}
Aorta	158 ± 8^{A}	1,292 ± 206^{B}	1,735 ± 633^{B}	1,255 ± 148^{B}	1,522 ± 290^{B}	212 ± 52^{A}
% Ester	5 ± .4^{A}	59 ± 8^{B}	64 ± 7^{B}	58 ± 3^{B}	54 ± 5^{B}	20 ± 6^{C}
Heart	118 ± 4^{A}	346 ± 24^{B}	418 ± 33^{BC}	413 ± 50^{BC}	523 ± 48^{C}	120 ± 11^{A}
% Ester	1 ± .6^{A}	35 ± 3^{B}	32 ± 5^{B}	33 ± 3^{B}	44 ± 3^{B}	3 ± 2^{A}
Muscle	46 ± 2^{A}	84 ± 7^{B}	97 ± 10^{B}	108 ± 25^{B}	104 ± 10^{B}	51 ± 5^{A}
% Ester	6 ± 5^{A}	19 ± 4^{AB}	26 ± 7^{B}	24 ± 6^{B}	23 ± 4^{B}	16 ± 7^{AB}
Liver	266 ± 22^{A}	2,043 ± 213^{BC}	3,102 ± 558^{B}	2,298 ± 212^{CD}	2,578 ± 253^{BD}	287 ± 37^{A}
% Ester	19 ± 7^{A}	63 ± 3^{B}	59 ± 7^{B}	57 ± 7^{B}	58 ± 3^{B}	17 ± 6^{A}

[a]Values bearing different superscripts differ significantly.

[b]N = chow; C = 2% cholesterol; H = 10% herd butter (from cows fed grain and hay); SOC = 10% SOC butter (from cows fed safflower oil-casein, grain, and hay); SOC-F = 10% SOC-F butter (from cows fed safflower oil-casein-formaldehyde, grain, and hay).

VII): The observed symptomology of cholesterol poisoning in some of the rabbits suggested that the 2% level of cholesterol in the diet was too high during the 12-week feeding period. A level of 0.5% cholesterol was, therefore, used in a second experiment, and the degree of atherosclerosis was determined after feeding for shorter periods of time. The rabbits were killed after 4, 8, or 12 weeks. There were few changes in body weight or feed consumption in any of the groups during the course of the experiment.

Aortic atheroma and heart weight (Table VIII): The feeding of cholesterol without added fat at a lower level produced much less atheroma than observed previously. Only 5-10% of the aortic surface was covered with plaque (group 8), as compared to ca. 25% with the 2% level of cholesterol (experiment 1, group 2). The presence of fat in the diet, whether saturated (group 9) or unsaturated (group 10), produced extensive atherosclerotic plaque. The degree of atheroma was as great when fat was combined with this lower cholesterol level as with the higher level of the first experiment.

Heart weights, as percentages of body weight, were not altered by cholesterol and/or fat feeding.

Changes in liver size and composition (Table IX): The addition of 0.5% cholesterol and/or fat increased liver weight ca. 35-40%. The liver enlargement was apparent after only 4 weeks of feeding. Gross liver pathology was similar to that observed when 2% cholesterol was fed.

Water and lipid changes accompanied the liver enlargement. Although the rabbits fed cholesterol alone exhibited a large increase in liver weight, increases in lipid content were small, in marked contrast to a 100% increase observed when 2% cholesterol alone was fed. Water content decreased and lipid content increased markedly in livers of rabbits fed 0.5% cholesterol and fat. There were no significant differences between rabbits fed the saturated or unsaturated butters (groups 9 and 10).

Time course of changes in plasma lipids during the development of atherogenesis: Four weeks after the addition of 0.5% cholesterol to the chow diet, plasma cholesterol increased ca. 10 times and increased still further during the next 8 weeks (Fig. 5). The inclusion of fat produced the synergistic effect noted earlier, and plasma cholesterol was elevated to ca. 30 times initial levels. There were no differences in plasma cholesterol levels in rabbits fed either saturated or unsaturated butter.

Plasma lipids increased ca. tenfold (Fig. 6) with the inclusion of 0.5% cholesterol. The rabbits receiving herd butter and cholesterol showed a similar increase, but those ingesting

TABLE VII

Body Weight and Feed Consumption of Rabbits Fed an Atherogenic Diet Containing 0.5% Cholesterol

Group[a]	Diet[b]	Body weight (kg) at week:[c] 0	4	8	12	Mean daily feed consumption (g/day)[c]
7	N	3.36 ± 0.07A	3.05 ± 0.17A	3.50 ± 0.12A	2.99 ± 0.05A	122.5 ± 3.1A
8	N+C	3.33 ± 0.05A	3.42 ± 0.14A	3.43 ± 0.12A	3.47 ± 0.05B	114.5 ± 7.4A
9	N+C+H	3.32 ± 0.04A	3.45 ± 0.13A	3.62 ± 0.66A	3.07 ± 0.57AC	117.2 ± 3.9A
10	N+C+SOC-F	3.34 ± 0.07A	3.61 ± 0.13A	3.27 ± 0.07A	3.45 ± 0.18AB	110.4 ± 7.3A

[a]Number of rabbits in each group: at weeks 0 and 4, 15; at week 8, 10; at week 12, 5.

[b]N = chow; C = 2% cholesterol; H = 10% herd butter (from cows fed grain and hay); SOC-F = 10% SOC-F butter (from cows fed safflower oil-casein-formaldehyde, grain, and hay).

[c]Values bearing different superscripts differ significantly.

TABLE VIII

Degree of Atheroma and Heart Weights of Rabbits on Atherogenic Diets[a]

Group[b]	Diet[c]	Aortic surface (%) covered by plaque at week: 4	8	12
7	N	0^{A}	0.4 ± 0.4^{A}	0^{B}
8	N+C	0^{A}	9.4 ± 6.8^{A}	4.8 ± 3.4^{B}
9	N+C+H	0^{A}	1.8 ± 0.8^{A}	28.7 ± 6.2^{A}
10	N+C+SOC-F	0^{A}	9.9 ± 6.0^{A}	28.2 ± 6.7^{A}

		Heart weights at week: 4		8		12	
		g	g/kg BW	g	g/kg BW	g	g/kg BW
7	N	5.56 ± 0.04^{A}	1.84	6.22 ± 0.29^{A}	1.78	5.52 ± 0.25^{A}	1.85
8	N+C	5.86 ± 0.10^{A}	1.72	6.54 ± 0.49^{A}	1.90	5.79 ± 0.18^{A}	1.67
9	N+C+H	6.88 ± 0.19^{B}	1.99	6.94 ± 0.24^{A}	1.92	6.77 ± 0.42^{A}	1.89
10	N+C+SOC-F	6.17 ± 0.21^{B}	1.86	5.72 ± 0.25^{A}	1.75	6.76 ± 0.26^{A}	1.97

[a]Values of dietary groups bearing different superscripts differ significantly.

[b]Each value is the mean of five rabbits.

[c]N = chow; C = 2% cholesterol; H = 10% herd butter (from cows fed grain and hay); SOC-F = 10% SOC-F butter (from cows fed safflower oil-casein-formaldehyde, grain, and hay).

unsaturated butter exhibited a significantly higher plasma level (20 times initial levels).

Plasma vitamin E exhibited changes (Fig. 7) parallel to those seen when 2% cholesterol and butter was fed (experiment 1). Vitamin E increased from 2 μg/ml to ca. 8 μg/ml with 0.5% cholesterol alone and reached levels of ca. 20 μg/ml with both butter and cholesterol in the diet.

Very high correlation coefficients were observed when all values for each of the three lipid constituents were plotted against each other. Correlation coefficients were cholesterol vs. lipids, r = 0.912; vitamin E vs. cholesterol, r = 0.961; vitamin E vs. lipids, r = 0.892.

Tissue cholesterol (Table X): Serum cholesterol levels increased 30-40 times in rabbits fed both 0.5% cholesterol and butter. These serum concentrations were as high as those attained with 2% cholesterol in the diet. The lowered dietary cholesterol, 0.5% instead of 2%, was successful in reducing the amount of the increase in the groups fed cholesterol alone. In rabbits fed only the 0.5% cholesterol, increases in serum and tissue cholesterol were only about half the concentration of rabbits receiving both cholesterol and butter. In the aorta of rabbits in the atherogenic groups, cholesterol increased only 6 times. This level was about half of the concentration when 2% cholesterol was fed, so the lower dietary load did limit the tissue increase.

In liver, the rabbits fed 0.5% cholesterol and butter had cholesterol concentrations which were very similar to levels when 2% cholesterol was fed. In the rabbits fed 0.5% cholesterol alone, however, the liver concentration was only half of the level seen when the diet contained 2% cholesterol. The lower dietary load was also successful in reducing the levels of heart and muscle cholesterol. When 2% cholesterol was fed, heart cholesterol levels increased 3-4 times to ca. 400-500 mg/100 g tissue. With 0.5% cholesterol, heart cholesterol only doubled to ca. 200 mg/100 g of tissue. And in contrast to the doubling of muscle cholesterol observed with the 2% dietary load, muscle cholesterol increased only slightly when 0.5% cholesterol and butter was fed.

Changes in ester cholesterol were similar to those observed when higher cholesterol levels were fed. Ester cholesterol increased markedly as cholesterol accumulated in the tissues. In control rabbits, almost all (85-100%) of the cholesterol was free. After 12 weeks of cholesterol feeding, 50-70% of the cholesterol in liver and aorta was ester cholesterol. In heart and muscle, 25-35% of the cholesterol was ester.

In all of these tissues, there were no significant differences in cholesterol levels in rabbits ingesting either saturated or unsaturated butter.

Liver vitamin E (Fig. 8): Liver vitamin E concentration increased when rabbits were fed 0.5% cholesterol and butter. The increases were very similar to those observed when 2% cholesterol was fed.

DISCUSSION

Our results demonstrated that there were no significant differences in the degree of atherosclerosis when either saturated or unsaturated

TABLE IX

Changes in Liver Size and Composition of Rabbits Fed Atherogenic Diets Containing 0.5% Cholesterol[a]

		Group:[b]	7	8	9	10
		Diet:[c]	N	N+C	N+C+H	N+C+SOC-F
Fresh weight (g) at week:	4		67.8 ± 5.6^{A}	96.1 ± 4.9^{A}	100.6 ± 10.8^{A}	109.7 ± 7.7^{A}
	8		71.1 ± 4.7^{B}	86.8 ± 3.1^{A}	100.9 ± 7.5^{A}	89.1 ± 4.4^{A}
	12		59.3 ± 1.7^{B}	96.6 ± 8.8^{A}	93.9 ± 8.9^{A}	101.6 ± 9.6^{A}
Weight (g/kg BW) at week:	4		22.2 ± 1.1^{A}	28.2 ± 1.3^{A}	28.9 ± 2.5^{A}	30.3 ± 1.4^{A}
	8		20.5 ± 3.7^{A}	25.4 ± 1.0^{A}	27.9 ± 2.0^{A}	27.3 ± 1.4^{A}
	12		19.8 ± 0.5^{A}	28.0 ± 2.8^{A}	26.0 ± 2.0^{A}	29.2 ± 1.7^{A}
Water (%) at week:	4		72.7 ± 0.9^{A}	69.3 ± 0.2^{B}	68.3 ± 1.0^{B}	69.0 ± 0.5^{B}
	8		72.2 ± 1.2^{A}	70.1 ± 0.8^{AB}	65.8 ± 1.3^{B}	67.5 ± 1.2^{AB}
	12		67.7 ± 1.2^{A}	68.6 ± 1.4^{A}	4.7 ± 1.3^{A}	63.5 ± 1.5^{A}
Lipid (%) at week:	4		2.96 ± 0.16^{A}	3.83 ± 0.11^{A}	6.57 ± 1.14^{A}	5.11 ± 0.34^{A}
	8		3.86 ± 0.40^{C}	5.90 ± 0.59^{BC}	10.27 ± 1.13^{A}	9.20 ± 1.32^{AB}
	12		3.60 ± 0.10^{A}	4.83 ± 0.11^{A}	9.43 ± 0.85^{B}	11.06 ± 0.90^{B}

[a]Values of dietary groups bearing different superscripts differ significantly.

[b]Each value is the mean of five rabbits.

[c]N = chow; C = 2% cholesterol; H = 10% herd butter (from cows fed grain and hay); SOC-F = 10% SOC-F butter (from cows fed safflower oil-casein-formaldehyde, grain, and hay).

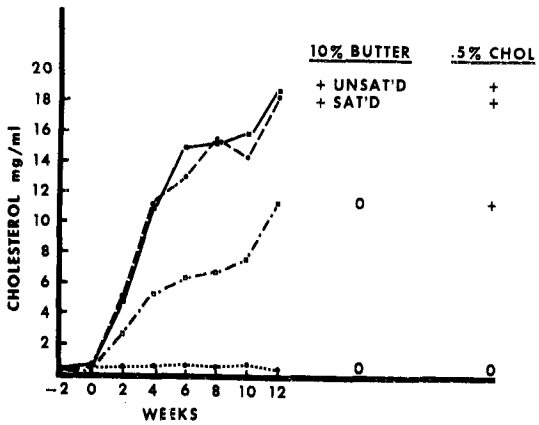

FIG. 5. Changes in plasma cholesterol of rabbits fed diets containing 0.5% cholesterol and/or 10% butter. SAT'D designates butter from cows fed grain and hay; UNSAT'D designates butter from cows fed safflower oil-casein-formaldehyde, grain, and hay.

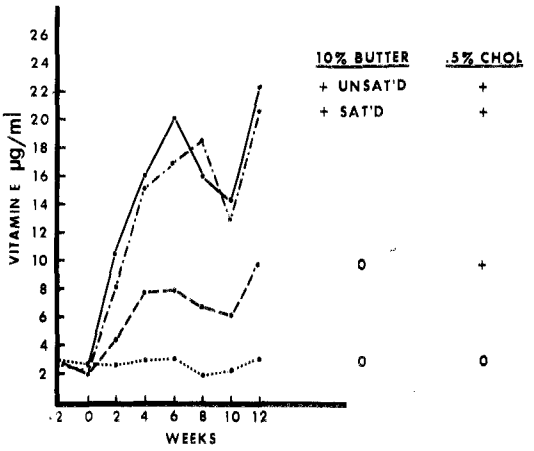

FIG. 7. Changes in plasma vitamin E of rabbits fed diets containing 0.5% cholesterol and/or 10% butter. SAT'D designates butter from cows fed grain and hay; UNSAT'D designates butter from cows fed safflower oil-casein-formaldehyde, grain, and hay.

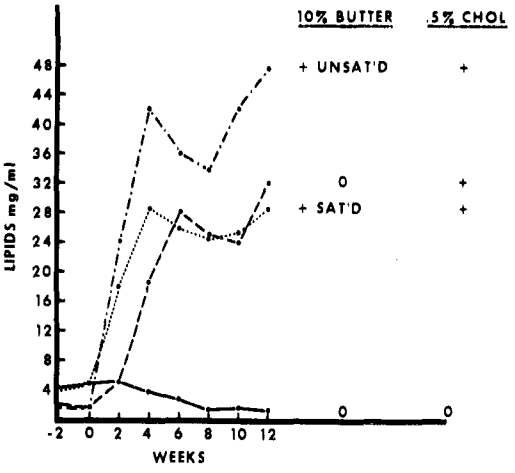

FIG. 6. Changes in plasma total lipids of rabbits fed diets containing 0.5% cholesterol and/or 10% butter. SAT'D designates butter from cows fed grain and hay; UNSAT'D designates butter from cows fed safflower oil-casein-formaldehyde, grain, and hay.

butter was fed to rabbits with cholesterol at a level of either 2% or 0.5%. This finding is in agreement with the report of Swell et al. (6), who found no differences in the degree of atherosclerosis when cholesterol diets containing corn oil (high in linoleic acid) and olive oil (low in linoleic acid) were fed. Feeding 2% cholesterol alone induced atherosclerosis to as great a degree as that produced when 0.5% cholesterol and 10% butter was fed and 25% of the aortic surface was covered with plaque. Feeding 0.5% cholesterol alone, however, induced a lesser degree of atherosclerosis, and only 5% of the aortic surface was covered with plaque.

Cholesterol feeding at the 2% level, either alone or with 10% fat, induced a 40-70% increase in liver size, and the 0.5% dietary level produced 25-40% increases. A similar large increase in liver weight (38-54%) was observed by Kritchevsky et al. (4) when 3% cholesterol, or 3% cholesterol and fat, were fed. Feeding 10% fat alone in our experiments or 9% fat alone by Kritchevsky et al. (4) did not cause any liver enlargement.

The inclusion of cholesterol in the rabbit's diet rapidly stimulated an increase in total blood lipids, cholesterol, and vitamin E. Blood cholesterol increased 20 times, blood lipids increased 10 times, and vitamin E increased 5 times. The inclusion of fat in the diet produced a synergistic effect, doubling these values to 40 times for cholesterol, 20 times for lipids, and 10 times for vitamin E, even though fat alone in the diet did not increase any of these lipid parameters. There were no differences in the increases achieved when either saturated or unsaturated fat and cholesterol was fed.

The extremely high correlations observed between these lipid constituents in the present experiment (0.81-0.97) are similar to relationships noted between these lipid constituents in several other species. Rubenstein et al. (21) found that hyperlipemia of diverse etiology in man was well correlated with elevated serum vitamin E levels. High correlations were obtained between serum vitamin E levels and various lipid fractions in patients with diabetes, hyperlipemia, and hypo- and hyperthyroidism.

TABLE X

Cholesterol Levels in Tissues (mg/100 g fresh tissue)[a]

Group[b]	Diet[c]	Week	Serum		Aorta		Liver		Heart		Muscle	
			mg/100 ml	% ester	mg/100 g	% ester	mg/100 g	% ester	mg/100 g	% ester	mg/100 g	% ester
7	N	4	65 ± 7^{C}	----	108 ± 3^{A}	0	258 ± 9^{A}	15 ± 2	123 ± 5^{A}	0	47 ± 4^{A}	7 ± 3
		8	53 ± 4^{C}	88 ± 1	124 ± 11^{A}	11 ± 6	268 ± 28^{B}	14 ± 4	97 ± 3^{A}	1 ± 1	39 ± 3^{A}	11 ± 10
		12	44 ± 9^{C}	----	94 ± 3^{A}	12 ± 5	240 ± 18^{D}	12 ± 4	85 ± 7^{A}	12 ± 5	43 ± 4^{A}	11 ± 3^{A}
8	N+C	4	530 ± 32^{B}	----	160 ± 9^{AB}	8 ± 6	594 ± 25^{A}	45 ± 3	169 ± 2^{A}	1 ± 1	50 ± 1^{A}	13 ± 5
		8	672 ± 36^{B}	82 ± 1	344 ± 75^{B}	29 ± 9	1,301 ± 214^{A}	55 ± 6	157 ± 20^{B}	17 ± 5	50 ± 3^{A}	10 ± 2
		12	1,126 ± 50^{B}	----	267 ± 46^{A}	40 ± 7	925 ± 102^{C}	62 ± 3	182 ± 5^{B}	26 ± 2	49 ± 1^{A}	9 ± 3^{A}
9	N+C+Herd	4	1,118 ± 87^{A}	----	284 ± 37^{C}	22 ± 5	1,230 ± 298^{A}	64 ± 5	248 ± 14^{B}	25 ± 3	52 ± 3^{A}	7 ± 3
		8	1,540 ± 205^{A}	84 ± 1	406 ± 31^{B}	44 ± 2	1,948 ± 273^{A}	68 ± 2	242 ± 11^{C}	33 ± 3	52 ± 3^{A}	6 ± 1
		12	1,834 ± 217^{A}	----	682 ± 69^{B}	54 ± 3	1,968 ± 205^{B}	70 ± 6	229 ± 20^{C}	29 ± 6	57 ± 3^{A}	34 ± 6^{B}
10	N+C+SOC-F	4	1,104 ± 86^{A}	----	246 ± 31^{BC}	16 ± 6	840 ± 117^{A}	57 ± 3	226 ± 14^{B}	25 ± 3	59 ± 8^{A}	7 ± 4
		8	1,516 ± 84^{A}	83 ± 1	552 ± 94^{B}	39 ± 8	1,688 ± 222^{A}	63 ± 5	208 ± 20^{BC}	30 ± 4	51 ± 4^{A}	5 ± 4
		12	1,878 ± 147^{A}	----	662 ± 39^{B}	54 ± 2	2,553 ± 218^{A}	71 ± 3	238 ± 8^{C}	35 ± 2	72 ± 7^{B}	42 ± 9^{B}

[a]Values of dietary groups bearing different superscripts differ significantly.

[b]Each value is the mean of five rabbits.

[c]N = chow; C = 2% cholesterol; Herd = 10% herd butter (from cows fed grain and hay); SOC-F = 10% SOC-F butter (from cows fed safflower oil-casein-formaldehyde, grain, and hay).

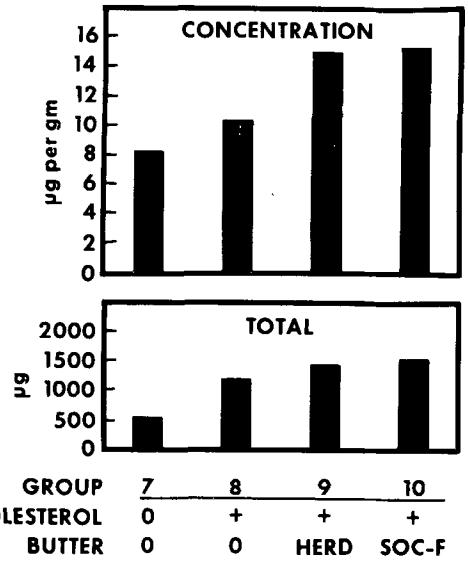

FIG. 8. Liver vitamin E concentration and content of rabbits fed diets containing 0.5% cholesterol and/or 10% butter. HERD denotes butter from cows fed grain and hay; SOC-F denotes butter from cows fed safflower oil-casein-formaldehyde, grain, and hay.

Horwitt et al. (22) have recently shown that rats fed atherogenic diets containing cholesterol and fat showed great elevations in blood lipids, vitamin E, vitamin A, cholesterol, and phospholipid after 2 or 3 weeks feeding. These investigators also studied the relationships among serum tocopherol, cholesterol, total lipids, triglycerides, and phospholipids in St. Louis University hospital patients. Total serum lipids were found to provide the highest correlation with serum tocopherol. Lewis and her co-workers (23) also found a high correlation between plasma tocopherol and cholesterol in diabetic human subjects.

An important and recurring question in atherosclerosis studies in man and animals concerns what happens to cholesterol in tissue pools when changes occur in blood cholesterol. If blood cholesterol is elevated, are tissue levels increased or has there been a shift in cholesterol out of tissues into the blood? Conversely, if blood cholesterol is lowered, is cholesterol lowered in the tissues, or is cholesterol moving out of the bloodstream and being deposited in the body tissues? In the current experiment we analyzed cholesterol in plasma, aorta, liver, heart, and skeletal muscle in an attempt to learn how changes in plasma cholesterol were reflected in tissue cholesterol levels.

We found that higher circulating levels of cholesterol caused increased tissue levels of cholesterol. When 2% cholesterol and fat were fed, liver and aorta cholesterol increased 10 times, heart 4 times, and muscle cholesterol doubled. The lower dietary load of 0.5% cholesterol was successful in limiting the amount of tissue cholesterol increase. Liver, aorta, heart, and muscle levels of cholesterol were only about half the concentration attained when 2% cholesterol was fed.

Variations in the susceptibility of different tissues to deposition of cholesterol has recently been reported in two studies by Ho et al. (24,25). The increases in tissue cholesterol we obtained agreed well, qualitatively and quantitatively, with those previously described except for muscle, which Ho et al. (24,25) found did not show any signficant change in cholesterol content. This difference may be due to difference in experimental design: only single rabbits were used, and the data of the Ho study consequently exhibited large variabilities in cholesterol levels; time periods were also different (25).

As atherogenesis proceeded, a greater proportion of the cholesterol that was deposited in the tissues was cholesterol ester. This finding is in agreement with the report of Swell et al. (6). Recently, Kritchevsky et al. (26) have reported that the enzyme cholesterol ester synthetase is elevated in the rabbit aorta after a short period of cholesterol feeding and before any atherosclerotic lesions are visible. No differences were found in the amount of ester in our rabbits fed either the saturated or unsaturated butter.

The tenfold increase in plasma vitamin E which we observed when rabbits were fed cholesterol and fat indicated that large changes in the distribution kinetics of vitamin E occurred. Since no supplemental vitamin E was fed, these large increases in vitamin E must reflect either (a) increased absorption of dietary vitamin E, (b) a redistribution of vitamin E in body tissues, or (c) a decreased destruction of vitamin E in the body. Intake of vitamin E was high in all dietary groups, being ca. 4.4 mg/day in the rabbits fed cholesterol (groups 2-5) and 10-20% more in rabbits of groups 1 and 6 (experiment 1). However, there was an increase only in those rabbits also ingesting cholesterol; thus, the high intake of vitamin E alone did not cause increases in plasma and tissue tocopherol. It is apparent that vitamin E, a fat soluble vitamin, increases when plasma lipids increase. The finding that tissue vitamin E was greatly elevated suggested that increased absorption of dietary vitamin E was the mechanism responsible for the tenfold increase in plasma vitamin and the threefold increase in liver vitamin E. Horwitt et al. (22) raised the possibility that serum tocopherol might give erroneous infor-

mation concerning the level of tocopherol in the tissues, since only ca. 1% of total body tocopherol may be in the blood. In the present experiment, plasma tocopherol and plasma cholesterol were found to accurately reflect tissue responses of these constituents.

The inability of fat alone to induce blood and tissue lipid increases in our rabbit experiments suggests that this herbivore is unable to absorb additional lipid from its diet. The addition of cholesterol resulted in large blood and tissue increases, indicating that the lipid mobilizing effect is mediated by cholesterol. Cholesterol is probably converted to bile acids and salts in the liver, which then enter the usual enterohepatic circulation. These bile salts then aid in micellar and chylomicron formation and thereby stimulate an increase in the intestinal absorption of cholesterol, lipid, and vitamin E from the diet.

There were few, if any, differences in the plasma levels of these three lipid constituents with either 0.5% or 2% cholesterol in the diet. The critical or threshold level for stimulation of lipid absorption, therefore, must lie below a dietary level of 0.5% cholesterol.

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Glycosphingolipids of Human Plasma Lipoproteins

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ABSTRACT

The content and structure of glycosphingolipids (GSL) in human plasma lipoproteins were studies. The quantitative distribution of the neutral GSL(Glc-Cer, Gal-Glc-Cer, Gal-Gal-Glc-Cer, and GalNAc-Gal-Gal-Glc-Cer) and the principal ganglioside (AcNeu-Gal-Glc-Cer) within the different lipoprotein classes was similar to that of whole plasma. The total amounts (μmol glucose/100 ml plasma) of GSL in the plasma lipoproteins of three normal subjects were VLDL (very low density lipoproteins) (trace to 0.46), LDL (low density lipoproteins) (1.08-1.48), HDL_2 (high density lipoproteins$_2$) (0.62-0.85), and HDL_3 (high density lipoproteins$_3$) (trace to 0.28). In subjects with Lp(a) lipoproteins, HDL_2 rather than HDL_3 contained most of the GSL in HDL. When the data were corrected for differences in the plasma concentrations of the lipoproteins, the total amounts of GSL(nmol glucose/mg lipoprotein cholesterol) were VLDL(trace to 21.20), LDL(11.70 - 15.36), HDL_2(8.50 - 9.10), and HDL_3(3.12). No GSL were detected in lipoprotein deficient plasma. Mass spectrometry of the trimethylsilyl derivatives of the GSL in LDL showed major fragment ions characteristic of their individual structural components. The elevated plasma levels of the GSL(2-18 fold), in a homozygote for familial hypercholesterolemia, resided in LDL which contained an absolute increase (per mg lipoprotein cholesterol) of GSL. Most, if not all, of the plasma GSL are associated with plasma lipoproteins and may have an important role in their biological functions.

INTRODUCTION

Human plasma lipoproteins are macromolecular complexes of specific lipids and proteins in relatively fixed proportions. The lipid and protein components of plasma lipoproteins have been the subject of intensive investigation. Very little, however, is known regarding the possible role of glycosphingolipids (GSL) in lipoprotein structure and metabolism. A physiologic relationship is suggested both by the hyperlipoproteinemia in patients with various GSL storage diseases (1) and the increase in plasma GSL in patients with familial hypercholesterolemia (FH) (2). This study provides information on the content and structure of the GSL of the plasma lipoproteins in normal subjects and in a patient homozygous for FH.

MATERIALS AND METHODS

Isolation and Characterization of Lipoproteins

Two units (350-522 ml) of citrated plasma were obtained by plasmaphoresis from each of three volunteers (R.S., 50 year old female; R.B and W.S., 22- and 24-year old males) who had normal concentrations of plasma lipids and lipoproteins (1). Plasma (50 ml) was prepared from D.D., a 31-year old female with homozygous FH who had total plasma and low density lipoprotein cholesterol levels of 700 and 640 mg/100 ml, respectively, xanthomas since the age of 2, and a history of hypercholesterolemia and premature ischemic heart disease on both the maternal and paternal sides of her family. Four major lipoprotein classes—VLDL (very low density lipoproteins, d<1.006), LDL (low density lipoproteins, d 1.022-1.055), HDL_2 (high density lipoproteins, d 1.063-1.12), and HDL_3 (high density lipoproteins, d 1.12-1.21)—lipoproteins of intermediate density and lipoprotein deficient plasma (LDP, d<1.21) were isolated from the plasma of these four subjects by preparative ultracentrifugation using KBr gradients (3). Each lipoprotein class and LDP were washed twice in the ultracentrifuge at their separation density and then dialyzed against three changes (4L) of 0.01 M NH_4HCO_3 containing 0.001 M EDTA at 4 C. Following immunoelectrophoresis (4), none of the lipoprotein fractions reacted against antisera to human albumin, and LDP did not react against antisera to LDL (Hyland Lab, Costa Mesa, CA.) or HDL (gift of Dr. Robert I. Levy [4]). LDL and HDL and their subfractions did not react against HDL and LDL antisera, respectively. The plasma concentrations (mg/100 ml) of Lp(a) lipoproteins were 9.3 and 16.6 in R.S. and W.S., respectively; none (<1.5) were detected in R.B. (kindly performed by Dr. John Albers [5]).

Isolation of Lp(a) Lipoproteins from HDL_2

HDL_2 lipoproteins (57.6 mg protein) from

W.S. were applied to a column (2.5 x 100 cm) of Sepharose 2B at 4 C (6). Fractions containing Lp(a) lipoproteins (4 mg protein) and Lp(a) deficient HDL_2 (45 mg protein) were eluted (6), pooled, dialyzed as described above, and the Lp(a) lipoprotein concentrations determined (5). Recovery of protein was 85%.

Isolation of Glycosphingolipids from Lipoproteins

Lipids were extracted from lyophilized whole plasma and lipoproteins in an Erlenmeyer flask (20 vol of chloroform:methanol [2:1, v/v] per 100 mg of protein) at 25 C. The extracts were filtered and the nonlipid residue reextracted (7). The pooled extracts were flash evaporated in vacuo, resuspended in 20 vol of chloroform:methanol (2:1, v/v), and partitioned into upper and lower phases as modified after Folch et al. (7,8). The upper phases were dried in vacuo, taken up in distilled water, and dialyzed against water for 48 hr at 4 C. The dialyzate was dried, resuspended in chloroform-methanol (2:1, v/v), and saved. The lower phase was dried under nitrogen, resuspended in chloroform (0.5 ml), and applied to a silicic acid column (1 cm x 22 cm) from which the neutral lipids, GSL, and phospholipids were eluted (9). Ganglioside and neutral GSL standards from human sources and aliquots of the total upper phase and neutral GSL containing fraction from the silicic acid column were studied by thin layer chromatography (TLC) on Silica Gel G plates (Analtech, Newark, DE). The solvent system for separating upper phase lipids was chloroform:methanol:ammonium hydroxide: water (60:35:1:7, v/v) (10). The plates were developed and dried in air. Gangliosides and the neutral GSL were identified by spraying the chromatograms with a resorcinol and aniline diphenylamine reagent respectively (10) and heating at 130 C for 10 min in an incubator. Following alkali-catalyzed methanolysis and neutralization (11), preparative TLC of the remainder of the upper phase and GSL fraction from the silicic acid column were performed as described above. The individual GSL were identified by spraying with methanol:water (1:1, v/v). Areas corresponding to standard GSL were scraped, eluted, and dried (12).

Gas-Liquid Chromatography (GLC) of Glycosphingolipids

Derivatives of the purified GSL were prepared for GLC as previously described (10,11). The 0-trimethylsilyl (0-TMSi) derivatives of methyl glycosides were separated on a glass column (2 m x 3 mm) containing 3% SP-2100 on 100/200 Supelcoport (Supelco, Inc., Bellefonte, PA) by temperature programming from 150 to 230 C at 3C/min. An F and M Hewlett Packard 400 gas-chromatograph with flame ionization detectors was used. Flash heater and detector temperatures were maintained at 275 C, and the carrier gas was nitrogen at a flow rate of 30 ml/min.

GLC of Fatty Acid Methyl Esters and Spingosines

Fatty acid methyl esters were converted to their TMSi derivatives to distinguish hydroxy fatty acids from nonhydroxy fatty acids (13) and analyzed by GLC as described above, except that the temperature programming was conducted from 120 to 250 C at the rate of 3 C/min. The sphingosine content in neutralized methanolysates was determined by the fluorometric methods of Naoi et al. (14) and by GLC at 210 C after re-N-acetylation and TMSI derivatization.

Mass Spectrometric Analysis of Glycosphingolipids

TMSi derivatives of GSL from LDL were analyzed by mass spectrometry (10,15). The sample inlet was Varian aerograph 2700 gas chromatograph attached to a Dupont model 21-491 mass spectrometer with a Bell and Howell datagraph model 5-134. Relative intensities of the major ions in mass spectra of these compounds were calculated manually from the datagraph sheets.

Other Methods

The concentrations of total cholesterol and triglycerides and plasma lipoproteins were determined by the ferric chloride and fluorometric techniques in a Technicon AA-I (16). Protein content was determined by the method of Lowry (17), using crystalline bovine albumin as a standard.

RESULTS AND DISCUSSION[1]

The distribution of the plasma GSL on the plasma lipoproteins and LDP was first studied by TLC. The GSL patterns from the three normal subjects had several common features. All the neutral GSL from plasma or plasma lipoproteins migrated as double bands. No GSL were detected in LDP. Finally, GM_3 was the predominant ganglioside with trace amounts of

[1] Abbreviations: G_{M3} = [AcNeu-α-(2→3)]-Gal-β-(1→4)-Glc-Cer; G_{M2} = GalNAc-β-(1→4)Gal-[AcNeu-α-(2→3)]-β-(1→4)-Glc-Cer; GD_{1a}=[AcNeu-α-(2→3)]-Gal-α-(1→3)-GalNAc-β-(1→4)Gal-[AcNeu-α-(2→3)]-β-(1→4)-Glc-Cer; GL-1a=Glc-β-(1→1)-Cer; GL-1b=Gal-β-(1→1)-Cer; GL-2a=Gal-β-(1→4)-Glc-Cer; GL-3=Gal-α-(1→4)-Gal-β-(1→4)-Glc-Cer; GL-4 = GalNAc-β-(1→4)-Gal-α-(1→4)-Gal-β-(1→4)-Glc-Cer.

GD_{1a}. Similar patterns were found in R.S. and W.S.; most of the GSL was in LDL and HDL_2, with only trace amounts in HDL_3 and the lipoproteins of intermediate density. R.B., however, contained easily detectable amounts of GSL on HDL_3 as well as on LDL and HDL_2. The trace (R.S.) to easily detectable (W.S., R.B.) amounts of GSL on VLDL were most likely related to individual differences since approximately equal amounts of lipid extracts from VLDL were studied by TLC.

The individual GSL were next isolated, partially characterized, and quantified (see Methods). The observed molar ratios (glc:gal: sphingosine) for GL-1a (1.00:0.09:0.89), GL-2a(1.00:1.14:0.89), GL-3 (1.00:1.76:0.91), GL-4 (1.00:1.80:0.92), and GM_3 (1.00:1.14:0.9) were close to those predicted (18). The ratio of glc to galNAc in GL-4 was 1.00:0.85; glc to AcNeu in GM_3 was 1.00:0.50.

The amounts of total plasma GSL and the relative proportions of GL-1a, GL-2a, GL-3, GL-4, and GM_3 (Table I) were in good agreement with those previously published (18,19). The total amounts (μmol glucose) of GSL in 100 ml plasma (Table I) in the plasma lipoproteins of normal subjects were VLDL (trace to 0.46), LDL (1.08 to 1.48), HDL_2 (0.62 to 0.85), and HDL_3 (trace to 0.28). The quantitative distribution of neutral GSL and GM_3 within the different lipoprotein classes was similar to that of whole plasma. When the data were corrected for differences in the plasma concentrations of the lipoproteins (Table I), the total amounts of GSL (nmol glucose/mg lipoprotein cholesterol) were VLDL (trace to 21.20) LDL (11.70 to 15.36), HDL_2 (8.50 to 9.10), and HDL_3 (3.12). The majority of the plasma GSL therefore was found on LDL, the plasma lipoprotein which is also richest in cholesterol (ca. 50% by wt compared to VLDL [15%] and HDL [20%]) (20).

The enrichment of GSL in HDL_2 might be attributed to the presence of the Lp(a) lipoproteins (d 1.060-1.090), which are isolated with HDL_2 rather than HDL_3. The GSL content in the HDL_2 of R.B., in whom no Lp(a) is detectable, indicates, however, that the presence of GSL in HDL_2 is not simply related to contributions from the Lp(a) lipoproteins. We successfully removed 92% of the Lp(a) from the HDL_2 of W.S. by gel chromatography. The Lp(a) deficient HDL_2 still contained most of the GSL and only immunochemically detectable amounts of Lp(a). Only trace amounts of GSL were present on the small amounts of isolated Lp(a) lipoproteins, but using the data of Simons and co-workers (6) on the GSL content of Lp(a) and the relative amounts of Lp(a) in the HDL_2 of W.S. (ca. 7.5% by protein), the Lp(a) most likely did not contribute any more than 10-20% of the GSL on HDL_2. The significant differences in the content of GSL in HDL_3 were not due to the sex of the donors or the amounts of their HDL_3 studied. The reason why only trace amounts of GSL were found on the HDL_3 of R.S. and W.S., compared to the measureable amounts on the HDL_3 of R.B., is not presently understood and requires further investigation.

The GSL in whole plasma from the patient (D.D.) with homozygous FH were 2-18 fold higher than those in normal subjects (Table I), and increase even more marked than that previously reported in heterozygotes (2). The abnormally high plasma GSL concentrations primarily resided in LDL, which contained an absolute (49.03 nmol glucose/mg lipoprotein cholesterol) as well as a relative (16.55 μmol glucose/100 ml plasma) increase in GSL (Table I). The LDL from this patient also had an increased ratio of cholesterol to protein (4.1), compared to the normal controls (mean 1.6). These data suggest that the increased plasma GSL in FH is not secondary to hypercholesterolemia and increased LDL but may be part of the phenotypic presentation of FH.

The precise structures of the GSL were not determined. More detailed structural studies, however, were performed by GLC and combined GLC-mass spectrometry on the individual GSL isolated from LDL of R.B. Mass spectrometry of their TMSi derivatives showed major fragment ions characteristic of their individual structural components (10,15). Sphingosine existed primarily as C-18 and C-20 sphingosine with trace amounts of phytosphingosine and had intense ions at m/e 299, 311, and 339; its predominant fatty acids were C_{16}, C_{18}, and C_{24}, with smaller amounts of other fatty acids in the C_{16}-C_{26} range. A major ion characteristic of monosaccarides was observed at m/e 204 for $[TMSiOCH - CHOTMSi]^+$. The predominant fragment ions at m/e 173 typical of N-acetamido sugars were seen in GL-4. GM_3 had peaks at m/e 186 and m/e 173 indicative of the presence of N-acetyl rather than N-glycoyl neuraminic acid. The GL-1a and GL_3 components of LDL contained $C_{22:0}$ fatty acids, which were absent in plasma and HDL_2; only GL-1a of LDL contained $C_{22:1}$. No detectable levels of hydroxy fatty acids were found in the GSL derived from LDL, an observation similar to that previously made on plasma GSL (19).

This study demonstrates that most, if not all, of the plasma GSL are associated with plasma lipoproteins. The similar composition of GSL and their constituent fatty acids in plasma (18,19), several blood cells (21), and lipoproteins suggests that the plasma GSL may undergo exchange and modification as they enter a

TABLE I

Glycosphingolipid (GSL) Content[a] of Human Plasma and Plasma Lipoproteins Derived from Three Normal Subjects and One Patient with Homozygous Familial Hypercholesterolemia (FH)[b]

	Normal female (R.S.)					Normal male (R.B.)					Normal male[d] (W.S.)			Homozygous FH[e] (D.D.)	
GSL[c]	Whole Plasma	VLDL	LDL	HDL_2	HDL_3	Whole Plasma	VLDL	LDL	HDL_2	HDL_3	Whole Plasma	VLDL	LDL	Whole Plasma	LDL
Gl-1a	4.77 (0.73)	Tr[f]	4.60 (0.35)	2.70 (0.20)	Tr	6.90 (0.90)	5.20 (0.10)	4.20 (0.45)	2.2 (0.25)	0.55 (0.05)	5.23 (0.80)	6.48 (0.14)	5.19 (0.50)	9.12 (6.00)	23.30 (7.87)
GL-2a	4.44 (0.68)	Tr	3.81 (0.29)	2.90 (0.21)	Tr	5.5 (0.72)	5.2 (0.10)	2.8 (0.30)	2.2 (0.20)	0.55 (0.05)	3.20 (0.49)	1.39 (0.03)	2.49 (0.24)	3.37 (1.14)	4.99 (1.68)
GL-3	1.96 (0.30)	Tr	2.71 (0.21)	0.55 (0.04)	Tr	3.80 (0.50)	2.60 (0.05)	2.30 (0.25)	1.80 (0.15)	1.10 (0.10)	2.75 (0.42)	2.31 (0.05)	2.90 (0.28)	7.0 (4.5)	9.29 (3.14)
GL-4	1.96 (0.30	Tr	1.30 (0.10)	1.38 (0.10)	Tr	3.00 (0.40)	2.60 (0.05)	1.40 (0.15)	1.80 (0.15)	0.55 (0.05)	3.59 (0.55)	8.33 (.18)	3.22 (0.31)	6.0 (3.9)	4.65 (1.57)
GM_3	1.76 (0.27)	Tr	1.70 (0.13)	0.97 (0.07)	Tr	2.60 (0.35)	1.40 (0.02)	1.00 (0.10)	1.10 (0.10)	0.37 (0.03)	2.61 (0.40)	2.78 (0.06)	1.56 (0.15)	7.6 (4.9)	6.80 (2.29)

[a]The data are expressed as nmol glucose/mg of lipoprotein cholesterol and within brackets as μmol glucose/100 ml plasma.

[b]VLDL = very low density lipoproteins (d<1.006); LDL = low density lipoproteins (d 1.022-1.055); HDL_2 = high density lipoproteins (d 1.063-1.12); HDL_3 = high density lipoproteins (d 1.12-1.21).

[c]Different structural moieties of GSL (see Footnote 1 in Results and Discussion).

[d]The content of GSL was not determined on HDL_2 and HDL_3 from W.S.

[e]No GSL were detected in VLDL, HDL_2, or HDL_3 from D.D.

[f]Tr = trace amounts.

particular blood cell type, the plasma pool, or its lipoproteins (22). The functional role of GSL as lipoprotein constituents remains to be established. The results from the patient with homozygous FH suggest that the association of GSL with LDL may be specific.

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Site of Bile Acid Absorption in the Rat

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ABSTRACT

Bile acid absorption was measured in the small intestine of the rat using ^{91}Y as a nonabsorbed reference substance. Some 50% of the secreted bile acids were absorbed in the proximal half of the small intestine. In situ incubations of ligated intestinal segments into which tauro(^{14}C-carbonyl)cholic acid was introduced confirmed the considerable uptake of bile acids in the jejunum. The in situ experiments indicated that serosal transport is the limiting stage of bile acid absorption in the jejunum but not in the ileum. Increasing bile acid concentrations in the in situ experiments did not affect the percentage disappearance of dose from the jejunum but reduced the percentage mucosal uptake in the ileum. It is concluded that, in the rat, the proximal small intestine is as important in the absorption of bile acids as the distal small intestine.

INTRODUCTION

Over 90% of the bile salts secreted into the small intestine are reabsorbed both in mammalian and avian species (1). On the basis of experiments involving the everted sac technique (2,3), the ileum has been implicated as the major site of bile acid absorption in the rat and in other species (4). This conclusion was further supported by experiments in which test solutions of bile acids were introduced into the intestine in situ (5,6). It was further shown that active transport of bile acids occurred only in the ileum, whereas passive diffusion was observed at all levels of the intestine (7). However, in vitro tests and measurements in vivo in isolated perfused intestinal segments do not provide information on the actual contribution of passive diffusion to total bile acid absorption under physiological conditions in intact animals (1).

The results of recent in situ experiments in human volunteers (8) have suggested that the jejunum may play a more important role in bile acid absorption than hitherto believed. Recent results from the authors' laboratory have shown that a considerable part of the bile acids secreted into the duodenum of chicks and hens is reabsorbed in the jejunum (9-12). These results were obtained in intact birds using ^{91}Y as a nonabsorbed reference substance.

The purpose of the present study was to reexamine the site and extent of absorption of bile acids in the rat small intestine. The nonabsorbed reference substance technique has been used to estimate the in vivo bile acid absorption. Independently, uptake and transfer of bile acids from ligated intestinal segments were measured in situ.

MATERIALS AND METHODS

Trials 1 and 2

Two experiments, using ^{91}Y as a nonabsorbable reference substance, were carried out with CR male rats. The animals were kept on a standard rat diet until they reached an average weight of 195 g in trial 1 and 155 g in trial 2. They were then given the experimental diets which had the following compositions (%):

Trial 1. Heated defatted soybean meal, 50.0; glucose monohydrate, 38.3; soybean oil, 4.0; cellulose, 4.0; methionine, 0.15; vitamin mixture (11), 0.2; mineral mixture (11), 0.1; choline chloride (50%), 0.2; dicalcium phosphate, 2.0; calcium carbonate, 1.0.

Trial 2. Heated defatted soybean meal, 35.0; fish meal, 7.0; glucose monohydrate, 47.5; soybean oil, 3.0; cellulose, 4.0; vitamin mixture (11), 0.2; mineral mixture (11), 0.1; choline chloride (50%), 0.2; dicalcium phosphate, 2.0; calcium carbonate, 1.0.

$^{91}YCl_3$ was added to both diets in amounts of 25-50 μCi/kg.

At the end of 4 days, the rats were killed with an overdose of sodium pentobarbital. The intestines were removed after the bile duct had been ligated. The small intestine was divided into five segments of equal length (15-20 cm), and the contents of each segment were gently extruded. The proximal two segments were further rinsed with cold 0.9% NaCl.

The intestinal contents were analyzed for ^{91}Y as described previously (9) and for bile acids as described by Singer and Fitschen (13). The latter technique involves precipitation of the bile acids as Ca salts, followed by deconjugation with sulfuric-acetic acid and spectrophotometric measurements at wavelengths specific for dihydroxy- and trihydroxycholanic acids. The method was calibrated with cholic

and chenedeoxycholic acids, and results were calculated as total di- + trihydroxycholanic acids.

The ratio of bile acids to ^{91}Y was calculated for the contents of each intestinal segment. This ratio, multiplied by the daily ^{91}Y intake, yielded the rate of transit of the bile acids through that particular segment and was expressed in mg/24 hr. The decrease in rate of transit between two consecutive segments represents the daily net amount of bile acids absorbed between these two segments. In the absence of exogenous bile acids, the calculated rate of transit through the first segment is taken to represent the net secretion of bile acids into this segment.

Trials 3 and 4

In these trials, mucosal and serosal transport of labeled taurocholic acid was measured in intestinal loops in situ. CR male rats weighing 120-200 g were anesthetized with sodium pentobarbital. The upper jejunum and lower ileum were exposed, and loops were formed as described previously (14). The distance of the loops from the pylorus, as measured at the end of the experiments, was 10-14 cm for the jejunal loops and 79-82 cm for the ileal loops. The length of both kinds of loop was 12-14 cm. The loops with their intact vascular and nervous systems were flushed with 0.9% NaCl at 37 C and loaded with 0.015 μCi tauro(^{14}C-carbonyl)cholic acid in 0.5 ml test solution.

Two kinds of test solution were employed. In trial 3, the test solution was the supernatant obtained after centrifugation of 2,000 g for 10 min of homogenates of intestinal contents taken from the corresponding intestinal sites of other rats, the homogenization being carried out with an equal amount of cold 0.9% NaCl. In trial 4, the test solutions were synthetic lipid-bile acid mixtures dispersed in bicarbonate buffer. The compositions of the test media are given in the corresponding tables in the Results section.

At the end of the incubation periods, the rats were killed with an overdose of sodium pentobarbital, and the contents of the intestinal loops were flushed out with cold 0.9% NaCl. The remaning intestinal tissue was homogenized. Aliquots both of the contents of the intestinal loops and of homogenized tissue were lyophilized, digested with Soluene (Packard Instrument Co., Downers Grove, IL), and counted in Instagel (Packard). Quench correction was applied using internal standards.

Mucosal uptake was defined as the amount (in mg or "absolute mucosal uptake," or as percentage of dose, i.e., "percentage mucosal uptake") of taurocholic acid disappearing from the intestinal lumen. Serosal transfer was taken to be the difference between mucosal uptake and the amount of taurocholic acid remaining in the intestinal wall at the end of the incubation and was expressed as "absolute serosal transfer" (in mg) or as percentage of dose ("percentage serosal transfer"). Statistical analyses were made according to Snedecor and Cochran (15).

RESULTS

Results of trials 1 and 2, in which ^{91}Y was used as a nonabsorbed reference substance, show that the secretion of bile acids into the duodenum was of the order of 9 g/kg body weight/24 hr in both trials (Table I). Ca. 50% of the bile acids found in the duodenum were absorbed in the proximal half of the small intestine, whereas the absorption of bile acids by the entire small intestine amounted to ca. 90%.

The results of the in situ incubations with intestinal supernatant are presented in Table II. Although the percentage of mucosal uptake was greater in the ileal as compared to the jejunal loop, the absolute mucosal uptake of bile acids was greater in the jejunal loop, probably owing to the higher concentration of bile acids in the jejunal supernatant. Serosal transfer was lower in the jejunal loop than in the ileum whether expressed as absolute or percentage transfer.

The results of in situ incubations with the synthetic lipid-taurocholic acid substrate at two concentrations of bile acids are presented in Table III. The percentage mucosal uptake in the jejunum remained constant at 28% at both concentrations of bile acids (4 mg/ml or 8 mg/ml). Percentage serosal transfer also remained constant at the two concentrations of bile acids tested in the jejunum.

In the ileal segment, doubling the bile acid concentration caused an increase in the absolute mucosal uptake and serosal transfer; but, in contrast to the situation prevailing in the jejunum, the percentage uptake was decreased.

The results of Tables II and III further reveal that, in both in situ trials, the proportion of serosal transfer to mucosal uptake was greater in the ileum (70-95%) than in the jejunal segment (40-50%).

DISCUSSION

When adding ^{91}Y to the feed as a nonabsorbed reference substance to measure bile acid absorption along the intestine, it is assumed that the rates of transit of bile acids and ^{91}Y are similar. In addition, the division of

TABLE I

In Vivo Absorption of Bile Acids

	Trial 1			Trial 2		
		Absorption[a]			Absorption[a]	
Segment	Rate of transit (mg/24 hr)	(mg/24 hr)	Amount secreted into segment 1 (%)	Rate of transit (mg/24 hr)	(mg/24 hr)	Amount secreted into segment 1 (%)
1	1,887 ± 382[b]	621	32.9	1,335 ± 145[c]	369	27.7
2	1,266 ± 160	406	21.6	966 ± 72	235	17.6
3	860 ± 60	311	16.5	731 ± 97	262	19.7
4	549 ± 107	336	18.3	469 ± 31	328	24.5
5	203 ± 48			141 ± 28		
Entire length		1,674	89.3		1,194	89.5

[a]Decrease in transit rate between two successive segments. Transit rate of bile acids through segment 1 represents net secretion.

[b]Mean ± SE for five rats weighing on the average 195 g at the beginning of the experiment.

[c]Mean ± SE for four rats weighing on the average 155 g at the beginning of the experiment.

TABLE II

In Situ Absorption Experiments with Intestinal Supernatant (Trial 3)[a]

Type of loop	Mucosal uptake		Serosal transfer	
	mg	% of dose	mg	% of dose
Jejunal	1.15 ± 0.10[b]	66.1 ± 6.0	0.59 ± 0.04	34.8 ± 3.5
Ileal	0.78 ± 0.03	92.3 ± 3.7	0.74 ± 0.06	87.5 ± 6.4

[a]The loops were emptied by flushing with 0.9% NaCl at 37 C and were then loaded with 0.5 ml intestinal supernatant having the following composition (mg/ml): *in the jejunum*, triglycerides 0.2, diglycerides 0.04, monoglycerides 0.06, free fatty acids 0.5, total di + trihydroxycholanic acids 3.3, pH 6.6; *in the ileum*, free fatty acids 0.2, total di + trihydroxycholanic acids 1.6, pH 7.0. To each 0.5 ml test medium 0.015 μCi tauro(^{14}C-carbonyl)cholic acid was added. Incubation time was 30 min.

[b]Mean ± SE from six animals.

TABLE III

In Situ Absorption Experiments with Synthetic Lipid Mixture (Trial 4)[a]

Type of loop	Taurocholic acid (mg/ml)	Mucosal uptake		Serosal transfer	
		mg	% of dose	mg	% of dose
Jejunal	4	1.14 ± 0.15[b]	28.5 ± 1.8	0.46 ± 0.10	11.7 ± 2.8
	8	2.23 ± 0.17	27.9 ± 2.3	0.88 ± 0.11	11.0 ± 1.9
Ileal	2	1.78 ± 0.11	72.7 ± 4.3	1.23 ± 0.04	61.3 ± 2.0
	4	2.50 ± 0.10	62.5 ± 2.5	1.78 ± 0.12	44.5 ± 5.6

[a]The loops were loaded with a mixture of lipids and taurocholic acids dispersed in bicarbonate buffer. The composition of the test media was (mg/ml): *in the jejunum*, triolein 3.0, diolein 0.3, monolein 0.5, oleic acid 1.7, lecithin 0.5, taurocholic acid 4.0 or 8.0, pH 6.5; *in the ileum*, oleic acid 3.0, taurocholic acid 2.0 or 4.0, pH 7.0. To each 0.5 ml test medium, 0.015 μCi tauro(^{14}C-carbonyl)cholic acid was added. Incubation time was 15 min.

[b] Mean ± SE from six animals.

the intestine into segments, as done here, measures only net secretion or absorption of bile acids and thus results in an underestimate of bile acid secretion into the duodenum and in an overestimate of ileal excretion. With these reservations in mind, secretion of bile acids into the duodenum was estimated to be close to 9 g/day/kg body weight. These values are of a magnitude similar to those previously reported in chicks and hens (5-11 g/day/kg body weight). Most of the values for total daily bile salt secretion reported in the literature using cannula techniques are of the order of 1-2 g/day/kg body weight (16-18). A secretion rate of cholic acid of as high as 4.5 g/day/kg body weight was reported by Light et al. (19). However, the results in surgically modified animals may not be directly comparable to those of intact animals.

The results obtained with the ^{91}Y technique indicate a considerable disappearance (90%) of bile acids in the small intestine. The jejunum (upper 60 cm of the small intestine) and the ileum contributed ca. 50% each to the total bile acid absorption. The magnitude of the absorption of bile acids in the proximal small intestine was confirmed independently by in situ incubation experiments involving intestinal loops. Using either the supernatant fraction of intestinal contents or a synthetic lipid-taurocholic acid mixture, considerable disappearance of bile acids from the jejunal lumen was observed.

Both the in vivo and the in situ experiments reported here support the previous findings with chickens (9-12) and man (8) indicating a significant contribution to bile acid absorption by the proximal small intestine.

Dietschy (2), after introducing a single dose of a liquid diet containing labeled bile acids and inulin into the stomach of rats, observed some 30% absorption in the proximal small bowel after 6 hr. The experimental circumstances were obviously very different from the approximately steady-state prevailing under normal feeding conditions.

Conflicting reports are found in the literature regarding the absorption of bile acids from ligated intestinal segments in situ. Weiner and Lack (6) and Sullivan (5) reported little or no uptake in the jejunum, whereas Webling (20) and Tidball (21) found considerable absorption

in this segment. All the above authors measured the uptake of bile acids from buffered solutions containing no lipids.

A critical discussion of the numerous physical-chemical factors bearing on the rate of absorption of bile acids has been published by Dietschy (2), who also showed that, in the presence of phospholipid in relatively large amount, the rate of passive mucosal-to-serosal flux of taurocholic acid across the rat jejunum is decreased, presumably due to micelle expansion. In the present in situ trials, an attempt was made to duplicate the composition of the lipid digestion products found in the upper and lower small bowel of the rat, and under these conditions the contribution of the jejunum to total bile acid absorption appeared to be considerable, in confirmation of the results obtained with the ^{91}Y technique in intact rats.

An additional point revealed by these trials concerns the observation that an increase in intraluminal bile acid concentration in ligated loops did not affect the percentage absorption of bile acids in the jejunum (while increasing the absolute amount transferred), whereas the ileum appeared to exhibit saturation characteristics. Despite the fact that conditions were not "steady-state," the greater accumulation of bile acids in the jejunal mucosa, as compared to the ileal segment, suggests that serosal transfer may be the rate-limiting step of bile acid absorption in the jejunum, but not in the ileum.

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Chemiluminescent Autoxidation of Linolenic Acid Films on Silica Gel

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ABSTRACT

Films of linolenic acid supported on chromatographic type silica gel undergo autoxidation at a rate which is slow compared to that in homogeneous phase at low surface coverage but comparable to it at coverages approximating a close-packed monolayer. The new observation reported here is that these surface phase oxidations are chemiluminescent. The quantum yield is ca. 10^{-12} quanta per molecule of fatty acid reacted for a close-packed monolayer and remains approximately constant with decreasing coverage. The emission intensity is temperature dependent, with an apparent activation energy of 10 kcal mol^{-1}, and shows an induction period for close-packed monolayers. The emission spectrum contains a component consistent with excited state ketone emission and a low energy component, ca. 600+ nm, which could be due to singlet oxygen emission. The existence of the emission means that surface autoxidation reactions can be followed in place; the nature of the emission may provide insight into the autoxidation mechanism beyond that which may be inferred from conventional rate measurements.

INTRODUCTION

There has been some interest in the autoxidation (defined as the slow, i.e., flameless, oxidation of an organic compound by oxygen [1]) of unsaturated fatty acids and related substances supported on adsorbents such as silica gel or other supports (2-7). Our observation (7) was that the autoxidation of polyunsaturated fatty acid esters is considerably slower when thinly spread on silica gel than when present as a relatively thick layer on glass (5) (as measured by the rate of disappearance of starting compound).

Homogeneous olefin autoxidation is well established as involving a radical chain mechanism (8-11), and the slowness of the surface reaction was considered as possibly due to inhibition of the chain carrying steps (7). It should be noted that in subsequent studies (12) increased oxidation rates were observed at surface loadings approaching that of a condensed monolayer.

Weak luminescence during the oxidation of solutions of organic compounds is well known (13-15). More specifically, such emission occurs during hydrocarbon autoxidation generally (16-18) and, in particular, has been reported for *n*-butyl oleate and linoleic acid (19,20) and for methyl oleate (21) (in solution, or neat). Emission also has been reported to accompany the formation of lipid hydroperoxides (22,23). While not well characterized, this type of emission is thought to derive from excited state carbonyl species formed by reaction of radical intermediates with oxygen (17,24). We can find no previous report of autoxidation chemiluminescence of surface adsorbed species.

It might be thought that emission at an interface would be quenched as a result of enhanced radiationless deactivation in the asymmetric field of intermolecular forces. This is not necessarily the case. Many instances of fluorescence of adsorbed molecules are known (25,26), including a coordination compound such as $Ru(bipyridine)_3^{2+}$ adsorbed on silica gel (A.W. Adamson, unpublished results; 27,28). Some instances of chemisorptive chemiluminescence on Si (29), and Al or Mg surfaces (30) have also been reported.

In summary, it appeared to be at least possible that autoxidation of adsorbed unsaturated fatty acids or their esters would be accompanied by detectable emission. The initial experiments, reported here, were carried out with the same type of silica gel as was used previously (7) but with linolenic acid as the unsaturated fatty acid.

EXPERIMENTAL PROCEDURES

Materials

Pentane, hexane, heptane, diethyl ether, methanol, and isopropyl ether were of reagent grade and were used without further purification. Likewise, the silica gel (J.T. Baker 3405, lot 28074) and the alumina were of reagent quality. Diisopropyl ether (MSC PX 1890) was run through a column of chromatographic alumina before use. The palmitic, arachidic, and linolenic acids were >99% pure (Nu-Chek

Prep., Elysian, MN) and were used directly.

Procedures

Samples of fatty acid coated silica gel were prepared in the same manner as in the previous work with esters (5). Silica gel was weighed into a 125 ml stoppered Erlenmeyer flask. A weighed mixture of linolenic acid and a saturated acid, palmitic or arachidic, was dissolved in pentane. Suitable volumes of this solution were added to a pentane slurry of the silica gel so as to provide coatings ranging from 3 to 100% of a condensed monolayer after solvent removal. After 15 min at room temperature, the pentane was removed under reduced pressure at ambient temperature in a Buchler flash evaporator. The coated samples were then stored at -80 C. The saturated acid, present in ca. 10% mol proportion, served as an internal standard for subsequent gas-liquid chromatographic (GLC) analyses; it was assumed that oxidation of saturated fatty acid would be negligible.

The emission measurements were made with a photon counter. The photomultiplier tube was a Hamamatsu R-292 (S-13 response) and was cooled to -20 C. The output was pulse shaped, amplified by a Sanborn 150 preamplifier, and recorded on a Sanborn 150 recorder. Samples were placed in a 1 in. diameter well, above the photomultiplier tube. Measurements with a ^{3}H standard provided by J.W. Hastings (31) gave an overall efficiency of ca. 6% in the region of 400 nm, that of maximum sensitivity of the photomultiplier. The standard was calibrated in quanta per sec and was reduced to pulses or counts per sec by dividing by 80, the average number of light quanta emitted by ^{3}H in a POPOP scintillation medium (31). Background count rates were ca. 20 photons per sec.

The samples were placed either in a 20 ml disposable scintillation vial (Kimble 74389) or in a small double walled test tube. The space between the test tube walls was evacuated, and the test tube acted as a Dewar flask; insulation was inefficient because of the small size, but hot samples cooled sufficiently slowly to allow observation of the decay of emission during cooling. Higher and longer heating of a sample was obtained by placing a heated stainless steel finger in the test tube. A thermometer well in the top of the finger allowed periodic estimation of the temperature.

Samples simply warmed from their -80 C storage temperature to room temperature showed negligible emission. One procedure, therefore, was to heat the sample either in a beaker on a hot plate, or in an oven, and then to transfer it to a scintillation vial or the Dewar test tube. In this case, a rapidly increasing count rate was observed as the sample heated, followed by a slow decrease as it cooled. A sample once heated showed a measurable emission rate for hours after returning to ambient temperature.

At the end of an emission experiment, the partially oxidized sample was frozen over dry ice and stored until GLC analysis. A 30-50 mg portion of fatty acid coated silica gel was placed on top of a tight plug of Pyrex glass wool which was wadded into the tip of a disposable Pasteur pipet. The acids were then extracted by pouring three 2 ml portions of diethyl ether through the pipet, and the ether was evaporated with nitrogen at room temperature. The extracted acids were converted to the methyl esters by ethereal diazomethane at room temperature, followed by room temperature evaporation of excess diazomethane and solvent. The esters were then dissolved in hexane or in benzene for the GLC analysis. This determination was of the ratio of unsaturated to reference esters. Comparison with the original ratio permitted calculation of the amount oxidized.

Solutions producing singlet oxygen were prepared by the ozonolysis of isopropyl ether (32). An ozone containing oxygen stream was bubbled through 5-10 ml of the liquid at dry ice-acetone temperature for 30-60 min. The ozone was produced by a 12,000 volt discharge through a stream of oxygen (33); the ozone concentration was ca. 1%. After ozonolysis, the liquid was purged with dry nitrogen gas for 15 min and stored at -70 C for use within the next few days. In use, the ozonized ether was warmed to -30 C and a 1-2 ml portion transferred to the sample container (scintillation vial or Dewar test tube). Singlet oxygen evolves from the hydrotrioxides of the ether.

The spectral characteristics of an observed emission were measured by interposing various Wratten cut-off filters between the sample container and the photomultiplier. These were in the forms of a disc of the filter cemented to a thin brass ring which closely fit the sample well. The filters (Wratten number and 50% absorbance wavelength in nm) are as follows: (2A, 410), (2E, 430), (3, 465), (4, 475), (8, 495), (9, 510), (15, 530), (21, 555), (23A, 585), (26, 605), (29, 625), and (70, 675). The typical wavelength variation of relative sensitivity of the photomultiplier tube is (wavelength in nm, relative sensitivity): (420, 1), (430, 1), (475, 0.92), (495, 0.90,) (510, 0.84), (530, 0.74), (555, 0.65), (585, 0.45), (605, 0.36), (625, 0.25), (660, 0.12), and (670, 0.006).

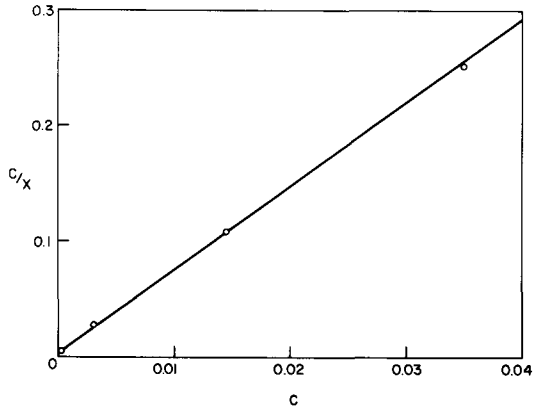

FIG. 1. Langmuir plot of adsorption data for hexane solutions of linolenic acid on silica gel at 25 C. Concentration (C) in g/cc and amount adsorbed (x) in g linolenic acid per g of silica gel.

The adsorption isotherm for linolenic acid on the silica gel was determined by equilibrating various concentration *n*-heptane solutions with a weighed amount of adsorbent and determining the change in concentration in the solution (34). Concentrations were determined before and after equilibrium by weighing the residue left on evaporation of the solvent from a aliquot.

RESULTS

It was found that easily measurable emission occurred from dry samples of coated silica gel. A series of runs was carried out to determine how the emission saturated with increasing depth of sample. The emission was found to be 75% saturated with a 1 mm depth (0.3 g) and essentially saturated with a 2 mm depth. Subsequent measurements were made with 1-2 mm depth samples, using the latter depth if reproducibility between runs was important.

An exploratory experiment was the following. Samples of silica gel coated with slightly more than a monolayer of linolenic acid plus 10% palmitic acid were suspended in mineral oil having nearly the same refractive index as the silica to allow light collection from a larger volume than the opaque dry silica. Light emission from suspensions preheated to 75 C with oxygen bubbling gave count rates of 100-200 per sec. Background averaged 20 cps. Mineral oil and uncoated silica suspensions were slightly higher than background and were not increased by heat-oxygen treatment. Since this procedure did not result in higher emission rates, all further experiments were made with dry samples.

Adsorption Data

The silica gel was of a type having BET nitrogen areas of around 300 m^2/g, but it was considered desirable to determine the approximate monolayer point through adsorption measurements using the linolenic acid itself. Figure 1 shows some data for 25 C, plotted according to the linear form of the Langmuir equation. From the slope and intercept, the monolayer capacity is 0.138 g linolenic acid per g of silica gel. If the molecular area of the fatty acid was effectively that of the carboxyl group, or ca. 20 $Å^2$/molecule, the silica gel area was only 60 m^2/g; if the fatty acid was lying flat, however, with a molecular area of ca. 143 $Å^2$/molecule (35), the calculated area of the silica gel became 430 m^2/g.

Induction and Temperature Effects

As noted above, samples brought from -80 C to room temperature showed negligible emission. If heated to 80-120 C, however, considerable emission was present, even after cooling back to room temperature. Reheating again gave a high, rapidly decaying emission (as the sample cooled) and a somewhat higher residual room temperature emission. It was apparent that an induction effect was present.

The temperature dependence was also of interest. Figure 2 shows the results of a run using the Dewar test tube with about a 1 mm depth of sample on which was seated the stainless steel finger, preheated to ca. 120 C. The initial rise in this case was due to the warming of the sample on contact with the hot finger. Cooling was now slow enough that the decay in emission could be followed easily; the temperature on the graph was obtained by periodic withdrawal of the Dewar test tube so that a thermometer could be inserted into the well of the finger. The results thus allow an activation energy calculation. The plot of log (emission intensity) vs. 1/T gives a slope of -5,000 deg^{-1}, hence the apparent activation energy is 5,000 R or 10 kcal mol^{-1}.

The induction period could be observed at higher temperature than ambient using the hot finger arrangement. On first heating to 120-125 C, the level of emission was ca. 10% of that finally obtained after three cycles of allowing the system to cool to ca. 40 C and then replacing the metal finger with a freshly heated one.

Emission Yield

The run of Figure 2 allowed an approximate calculation of the emission yield. Over the entire set of temperature cycling, the total photon count was 8.5×10^6, or, corrected for

the 6% geometric efficiency factor, ca. 1.4×10^8 quanta. The sample, initially coated with 1×10^{-4} mol of linolenic acid, was 55% oxidized at the end of the run. The quantum yield is thus $(1.4 \times 10^8/6.02 \times 10^{23})/(0.55 \times 10^{-4})$, or ca. 5×10^{-12}. Much of the emission was at wavelengths for which the response efficiency of the photomulitplier was low (see further below). The quantum yield of 5×10^{-12} is thus a minimum one.

Effect of Degree of Surface Coverage

A series of experiments was made in which silica gel samples having successively smaller degrees of surface coverage were used. Each was given the same preheating treatment and transferred to the Dewar test tube after the same elapsed time following the heating. In each case, the usual rapid decay of emission occurred, followed by a residual, very slowly decaying emission. The measurements used were taken 15 min after the start of the emission measurement or during this slowly decaying period. The results were as follows (% of monolayer, emission in photons/sec): (110, 52), (75, 55), (40, 16), (15, 1.7). On plotting, an S-shaped curve of emission rate vs. degree of surface coverage results. That is, the rate is not linear in coverage but appears to increase autocatalytically.

Emission Spectrum

A sample of silica gel coated to a full monolayer of linolenic acid was heated briefly to ca. 150 C and then transferred to the Dewar test tube. After the intial rapid decay, the residual, slowly decaying emission rate was ca. 200 photons per sec. The emission was measured through Wratten filter 2A, then through another filter of the series, then through 2A again, and so on, until a complete series of attenuations was obtained, each referenced to bracketing measurements with filter 2A. In a second series of measurements, emission through 2A was redetermined every third rather than every second time. The two series gave very similar results; these are shown in Figure 3, along with the corresponding differential curve calculated from the smoothed integral curve.

There is a peak in emission at 530-540 nm. The spectrum is severely distorted for wavelengths longer than 600 nm because of the failing photomultiplier tube efficiency, but, clearly, important emission is occurring in this region. The data are consistent with a second emission peak in the region of 630 nm.

The analogous procedure was followed with ozonized isopropyl ether. These results are included in the figure. Here a peak occurs at ca.

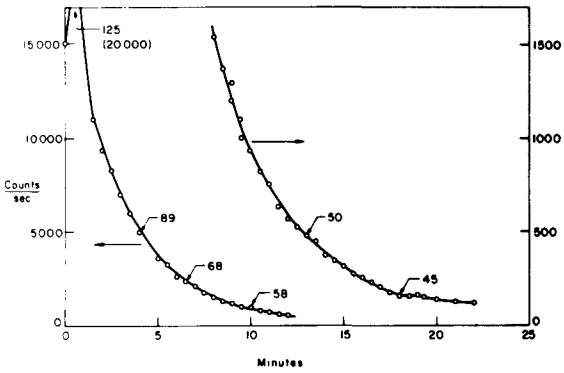

FIG. 2. Decay of emission from the autoxidation of linolenic acid in a monolayer coating on silica gel. The decay is due to the cooling of the sample; the temperatures measured (in C) are shown at various points along the decay curve.

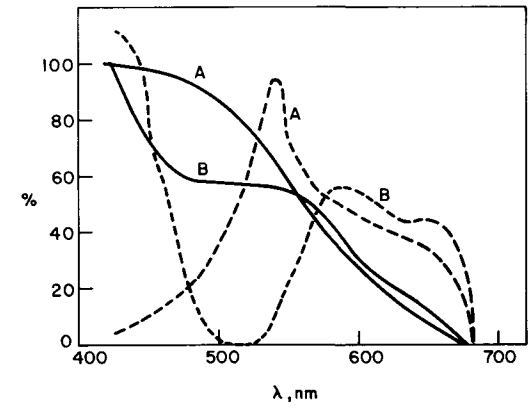

FIG. 3. Integral emission spectra (full lines) for A, linolenic acid monolayer on silica gel, and B, ozonized isopropyl ether (assuming 100% of all emission is at 420 nm or more in wavelength). Dashed curves are the respective differential emission spectra (ordinate scale arbitrary).

430 nm, and there is a second region of strong emission in the 480-690 region. The shape of the integral curve is, in this region, within experimental uncertainty, the same as that for the emission accompanying the autoxidation of the linolenic acid samples.

DISCUSSION

Most of the observations are self-explanatory. The autoxidation of linolenic acid adsorbed on silica gel is indeed chemiluminescent. The minimum quantum yield estimate of 5×10^{-12} is in the range reported for homogeneous autoxidations, 10^{-8} to 10^{-15} (17,19,21). The emission rate varies with degree of surface coverage in qualitatively the same way as has

previously been reported for chemical rate measurements (5,7,12). That is, the emission rate rises autocatalytically in going from ca. 0.1 to ca. 1.0 of a complete monolayer.

The temperature dependence of the emission intensity (10 kcal/mol) appears to be about the same as that found for low surface coverages from chemical rate measurements, 11 kcal/mol (7). Both figures are smaller than the ca. 20 kcal/mol value reported for homogeneous autoxidation of methyl linoleate (11).

The spectral information is suggestive. The peak around 530 nm corresponds to that reported for chemiluminescence from peroxy radicals (17) and assigned to excited state ketone emission. However, the indication of strong emission around 630 nm is suggestive of singlet oxygen (36-38). This is an emission ascribed to bimolecular reaction of two $^1\Delta_g$ oxygens (37). (Direct or spontaneous emission from the $^1\Sigma_g^+$ and $^1\Delta_g$ states would be at 762 nm and 1,268 nm, respectively, and undetected by our equipment).

Both assignments, excited state ketone at 530 nm and singlet oxygen at 630 nm, would carry mechanistic implications. Both involve processes customarily written as bimolecular reactions. As already noted, the 630 nm emission calls for interaction between a pair of $^1\Delta_g$ oxygen molecules; and excited state ketone formation has been written as a cleavage product (symmetry controlled) of two associated $R_2CHOO\cdot$ radicals (17). One may reasonably question whether such bimolecular processes are likely on a silica gel surface. As one alternative, the 650 nm emission could come from an excited state aldehyde or other intermediate.

Our emission spectra were obtained, however, with a full monolayer coverage. Under this condition, chemical autoxidation rates become comparable to those in homogeneous systems (12) and, moreover, show induction and inhibition behavior consistent with the type of radical chain mechanism postulated for homogeneous systems. In our experiments, heating was necessary to initiate emission, which would continue even on cooling back to room temperature. It may thus be that, with high loadings of the silica gel, the linolenic acid is present in a two-dimensional liquid state of appreciable surface mobility; it could, in fact, be at least partly present in bi- or multilayer patches.

At low surface coverages, the autoxidation rate as determined chemically is much reduced over that at high coverage (7,12) and there does not seem to be an induction period. The mechanism may thus be different; the chemical rates may now reflect the initiation rather than the propagation steps. It is thus quite possible that the emission during autoxidation at low surface coverages will change its nature; there may no longer be an induction period, for example, and the emission spectrum may be different. Such measurements are planned with later modifications of the equipment.

In summary, the present results discover chemiluminescence in a surface autoxidation reaction and indicate that the phenomenon will be useful both as a means of following reactions in situ and in answering mechanistic questions. In particular, the nature of the emission may allow an appraisal of the relative importance of bimolecular reactions involving reactive intermediates.

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Esterified Alkan-1-ols and Alkan-2-ols in Barley Epicuticular Wax

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ABSTRACT

The epicuticular wax on all barley organs is characterized by the presence of long chain esters which have alkan-1-ols as their alcohol moiety (mainly C_{38}-C_{48}). A second type of long chain ester in which alkan-2-ols serve as the alcohol moiety has been identified in the wax from all organs except the awns and leaf blades (mainly C_{33}-C_{35}). Utilizing Silica Gel H column chromatography, a 95% separation of the two ester types was achieved. Unlike alkan-1-ols, the alkan-2-ols (primarily C_{13} and C_{15}) do not occur free in the wax. Esters isolated from spike minus awn wax consisted of 62% alkan-2-ol and 38% alkan-1-ol containing esters. The isomeric composition of each ester was determined with the aid of gas liquid chromatography-mass spectrometry. In the mass spectra of the alkan-2-ol containing esters, mass ions were absent and the relative intensities of the $RCO_2H_2^+$, RCO_2H^+, $[R'\text{-}1]^+$, and RCO^+ ions were markedly different from those characteristic for alkan-1-ol containing esters. Since esterified alkan-2-ols occur only in those waxes having β-diketones, a close biosynthetic relationship between these two lipid classes is suggested.

INTRODUCTION

In barley the major lipid classes of the epicuticular wax include hydrocarbons, esters, β-diketones, aldehydes, primary alcohols, hydroxy-β-diketones, and fatty acids (1). The deposition of these lipid classes on the cuticular surfaces of individual organs–leaf blade, spike, and leaf sheath plus internode–is controlled by at least 59 genes (2). These *eceriferum (cer)* loci provide a valuable asset for attacking various problems of epicuticular wax biosynthesis, such as (i) the mode of synthesis of a given wax class and its relationship to the other wax classes, (ii) the question of chain length specificities of the various enzymes, or (iii) the contribution of enzyme and/or substrate compartmentalization to the observed wax composition (e.g., 1, 3-8). Thus the chemical composition of the wax present on individual organs of the wild type and selected *cer* mutants is being determined. During the course of these investigations, two prominent short chain components frequently appeared in gas liquid chromatograms of ester alcohols isolated from spike or leaf sheath plus internode wax but never from leaf blade wax. In this paper these unknowns are shown to be alkan-2-ols, the isomeric composition of both alkan-1-ol and alkan-2-ol containing esters is determined, and the biosynthetic relationship of the alkan-2-ols to the other wax classes is discussed.

MATERIALS AND METHODS

Plant Material and Wax Isolation

The mutant *cer-u*69 was selected for study since its spike wax contained the unknown short ester alcohols and it was already being grown in large amounts for biosynthetic studies (3). It differs from the wild type barley (*Hordeum vulgare* L.) cv. Bonus by lacking hydroxy-β-diketones and having a compensatory increase in the amount of β-diketones (1). Seeds were planted and grown for 8 weeks in the Phytotron at the Royal College of Forestry, Stockholm. At this time the first two to three spikes on a plant have completely emerged from their leaf sheaths. The methods of plant culture and isolation of the epicuticular wax from the intact spikes have been previously published (9). For certain analyses immediately after a spike was cut from the stem each of its 22-27 awns was clipped off at its base. Wax was collected separately from the awns and the spikes minus awns by dipping in chloroform as described earlier for the intact spikes (9). A small quantity of internal lipids was probably extracted with the epicuticular wax from the spikes minus awns since it is not possible to avoid contact between the cut surfaces and the chloroform.

Separation of Esters by Column Chromatography

Prior to separating the esters from a wax sample, the fatty acids and β-diketones were removed by complex formation as previously described (3). The remaining wax mixture was dried and the lipids (64 mg) taken up in carbon tetrachloride and applied to a 60 x 1.8 cm (internal diameter) column containing Silica Gel Type 60 (E. Merck, Darmstadt, West

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Germany). The latter had been washed in ethyl acetate, dried, activated at 110 C overnight, and suspended in carbon tetrachloride, which was then degassed under vacuum, before being packed into the column. Thirty minute fractions of 5.5-6.0 ml each were collected. Carbon tetrachloride eluted the hydrocarbons (23 mg) in fractions 23-34 and the esters (16 mg) in fractions 120-190. The earlier eluting esters in fractions 120-152 were pooled to give the ester-1 fraction (9 mg), and the latter eluting ones in fractions 153-190 were pooled to give the ester-2 fraction (7 mg). The remaining wax classes were eluted using benzene (900 ml) and diisopropyl ether (900 ml). The last solvent gave a better resolution of the more polar lipid classes than ethyl acetate which was used previously (10,11).

Thin Layer Chromatography (TLC)

TLC was used to (i) determine the lipid class composition of the waxes, (ii) monitor the various column chromatographic separations of the lipid classes, and (iii) follow the course of and purify the products of chemical reactions. In all cases the adsorbent was Silica Gel H (Merck), the plates were activated for 1 hr at 110 C, and the developing solvent was either benzene or hexane:diethyl ether:acetic acid (70:30:2, v/v/v) (9).

Gas Liquid Chromatography (GLC)

GLC was carried out using a Varian Aerograph model 1700 instrument with flame ionization detectors coupled to an Infotronics Model CRS-100 digital electronic computer. Three sets of stainless steel columns were used: (i) 152.4 x 0.22 cm (internal diameter) columns containing 7% SE-30, GC grade on Chromosorb W (DMCS) 60/80 mesh (Applied Science Laboratories, State College, PA); (ii) 91.4 x 0.22 cm columns containing 2% SE-30, GC grade on Anakrom ABS 100/120 mesh (Analabs, North Haven, CT); and (iii) 152.4 x 0.22 cm columns containing 15% HI EFF-2BP on Chromosorb W (AW) 80/100 mesh (Applied Science Laboratories). Isothermal and programmed chromatograms were run using column temperatures from 100 to 300 C as required, while nitrogen, hydrogen, and air streams were adjusted to yield optimum sensitivity. Chain lengths of the ester acids as methyl esters and ester alcohols as alcohols were established using internal standards either purchased from Applied Science Laboratories or synthesized as detailed below. The chain length distributions as % by wt of the esters, ester aicds as methyl esters, and ester alcohols as acetates were converted to mol % using the effective carbon numbers of Sternberg et al. (12).

Gas Liquid Chromatography-Mass Spectrometry (GLC-MS)

GLC-MS was performed to establish the chain length and isomeric composition of the esters and to aid in identifying the alkan-2-ols. An LKB type 9000 instrument (LKB Produkter AB, Stockholm, Sweden) was used with an ionizing potential of 70 eV and ionizing current of 60 μA. The esters were separated on a 100 x 0.22 cm (internal diameter) column containing 3% OV-1 temperature programmed from 240-300 C. The alcohols were separated on a 30 x 0.22 cm column containing 3% OV-1 at 90 C.

Chemical Reactions

The esters were saponified (9) and the resulting acid and alcohol moieties separated using a 1.5 x 2.4 cm (internal diameter) column containing 10% finely ground NaOH/Gas Chrome Q, 100/120 mesh. The alcohols were eluted with 200 ml petroleum ether (bp 60-80 C) and the bound acids recovered as described previously (8). Fatty acid methyl ester and alcohol acetate derivatives were prepared (9) for GLC analyses. An aliquot of the ester alcohols which comprises alkan-1-ols and alkan-2-ols, both having an R_f of 0.07 in benzene, was oxidized to the corresponding fatty acids and methyl ketones as follows. The alcohols were transferred to a tube with a teflon lined screw cap and taken to dryness. Next, 1 ml of acetone plus 100 mg of finely ground $KMNO_4$ were added, and the tube was allowed to shake gently at room temperature overnight. The solution was acidified by adding 1 ml 5N H_2SO_4 and the remaining permanganate plus manganese dioxide destroyed by adding an excess of solid $FeSO_4 \cdot 7H_2O$. The methyl ketones were extracted with three aliquots of diethyl ether, dried over anhydrous Na_2SO_4, and purified via TLC.

Tridecan-2-one and pentadecan-2-one purchased from Applied Science Laboratories were reduced to tridecan-2-ol and pentadecan-2-ol using $LiAlH_4$. This was carried out by adding an excess of $LiAlH_4$ in ether to the methyl ketones in ether and allowing the reaction to proceed for 1 hr at room temperature. After the excess $LiAlH_4$ had been destroyed by adding ethyl acetate and the solution acidified by adding 1N H_2SO_4, the alkan-2-ols were extracted using three aliquots of diethyl ether, dried over anhydrous Na_2SO_4, and purified via TLC.

RESULTS AND DISCUSSION

Esters isolated from the epicuticular wax of the spikes of *cer-u*[69] ran as a single spot on TLC plates. Semilog plots of apparent carbon number vs. retention time from isothermal GLC gave two different straight lines. That is, the retention times of the four shortest esters were slightly less than those expected if all the esters belonged to a single homologous series whose members differed by two carbons. By dividing the spike into two parts, namely the awn and the spike minus awn, before wax isolation, it was found that the four shortest esters were present only on the spike minus awn whereas the longer esters were present on both parts of the spike. The R_f of the awn esters in benzene is slightly greater (0.02) than that of the esters from the total spike or the spike minus awn.

A 95% separation of the two types of esters was obtained via column chromatography; the longer ones eluting first (ester-1 fraction) and the shorter ones thereafter (ester-2 fraction). Both ester fractions were saponified and the resulting acid and alcohol moieties, as methyl esters and acetates respectively, analyzed via GLC. No qualitative differences were apparent between the two fractions in their acid moieties, which were primarily n-C_{16} to n-C_{22}. On the other hand, the alcohol moieties of the two fractions differed considerably. Those from the ester-1 fraction were almost entirely the long chain alcohols n-C_{20} to n-C_{28}, whereas those from the ester-2 fraction were dominated by two short chain alcohols having slightly longer retention times than n-C_{12} and n-C_{14} on SE-30 columns.

To identify the two short alcohols, they were subjected to GLC-MS. The molecular ions of the shorter alcohol at m/e 200 and of the longer one at m/e 228 were both very small compared to the M - 18 (M - H_2O) peak, as expected for alcohols. The relatively small intensity of the M - 46 [M - (H_2O + CH_2=CH_2)] peaks and large intensities of the M - 60 [M - (H_2O + C_3H_6)] and M - 15 (M - CH_3) peaks combined with a very large peak at m/e 45 ($CH_3CH = O^+H$) indicated that the two alcohols were tridecan-2-ol and pentadecan-2-ol, respectively (13,14). MS of authentic compounds gave the same expected diagnostic peaks. The identification of the short alcohols was supported by their co-GLC with authentic alkan-2-ols on both SE-30 and HI EFF columns. In addition, the short alcohols were oxidized with $KMNO_4$ to the corresponding methyl ketones which co-chromatographed in both TLC and GLC systems with authentic tridecan-2-one and pentadecan-2-one.

On the basis of these results, the esters of *cer-u*[69] spike minus awn wax consist of two homologous series differing in that the fatty acids are esterified with either alkan-2-ols (C_{31}-C_{37} esters) or alkan-1-ols (C_{36}-C_{48} esters). The latter type of ester is also characteristic of other cereals such as wheat, oats, and rye, although alkan-2-ol containing esters have not been reported in these waxes (15-20). In wheat and rye, however, a second series of long chain esters (C_{42}-C_{46}) is present in which unsaturated fatty acids (trans-2-docosanoic and trans-2-tetracosanoic) are esterified with alkan-1-ols (15-17,19).

The fractionation of the two types of esters by column chromatography is probably due to differences in the overall chain lengths, although inductive and stearic affects may also be involved. Similar separations have been reported in selected TLC systems in which the chain lengths of the acid moieties differ while those of the alcohol moieties remain constant (21-24). By using solvent systems other than those employed in the present study, the two types of esters should also be resolved on TLC plates (10).

Alkan-2-ols have been previously reported only in *Eucalyptus* waxes (25) and, as in the present case, were found in long chain esters, but not as free alcohols. This is in marked contrast to the long chain secondary alcohols commonly found in plant waxes, such as nonacosan-15-ol in *Brassica oleracea* (26) or hentriacontan-9-ol in *Rosa damascena* (27), which occur free but not esterified. On the other hand, primary alcohols are found both free and esterified. Such occurrences intimate the presence of one or more highly selective esterification systems and/or a differential availability of substrates to the enzymes due to compartmentalization, for example. To what extent such factors are involved in the synthesis of the several types of long chain wax esters found on a plant remains to be determined. In *B. oleracea*, three different mechanisms for synthesis of long chain esters in vitro have been demonstrated (28,29).

Esters isolated from the spike minus awn of *cer-u*[69] were chosen for further analyses since both the alkan-1-ol and alkan-2-ol containing esters are present in good quantities, amounting to 38 and 62%, respectively (Table I). To estimate the isomeric composition of each ester, the esters were subjected to GLC-MS and the MS analyzed. The MS showed molecular ions for all alkan-1-ol containing esters but not for the alkan-2-ol containing esters, indicating that fragmentation occurs more readily in the latter type of ester. Good estimates of the

TABLE I

Composition of Esters and Their Isomers from *cer-u*[69] Spike Minus Awn Epicuticular Wax

Ester[a]	Mol %[b]	Carbon number		Mol %			
		Acid moiety	Alcohol moiety	Found[c]		Calculated[d]	
C_{29}						1.8	
C_{31}	1.9	16	15	0.2		2.6	
		18	13	1.0		2.9	
		20	11	0.7		0.4	
C_{33}	22.1	18	15	6.6		4.5	
		20	13	15.5	62.3	12.7	55.2
C_{35}	33.8	20	15	24.8		19.8	
		22	13	9.0		4.0	
$C_{37}+C_{36}$	4.8	20	17	0.4		0.2	
		22	15	4.1		6.3	
		20	16	0.3		0.2	
C_{38}	3.9	16	22	3.2		1.3	
		18	20	0.4		0.3	
		20	18	0.4		0.2	
C_{40}	6.9	16	24	3.8		1.1	
		18	22	2.1		2.2	
		20	20	1.0		1.4	
C_{42}	10.7	16	26	2.3		0.7	
		18	24	1.7	37.7	1.9	44.8
		20	22	6.0		9.6	
		22	20	0.7		0.4	
C_{44}	11.3	16	28	0.8		0.1	
		18	26	2.3		1.3	
		20	24	5.7		8.5	
		22	22	2.5		3.0	
C_{46}	4.5	18	28	0.6		0.2	
		20	26	2.9		5.6	
		22	24	0.7		2.7	
		24	22	0.3		0.0	
C_{48}	<0.1					2.9	
Others						1.2	

[a] C_{31}-C_{37} are alkan-2-ol containing esters, and C_{36}-C_{48} are alkan-1-ol containing esters.

[b] Determined from gas liquid chromatography.

[c] The relative isomer content of each ester was determined from an analysis of its mass spectrum (31). These values were multiplied by the mol % of the respective ester determined by gas liquid chromatography to give the mol % of each isomer.

[d] Expected composition assuming that the fatty acids and alcohols (Table III, MS) were esterified randomly.

isomeric composition of C_{28}-C_{36} esters were obtained by Streibl et al. (30) using the relative intensities of the base ion $RCO_2H_2^+$ only. Aasen et al. (31) have shown for saturated straight chain esters in which the alcohol and acid moieties vary from 10 to 18 carbons, however, that the isomeric composition of an ester can be best determined from MS if the relative intensities of three ions are known. Two of these ions, namely, RCO_2H^+ and $RCO_2H_2^+$, arise from the acid moiety of the ester, and the third, $[R'\text{-}1]^+$, comes from the alcohol moiety. Table II contains the intensities of these ions arising from the predominant isomer of each investigated ester relative to its base peak. The relative intensities of a fourth ion, RCO^+, are also included. In the alkan-1-ol containing esters, the $RCO_2H_2^+$ ion was the base peak in all cases, as expected (31), and the observed relative intensities of the other three ions were similar to those reported by Aasen et al. (31) for C_{34} and C_{36} esters (Table II). As shown in Table II, however, the presence of the methyl group in the alkan-2-ol containing esters alters considerably the relative intensities of the four ions, either the RCO_2H^+ or RCO^+ ion being the base peak. Further work should elucidate the mechanisms underlying these changes in relative intensity. Aasen et al. (31) describe an additional four characteristic ions in

TABLE II

Intensities of Four Characteristic Ions in the Mass Spectra of Alkan-1-ol and Alkan-2-ol Containing Esters[a]

Esters containing	$RCO_2H_2^+$	RCO_2H^+	$[R'-1]^+$	RCO^+
Alkan-1-ols				
C_{38}-C_{46}	100	21-27	7-16	8-10
C_{34}-C_{36}[b]	100	23-25	17-23	10-11
Alkan-2-ols				
C_{31}	72	87	98	100
C_{33}	75	100	78	76
C_{35}	95	90	63	100
C_{37}	75	100	69	82

[a]The intensities given are for the predominant isomer in each ester (see Table I) and are expressed as % of the base peak in the MS.

[b]From Aasen et al. (31). Acid and alcohol moieties have 16 or 18 carbon atoms.

TABLE III

Composition of the Ester Acids and Alcohols from *cer-u*[69] Spike Minus Awn Epicuticular Wax

	Ester acids (mol %)		Ester alcohols (mol %)	
Carbon number	GLC[a]	MS[b]	GLC[a]	MS[b]
11				0.6
13			13.1	21.4
15			31.8	33.3
17			<0.1	0.3
16	11.6	7.8		0.3
18	12.6	13.5		0.3
20	54.3	59.5	1.8	2.3
22	19.8	18.9	20.9	16.1
24	1.7	0.3	22.1	14.2
26			9.6	9.4
28			0.7	1.8

[a]Determined from gas liquid chromatography (GLC) of the appropriate derivatives of the ester saponification products.

[b]MS = mass spectrometry. The relative contributions to mol % (Table I, column 5) made by the acid and alcohol moieties of each isomer were calculated. Then the mol % of a given alcohol or acid was obtained by summing the percent of that chain length found in each of the esters.

the MS of their esters. We observed similar relative intensities for these latter ions in both alkan-1-ol and alkan-2-ol containing esters. To approximate the isomeric composition of both types of esters given in Table I, we used the formula of Aasen et al. (31). Inclusion of the RCO^+ ion made no marked difference to the results.

The MS showed that only one GLC peak contained both types of esters, namely, the $C_{37} + C_{36}$ peak, which equalled 4.8% of the esters (Table I). In the MS of this peak, $[R'-1]^+$ ions occur corresponding to three alcohol moieties: C_{15}, pentadecan-2-ol (m/e = 210, I = 69); C_{17}, heptadecan-2-ol (m/e = 238, I = 7); and C_{16}, hexadecan-1-ol (m/e = 224, I = 5). This indicated the presence of three esters, 15:22, 17:20, and 16:20 (alcohol moiety:acid moiety, respectively). In calculating the percentages of the C_{22} acid (4.1%) vs. C_{20} acid (0.7%) containing isomers in the $C_{37} + C_{36}$ peak, the formula of Aasen et al. (31) was again used with the modification that the relative intensities of both $[R'-1]^+$ ions associated with the C_{20} acid were included. Then the proportion of the 17:20 (0.4%) and 16:20 (0.3%) isomers was estimated from the ratio of the intensities of the appropriate $[R'-1]^+$ ions.

The compositions of the ester acids and alcohols were determined from the MS isomeric analysis of each ester. These results are compared with those from GLC analyses of the ester saponification products in Table III. The agreement between the chain length distribu-

tions is not as good as could be expected for the alcohols. Streibl et al. (30) obtained similar distributions using both methods. Only one type of ester, however, was involved in their study (30) compared with two types in the present analysis. We attribute our poorer fit to losses of the more volatile short alkan-2-ols during their preparation for GLC. A recalculation of the alkan-1-ol GLC distribution, so that it totals 44.4% as in the MS analysis, yields a distribution quite similar to that from the MS analysis. Our results support the proposal made above that the formula of Aasen et al. (31) for determining the isomeric composition of an ester can be extended to alkan-2-ol containing esters.

The expected isomeric composition of the esters was generated assuming a random combination of the fatty acids with both types of alcohols (Table I). The calculated distribution does not fit very closely to the observed isomeric composition of the esters. An excess of alkan-2-ol containing esters (C_{31}-C_{37}) and a deficit of alkan-1-ol containing esters (C_{36}-C_{48}) is apparent in the experimental results. This may reflect differences in the available amounts of the two types of alcohols and/or, as mentioned above, their compartmentalization or esterification by separate enzyme systems. When the two types of alcohols are tested separately for randomness of esterification, a slightly improved fit of calculated to found occurs in both cases. Although the latter fits are similar to (32) or better than (33,34) those reported by others in support of a random esterification pattern, the agreement is still not as good as could be expected (35,36). A closer examination of the data in Table I reveals that within the alkan-2-ol containing esters an excess of isomers having a C_{20} acid moiety and a deficit of those having a C_{16} acid moiety occur. The opposite situation is seen in the alkan-1-ol containing esters. These observations suggest that the enzymes involved in the esterification of the alkan-1-ols and alkan-2-ols differ in their chain length specificities for the acid moieties. The esterification system(s) giving rise to the saturated and unsaturated types of long chain esters in wheat are apparently selective for given chain lengths (17). Namely, the unsaturated C_{22} and C_{24} fatty acids are preferentially esterified with C_{20}-C_{24} alcohols whereas the saturated C_{14}-C_{32} fatty acids are predominantly esterified with octacosanol.

Since esterified alkan-2-ols are found only on those parts of the barley plant, spike minus awn and leaf sheath plus internode, which can synthesize β-diketones, a close biosynthetic relationship between these two lipid classes may exist. In the wax of the two *Eucalyptus* species in which esterified alkan-2-ols have been reported, β-diketones are also present (25). Our recent experiments indicate that the β-diketone hentriacontan-14,16-dione is most likely synthesized via elongation from the C_{31} end of the molecule (3). Whether the carbonyl groups are inserted after elongation in an analogous manner to nonacosan-15-one synthesis in *Brassica oleracea* (37,38) or during elongation remains to be determined.

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Specific Inhibition of Hepatic Fatty Acid Synthesis Exerted by Dietary Linoleate and Linolenate in Essential Fatty Acid Adequate Rats[1]

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ABSTRACT

Dietary linoleate and linolenate were investigated for their ability to specifically inhibit liver and adipose tissue lipogenesis in meal-fed (access to food 900-1,200 hr), essential fatty acid (EFA) adequate rats. Supplementing a high carbohydrate diet containing 2.5% safflower oil with 3% palmitate 16:0, oleate 18:1, or linoleate 18:2 did not affect in vivo liver or adipose tissue fatty acid synthesis. However, 18:2 addition to the basal diet did result in a significant ($P<0.05$) decline of liver fatty acid synthetase (FAS) and glucose-6-phosphate dehydrogenase (G6PD) activities. When the safflower oil content of the basal diet was reduced to 1%, the addition of 3% 18:2 or linolenate 18:3 significantly ($P<0.05$) depressed hepatic FAS, G6PD, and in vivo fatty acid synthesis by 50%. Addition of 18:1 caused no depression in hepatic FAS activity but did result in a significant ($P<0.05$) decline in liver G6PD activity and fatty acid synthesis which was intermediate between basal and basal + 18:2- or +18:3-fed animals. Adipose tissue rates of lipogenesis were completely unaffected by dietary fatty acid supplementation. Similarly, the addition of 3 or 5% 18:3 to a basal diet for only one meal resulted in no change in lipogenesis relative to that in animals fed the basal diet. The data indicate that, like rats fed EFA-deficient diets, dietary 18:2 and 18:3 exert a specific capacity to depress rat liver FAS and G6PD activities and rate of fatty acid synthesis.

INTRODUCTION

Ingestion of large amounts of either unsaturated or saturated fat sources markedly inhibit rat liver and adipose tissue fatty acid synthesis (1-3). Unsaturated fatty acids may be more effective in inhibiting rat liver lipogenesis than are saturated fatty acids (1,3), but detecting the mode of action of this specific effect may be masked when using high dietary levels of fat.

When linoleate (18:2), linolenate (18:3), and arachidonate (20:4) are supplemented to a fat-free diet of rats and mice, they exert a specific inhibitory action on hepatic lipogenesis (4-8). Furthermore, the oral administration of methyl 18:2, 18:3, or 20:4 to rats fed a fat free diet for 7 days caused a decline in liver glucose-6-phosphate dehydrogenase (G6PD) and fatty acid synthetase (FAS) activities within 2 days (5).

Feeding an essential fatty acid (EFA) deficient diet to rats leads to marked changes in total liver and hepatic organelle fatty acid composition (4,9). Rats previously fasted for 48 hr displayed large increases in mitochondrial, microsomal, and nuclear content of palmitoleic and oleic (18:1) acids and decreases in 18:2 and 20:4 content within 12-18 hr after refeeding a fat-free diet (4). In addition, the ability of rat liver mitochondria to translocate anions and cations, as measured by swelling properties, and the activities of glutamate dehydrogenase, β-hydroxybutyrate dehydrogenase, and cytochrome C oxidase appeared to be lowest in rats fed diets low in EFA for prolonged periods of time (9). Most earlier studies examining the effect of various dietary fatty acids on rat liver and adipose tissue lipogenesis rates have involved supplementation of EFA-deficient diets (4-8), which are known to greatly alter liver organelle fatty acid composition (4,9) and possibly mitochondrial function (9). To avoid this abnormal state, the experiments in this report were designed to investigate the ability of dietary 16:0, 18:1, 18:2, and 18:3 to inhibit rat liver and adipose tissue lipogenesis when supplemented to EFA-adequate diets.

METHODS AND MATERIALS

Gozukara et al. (10) have proposed that polyunsaturated fatty acids (PUFA) exert an inhibitory action on rat liver fatty acid (FA) synthesis by precipitating a depression in carbohydrate intake. Therefore, the male Sprague-

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TABLE I

Basal Diet Ingredients

Ingredient	Percent	
	Expt. I	Expt. II
Glucose	69.5	71.0
Casein	20.0	20.0
Nonnutritive fiber[a]	3.0	3.0
Mineral mix[b]	4.0	4.0
Vitamin mix[c]	0.4	0.4
Choline-chloride	0.3	0.3
Methionine	0.3	0.3
Safflower oil	2.5	1.0

[a]Solka-floc, Brown Company, Berlin, NY 03570.

[b]Rat mineral mix #4164, Teklad Test Diets, 2826 Latham Dr., Madison, WI 53713.

[c]See Y.Y. Yeh and G.A. Leveille, J. Nutr. 98:356 (1969).

Dawley rats (Spartan Research Animals, Haslett, MI) utilized in these studies were adapted to a 3 hr/day (900-1,200 hr) meal-eating regimen to facilitate equalization of food intake among treatments. A high glucose-casein basal diet (Table I) containing either 2.5% safflower oil (Experiment I) or 1% safflower oil (Experiment II) was utilized to ensure adequate EFA status (11). Following adaptation to meal eating, blocks of four animals per block (8), carefully matched for body weight and food intakes, were assigned to respective treatments. Within each block of animals, food intake was constant among treatments. Methyl esters (99% purity, Sigma Chemical Co., St. Louis, MO) of palmitate (16:0), 18:1, 18:2, or 18:3 were supplemented to the basal diet as 3% of the daily food allotment. In Experiment I, the esters were mixed into the diet daily and fed for 7 days prior to sacrifice. In Experiment II, the esters were intubated prior to each meal for 10 days before sacrifice.

In addition to the animals supplemented with various esters for 7 or 10 days, a second set of rats in each experiment was concurrently maintained on the basal diet until the day of sacrifice. These animals were then fed one meal containing 3% (Experiment I) or 5% (Experiment II) of the day's food allotment as methyl 18:3.

Liver and adipose tissue FA synthesis rates were ascertained by intraperitoneal injection of 1.5 mCi ^3H_2O (New England Nuclear, Boston, MA) (8). G6PD (EC 1.1.1.49) nicotinamide adenine dinucleotide phosphate-malic enzyme (EC 1.1.1.40) (ME), and FAS activities were quantitated as described previously (12-14). Protein content of supernatant fractions was determined by the method of Lowry et al. (15).

To determine the digestibility of 16:0, the rats (Experiment I) were placed in metabolic cages for days 2-6 of the ester feeding period and feces were collected. The feces were extracted using 1 N HCl and chloroform: methanol (8).

The data were analyzed with analysis of variance for a randomized complete block design, and treatment differences were ascer-

TABLE II

Effect of 16:0, 18:1, or 18:2 Supplementation to Essential Fatty Acid Adequate Diet (2.5% Safflower Oil) on Rat Liver Lipogenesis

	Dietary fatty acid			
	Basal	+3% 16:0	+3% 18:1	+3% 18:2
Body weight (g)[a]	149 ± 2	151 ± 3	151 ± 3	151 ± 2
Total weight gain (g)	21 ± 1	22 ± 1	23 ± 1	23 ± 1
Daily food intake (g)	10.5	10.5	10.5	10.5
Ester digestibility (%)	---	57	88[b]	87[b]
Liver weight (g)	6.6 ± 0.2	6.5 ± 0.2	6.7 ± 0.2	6.8 ± 0.1
Epididymal fat weight (g)	0.82 ± 0.08[c,d]	0.74 ± 0.05[c]	1.03 ± 0.04[d]	0.98 ± 0.05[c,d]
Enzyme activities[a]				
FAS[e]	6.7 ± 0.7[d]	6.7 ± 0.7[d]	7.4 ± 0.7[d]	5.2 ± 0.6[c]
G6PD[f]	65 ± 4[d]	63 ± 10[d]	69 ± 10[d]	43 ± 7[c]
ME[f]	44 ± 2[c,d]	43 ± 8[c,d]	50 ± 5[d]	32 ± 3[c]
In vivo FA-synthesis[g]	7,221 ± 1,911[c]	10,153 ± 587[c]	10,652 ± 1,578[c]	8,649 ± 860[c]

[a]Growth parameters and enzyme activities are mean ± SEM, n=8.

[b]Determined by Clarke et al. (8) in ester supplementation of a fat-free diet.

[c,d]Those values with different superscripts are significantly different ($P<0.05$).

[e]nMol reduced nicotinamide adenine dinucleotide phosphate oxidized min^{-1} mg $protein^{-1}$ at 37 C. FAS = fatty acid synthetase.

[f]nMol nicotinamide adenine dinucloetide phosphate reduced min^{-1} mg $protein^{-1}$ at 25 C. G6PD = glucose-6-phosphate dehydrogenase, ME = NADP malic enzyme.

[g]Mean ± SEM, n=7; dpm ^3H incorporated into fatty acids per g liver in 15 min.

TABLE III

Effect of 18:1, 18:2, and 18:3 Supplements on Liver Lipogenesis in Rats Fed an Essential Fatty Acid Adequate Diet (1% Safflower Oil)

	Dietary fatty acid			
	Basal[a]	+3% 18:1[a]	+3% 18:2[b]	+3% 18:3[b]
Body weight (g)	150 ± 2[c]	156 ± 2[c,d]	161 ± 2[c]	159 ± 3[c]
Total weight gain (g)	22 ± 1[c]	27 ± 3[c,d]	29 ± 1[d]	27 ± 2[c,d]
Daily food intake (g)	9.6	9.5	9.8	9.5
Liver weight (g)	6.9 ± 0.2[c]	7.1 ± 0.3[c,d]	7.4 ± 0.3[c,d]	7.8 ± 0.2[d]
Enzyme activities				
FAS[f]	12.3 ± 0.7[d]	11.3 ± 0.5[d]	7.6 ± 0.5[c]	7.6 ± 0.4[c]
G6PD[g]	110 ± 12[e]	82 ± 6[d]	47 ± 5[c]	50 ± 5[c]
In vivo FA-synthesis[h]	4,207 ± 238[e]	3,105 ± 234[d]	2,455 ± 185[c,d]	1,990 ± 107[c]

[a]Mean ± SEM, n=7.

[b]Mean ± SEM, n=9.

[c,d,e]Those values with different superscripts are significantly different ($P<0.05$).

[f]nMol reduced nicotinamide adenine dinucleotide phosphate oxidized min^{-1} mg $protein^{-1}$ at 37 C. FAS = fatty acid synthetase.

[g]nMol nicotinamide adenine dinucleotide phosphate reduced min^{-1} mg $protein^{-1}$ at 25 C. G6PD = glucose-6-phosphate dehydrogenase.

[h]Dpm ^3H incorporated into fatty acids per g liver in 10 min.

TABLE IV

Influence of Dietary 16:0, 18:1, 18:2, or 18:3 on Adipose Tissue Lipogenesis in Essential Fatty Acid Adequate Rats

	Body weight (g)	FAS[f]	G6PD[g]	In vivo FA-synthesis[h]
Experiment I[a]				
Basal[b]	142 ± 2[d]	28 ± 3[d]	128 ± 14[d]	19,329 ± 6,600[d]
+16:0[b]	151 ± 3[d]	31 ± 1[d]	128 ± 14[d]	29,972 ± 3,485[d]
+18:1[b]	151 ± 3[d]	30 ± 3[d]	144 ± 14[d]	28,409 ± 5,796[d]
+18:2[b]	151 ± 2[d]	30 ± 1[d]	128 ± 14[d]	28,221 ± 7,491[d]
Experiment II[a]				
Basal[b]	150 ± 2[d]	25 ± 9[d]	---	17,567 ± 6,004[d]
+18:1[b]	156 ± 2[d,e]	34 ± 3[d]	---	9,704 ± 2,927[d]
+18:2[b]	161 ± 2[e]	37 ± 5[d]	---	9,230 ± 2,537[d]
+18:3[b]	159 ± 33[e]	36 ± 4[d]	---	9,722 ± 1,543[d]

[a]Basal diet contained 2.5% and 1% safflower oil in Experiments I and II, respectively.

[b]Mean ± SEM, n=7.

[c]Mean ± SEM, n=9.

[d,e]Those values within an experiment with different superscripts are significantly different ($P<0.05$).

[f]nMol reduced nicotinamide adenine dinucleotide phosphate oxidized min^{-1} mg $protein^{-1}$ at 37 C. FAS = fatty acid synthetase.

[g]nMol nicotinamide adenine dinucleotide phosphate reduced min^{-1} mg $protein^{-1}$ at 25 C. G6PD = glucose-6-phosphate dehydrogenase.

[h]Dpm ^3H incorporated into fatty acids per g liver in 15 min for Experiment I and 10 min for Experiment II.

tained by Duncan's multiple range (Experiment I) and Tukey's *t*-test (Experiment II). Data for the one meal of 18:3 were compared to basal groups using a Student's *t*-test (16).

RESULTS AND DISCUSSION

Growth Parameters

Because the animals in these experiments (Tables II-V) were pair-fed, no differences existed in food or carbohydrate intakes. Final body weight and total weight gain for 7 days were comparable among treatments after 7 days (Table II), but 10 days of ester supplementation resulted in greater body weight and weight gain (Table III). The heavier fat pad weights for the 18:1 and 18:2 groups (Table II) are in accord with slightly greater energy intakes of

TABLE V

Effect of One Meal Containing 18:3 on Hepatic and Adipose Lipogenesis in Essential Fatty Acid Adequate Rats

	Experiment I[a]		Experiment II[a]	
	Basal	+3% 18:3[b]	Basal	+5% 18:3[b]
Body weight (g)	149 ± 2[c]	146 ± 6[c]	150 ± 2[c]	160 ± 6[c]
Total weight gain (g)	21 ± 1[c]	16 ± 1[d]	22 ± 1[c]	18 ± 1[c]
Daily food intake (g)	10.5	10.2	9.6	9.6
Liver weight (g)	6.6 ± 0.2[c]	6.4 ± 0.2[c]	6.9 ± 0.2[c]	7.1 ± 0.3[c]
Epididymal fat weight (mg)	818 ± 82[c]	812 ± 89[c]	---	---
Hepatic in vivo FA-synthesis[d]	7,221 ± 1,911[c]	4,630 ± 1,943[c]	4,207 ± 238[c]	3,657 ± 238[c]
Adipose in vivo FA-synthesis[e]	19,329 ± 6,600[c]	17,349 ± 7,974[c]	17,567 ± 6,004[c]	8,750 ± 1,700[c]

[a]Basal diet contained 2.5% and 1% safflower oil in Experiments I and II, respectively.

[b]Mean ± SEM where n=7 in Experiment I and n=6 in Experiment II.

[c,d]Those values with different superscripts within an experiment are significantly different ($P<0.05$).

[e]Dpm ^3H incorporated per g tissue in 15 min in Experiment I and in 10 min in Experiment II.

these animals due to the high digestibility of 18:1 and 18:2 (8). Although the digestibility of 16:0 was poor (57%), the inclusion of 2.5% safflower oil did improve the value over that determined in a fat-free diet (8).

Liver

Dietary 18:2 and 18:3 were very effective in depressing FAS and G6PD activities (Tables II and III), with 18:2 and 18:3 having equal efficacy (Table III). When the basal diet contained 2.5% safflower oil, 18:2 supplementation depressed ME activity the most but was only significantly ($P<0.05$) lower than the 18:1 group (Table II). The specific action of dietary 18:2 on liver FAS, G6PD, and ME activities in EFA-adequate rats is similar to its effect in rats fed fat-free diet supplemented with 18:2 (4-8). Contrary to the hepatic enzyme activity pattern in Experiment I, the in vivo rate of ^3H_2O incorporation into hepatic fatty acids showed no statistical change from basal due to dietary fatty acids (Table II). Such lack of statistical difference was partially the product of large experimental variation as indicated by an experimental coefficient of variation of 44%. Unlike Experiment I, supplementation of a basal diet containing 1% safflower oil (Experiment II) with 3% 18:2 or 18:3 resulted in a dramatic drop in the rate of ^3H_2O incorporation into liver FA and a significant ($P<0.05$) decline in hepatic FAS and G6PD activities (Table III). In addition, 18:1 supplementation precipitated a rate of liver FA synthesis and hepatic G6PD activity intermediate to the basal and basal plus PUFA treatments (Table III). Such a response for 18:1 was not found in Experiment I or in rats previously fed fat-free diet for 10 days and then intubated with oleate ester for 3 days (6). However, an intermediate effect of 18:1 has been found in rats pair-fed fat-free diet plus 18:1 for 7 days (5). The inability to detect an effect of 18:1 in Experiment I may be related to the higher level of safflower oil in the basal diet, which could have depressed hepatic lipogenesis to such a low point as to mask the influence of 18:1. In addition, the longer duration of Experiment II may have contributed to observing slight differences (6). The higher level of oil in Experiment I and the poor digestibility of 16:0 may also have prevented detecting an influence of 16:0 on liver lipogenesis (Table II).

Linoleate has been reported to be more effective than 16:0 or 18:1, but 18:3 and 20:4 supposedly have the greatest efficacy in dampening fatty acid synthesis (6,17). Data in Table III would suggest this is true, but its biological significance requires further elucidation. Using changes in G6PD activity as an indicator of the rate of rat liver FA synthesis, Gozukara et al. (10) have proposed PUFA do not have a specific inhibitory effect on hepatic FA synthesis but rather cause a reduction in carbohydrate intake which in turn lowers the rate of rat liver FA synthesis. Since animals among treatments in these studies had similar basal diet intakes, the action of 18:2 and 18:3 must be independent of carbohydrate intake (7,8,17).

Rat liver G6PD and ME activities appear to be closely related to dietary PUFA intakes (18). In addition to providing reduced nicotinamide adenine dinucleotide phosphate (NADPH) for FA synthesis, G6PD and ME provide reducing equivalents for desaturation and fatty acid chain elongation (19,20). Dietary 18:2 and 18:3 not only depress FA synthesis but also reduce the need for NADPH in chain elongation and desaturation. Conceivably, this reduced need for NADPH production could result in lowered enzyme activities (Tables II and III,

19). A similar but less pronounced phenomenon might accompany dietary 18:1, thus accounting for the intermediate values seen in Experiment II (Table III).

Adipose

Unlike rat liver, supplementing low-fat:EFA adequate diets (Tables II and III) or fat-free diets (8) with 16:0, 18:1, 18:2, or 18:3 does not precipitate a change in FAS or G6PD activities or FA synthesis rates. Rates of adipose tissue FA synthesis in these experiments (Tables II and III) are complicated by large degrees of variation between basal and fatty acid treatments. However, in both experiments the fatty acid supplemented groups within an experiment have very similar rates of lipogenesis. The level of dietary fat (3%) in our studies with meal-fed rats may be too low to exert an inhibitory effect on fatty acid synthesis in adipose tissue. The influx of glucose into adipose tissue and production of glyceride glycerol may be sufficiently large to permit rapid esterification of incoming fatty acids generated by lipoprotein lipase (21). Such action would prevent free fatty acids or their CoA derivatives from exerting an inhibitory influence on adipose tissue lipogenesis rates (22).

One Meal 18:3

Two further experiments were conducted in an attempt to explain the specific action of 18:3 on rat liver FA synthesis and to ascertain the length of time required to elicit such a response. Although the absolute rates of liver and adipose tissue FA synthesis were lower for the basal plus 3 or 5% 18:3 diets, neither level of 18:3 significantly ($P<0.05$) depressed rat liver or adipose tissue FA synthesis. Hill et al. (23) found that rats fed ad libitum and intubated with 2.0 ml corn oil displayed a tremendous drop in liver slice lipogenesis within 3 hr, and this inhibition continued for several hours. The level of 18:3 fed in Experiment II was 0.75 ml. Not only was the amount of fat consumed substantially less in these experiments, but, as previously discussed, the large simultaneous influx of carbohydrate may prevent a significant increase in plasma and tissue FFA. Therefore, the meal-eating animal may be less sensitive to dietary fat inhibition of lipogenesis than the nibbling rat.

Similar to effects found in rats consuming an EFA-deficient diet, 18:2 and 18:3 possess a capacity to elicit a specific suppression of rat liver FA-synthesis within 7-10 days (Tables II and III). However, this effect is not immediate since one meal containing 3 or 5% 18:3 did not significantly alter the rate of liver FA synthesis (Table V). The reason for this lack of influence of one meal may be due to very rapid clearance by peripheral tissues (e.g., adipose) of the absorbed fat so that the potential FFA effectors do not reach the liver in significant quantities. Unlike liver tissue, adipose tissue of meal-fed rats consuming EFA-adequate or EFA-deficient diets (8) is insensitive to dietary 18:2 or 18:3. The reason for this observation may be related to the rapid influx of carbohydrate and subsequent production of α-glycerophosphate.

Since animals in these experiments were fed equal amounts of carbohydrate and EFA-adequate diets, the effects of 18:2 and 18:3 on liver lipogenesis appear to be specific and independent of carbohydrate intake and EFA status.

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Lipid Synthesis by Perfused Lung

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ABSTRACT

An isolated lung ventilated with pulses of negative pressure and perfused through the pulmonary vasculature was utilized for the study of 3-sn-phosphatidylcholine synthesis. The perfusion fluid consisted of a Krebs-Ringer phosphate buffer with 6% bovine serum albumin, pH 7.4, and the appropriate substrate. The simultaneous incorporation of (1-^{14}C) palmitate and (2-^{3}H) glycerol and the simultaneous incorporation of (CH_3-^{14}C) choline and (CH_3-^{3}H) methionine were examined. From these experiments it is concluded: 1) lung tissue incorporates (2-^{3}H) glycerol into 3-sn-phosphatidylcholine to a greater extent than any other lipid examined; 2) both choline and methionine contribute to the synthesis of 3-sn-phosphatidylcholine, and 50-70% of the label in its nitrogen base is derived from choline and 30-50% from methionine; and 3) a high PO_2 appears to reduce the synthesis of 3-sn-phosphatidylcholine.

INTRODUCTION

The prevention of alveolar collapse during expiration is primarily due to the presence of a surface active substance known as lung or pulmonary surfactant (1,2). Under certain pathological conditions, inadequate surfactant or abnormal surfactant appears to occur. To understand these pathologies, it may be necessary to examine the normal lung tissue synthesis of 3-sn-phosphatidylcholine (PC) and to identify the regulatory mechanisms involved.

Most of the surface active properties of lung surfactant are due to the saturated phospholipid component of the material, dipalmitoyl-3-sn-phosphatidylcholine (dipalmitoyl-PC) (3). There are contradicting reports in the literature as to whether lung tissue synthesizes this material primarily do novo, or whether it modifies (as to fatty acid composition) the PC supplied by the blood (4-7). Blood PC containing at least one unsaturated fatty acid might be modified in the lung by a combination of a deacylation-acylation cycle and lung lipase (8). It has also been suggested that blood 3-sn-lyso-phosphatidyl-choline (lyso-PC) may be converted to PC by the lung (7). Lung tissue has the ability to synthesize PC de novo, but disagreement exists as to the relative contributions of the two main pathways (i.e., methylation vs. cytidinemonophosphate-phosphorylcholine) to the formation of the nitrogenous base in the de novo synthesized PC (9-12).

The synthesis of PC has been studied in subcellular particles, lung tissue slices, and whole animals. It is possible that these previous studies concerning themselves with systems that no longer are integrated (subcellular particles and tissue slices) or with systmes that cannot be easily controlled (whole animals) do not reflect the PC synthesizing ability of the lung as an organ.

In this communication, an isolated, perfused lung preparation has been employed to examine the incorporation of glycerol, palmitate, choline, and the methyl group of methionine into PC. Similar preparations have been utilized r ently by others for biochemical studies (13). It is concluded from the present experiments: 1) lung tissue has a high capacity to synthesize phosphatidylcholine de novo, 2) ca. 1/3 of the nitrogenous bases of the newly synthesized PC are formed via the methylation pathway, and 3) enriched oxygen atmospheres tend to inhibit the de novo synthesis of the PC at the level of nitrogenous base formation.

MATERIALS AND METHODS

Animals

Two- to three-month old female Wistar rats and Syrian golden hamsters were used. The Wistar rats came from Hilltop Laboratory Animals (Scottsdale, PA). Hamsters were from Engle Laboratory Animals, Inc. (Farmersburg, IN).

Animals were isolated for acclimation and for detection of incipient infections at least 3 weeks before being used. They were fed water ad libitum and Wayne Lab-Blox from Allied Mills, Inc. (Chicago, IL). The Lab-Blox contains 24.5% protein, 4.15% fat, and 49.7% nitrogen-free extract.

Materials

Choline chloride (4.99 mCi/mmol) (1,2-^{14}C) and glycerol (500 mCi/mmol) (2-^{3}H) were purchased from New England Nuclear (Boston,

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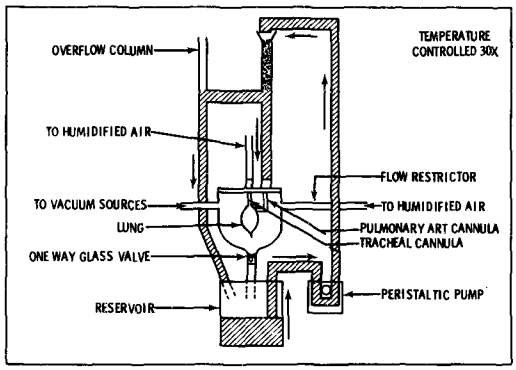

FIG. 1. Schematic diagram of perfused lung preparation.

MA); methionine (1,000 mCi/mmol) (L-Me-^{3}H) from International Chemical and Nuclear Co. (Irvine, CA); and palmitate (45.8 mCi/mmol) (1-^{14}C) from Volk Radiochemical Co. (Burbank, CA).

Unlabeled choline chloride was purchased from Sigma Chemical Co. (St. Louis, MO); L-methionine from Nutritional Biochemical Co. (Cleveland, OH); glycerol from Mallinckrodt Chemical Co. (St. Louis, MO); and palmitic acid from Fisher Scientific Co. (Fairlawn, NJ). The unlabeled materials were used for maintaining approximately optimal concentrations of the substrates in the study.

Bovine serum albumin fraction V was obtained from Miles Lab., Inc. (Kankakee, IL); silica gel without binder for the thin layer chromatography (TLC) of phospholipids from Camag (Muttenz, Switzerland); and Silica Gel G from TLC of neutral lipids from E. Merck, A.G. (Darmstadt, West Germany).

Neutral lipid standards used for TLC were purchased from The Hormel Institute (Austin, MN) and phospholipid standards from Applied Science Laboratories, Inc. (State College, PA). The Aquasol scintillation cocktail used for radioassay was purchased from New England Nuclear Pilot Chemical Division (Boston, MA).

Lung Perfusion

The basic lung perfusion preparation and methods of Rosenbloom and Bass (14) were used. The preparation consisted of the excised lung and trachea of a hamster or rat, ventilated with pulses of negative pressure, while being perfused through the pulmonary vasculature. A simplified schematic drawing of the preparation is shown in Figure 1. The lung was housed in a glass container and suspended from the plexiglass lid through which pass the cannulae for the trachea and pulmonary artery. Pulses of negative pressure were exerted in the chamber, causing the lung to inflate. Deflation was allowed by bleeding air back into the chamber between inflations. The trachea was attached to a humidified supply (at ambient pressure) of either room air or oxygen-enriched air. The perfusion fluid was drawn from a reservoir beneath the lung by a peristaltic pump and passed to the top of the H-shaped column. The fluid filled the column to the horizontal bar of the H. The height of this bar maintained the constant perfusion pressure, while media in excess of that flowing through the lungs returned to the reservoir via the other leg of the H column. The perfusion fluid entered the lung through a cannula tied into the pulmonary artery. The fluid was oxygenated through the lung as occurs physiologically. After coursing throughout the lung vasculature, it left the lung via the pulmonary veins and dropped to the reservoir. The entire system was enclosed in a temperature-controlled box. The basic perfusion fluid consisted of a Krebs-Ringer phosphate or bicarbonate buffered solution containing 6% bovine serum albumin. Two potential energy and carbon sources were used in the course of these studies: glucose (0.05 M) in the case of nitrogenous base incorporation, and citrate (0.05 M) in the case of glyceride and palmitate. Rates of either bases or palmitate and glycerol incorporation into glycerides were approximately the same. This would imply that either (a) both materials serve as carbon source, or (b) endogenous levels of carbon and energy sources in the cells are such that added glucose or citrate did not affect energy or carbon requiring processes.

In the experiments described here, the preparations were maintained at 30 ± 2 C, and the perfusion pressure was a constant 15 cm H_2O. The tidal volume was 1-1.5 cc for hamsters and 2-2.5 cc for rats, and the respiration rates were 70 ± 5/min for hamster lungs and 60 ± 5/min for rat lungs. The perfusion flow rates were 4.0-4.5 ml/min for rats and 2.0-2.5 ml for hamsters. The volume of perfusate was 15 ml for each lung. The concentration of the substrates in the perfusion fluid were 1.4 mM palmitate, 5 mM glycerol, and 2 mM nitrogenase base or methyl donors.

In preliminary experiments in our laboratory, representative sections of lung tissue were taken from perfusion preparations after either 10, 20, 30, 45, 60, or 90 min of perfusion. Sections were placed in 10% neutral buffered formalin and subsequently processed by standard procedures and stained with hematoxylin and eosin. Light microscopic examination failed to reveal any abnormal tissue and cell structure. As an indication of metabolic

TABLE I

Simultaneous Incorporation of (1-^{14}C) Palmitate and (2-^{3}H) Glycerol into Lipids by Perfused Hamster Lung[a]

Perfusion period (min)	Number of experiments	Labled substrate	nmol/500 mg lung tissue[b]			
			1,2-DG	TG	PC	PE
10	3	(2-^{3}H) Glycerol	2.3 ± 1.7	2.7 ± 1.3	21.8 ± 4.2	5.8 ± 0.9
10	3	(1-^{14}C) Palmitate	9.2 ± 3.9	5.5 ± 1.9	20.3 ± 6.8	5.8 ± 0.8
10	3	(2-^{3}H) Glycerol[a] / (1-^{14}C) Palmitate	0.2 ± 0.1	0.2 ± 0.1	0.8 ± 0.05	1.2 ± 0.2
40	5	(2-^{3}H) Glycerol	3.5 ± 1.2	6.5 ± 2.0	76.5 ± 2.7	17.2 ± 2.1
40	5	(1-^{14}C) Palmitate	10.7 ± 1.7	40.6 ± 7.8	107.0 ± 8.4	18.7 ± 1.8
40	5	(2-^{3}H) Glycerol[c] / (1-^{14}) Palmitate	0.3 ± 0.1	0.2 ± 0.5	0.7 ± 0.03	1.0 ± 0.1

[a]Perfusion conditions are described in Materials and Methods. Phosphate buffer was used as the perfusion fluid containing 6% bovine serum albumin, 0.05 M citrate, 1.4 mM palmitate, and 5 mM glycerol. The flow rate was 2.0-2.5 ml/min and the total perfusate volume 15 ml. Results are expressed as means ± SEM between experiments.

[b]1,2-DG = 1,2-diacyl-sn-glycerol, TG = triacyl-sn-glycerol, PC = 3-sn-phosphatidylcholine, PE = 3-sn-phosphatidylethanolamine.

[c]The ratio was calculated from the individual observations.

integrity, the linearity of acetate incorporation into fatty acids and the lactic acid production from glucose by the perfused lung were examined. Weights of the lung preparations were taken before and after perfusion as an indication of edema. During the actual experiments, the integrity of the lung was judged by its external appearance, ease and modality of respiration, and changes in weight. Results are reported only from those lungs which appeared normal during perfusion in all criteria examined.

Analytical Procedures

At the end of the experimental perfusion period, the lungs were perfused with saline to remove the labeled perfusion fluid from the large vessels. The lungs were then stripped to remove the trachea and large bronchi and homogenized in chloroform:methanol (2:1). Extraction of lipids, their fractionation by TLC, and radioassay of samples were performed as previously described (15). Simultaneous measurement of ^{3}H and ^{14}C in the double-label experiments was made in a two-channel liquid scintillation counter (5).

The fatty acid composition of the individual lipid classes was determined from the corresponding bands of the TLC plates. By using long (40 cm) TLC plates, we avoided contamination of the examined lipid classes with any detectable amount of ether lipids. Silica gel bands containing the lipids were scraped into screw-capped test tubes containing 4% H_2SO_4 in anhydrous methanol and were left overnight at room temperature. Water, followed by petroleum ether, was then added to the tubes. The upper phase containing the esters was then evaporated to dryness under a stream of nitrogen. The esters were analyzed using a gas chromatograph equipped with dual flame ionization detectors. The glass column, 1.83 m by 2 mm inside diameter, packed with 15% diethylene glycol succinate on 100-120 mesh Gas Chrom P, was operated at 190 C with an inlet pressure of 1.1 kg/cm^2. Quantitative data were calculated by the formula Rt x h, where Rt is the retention time and h is the peak height (16).

RESULTS

For clarity, the results have been divided into two main parts. The first part includes results on the mode of formation of the 1,2-diacyl-sn-glycerol (1,2-DG) moiety of lipids and the second with the mode of formation of the nitrogenous base of PC and the effect of high PO_2 on its formation.

Simultaneous Incorporation of (2-^{3}H) Glycerol and (1-^{14}C) Palmitate

If the incorporation of (2-^{3}H) glycerol is indicative of de novo synthesis of glycerides, then the lung tissue synthesizes over 5 times more phospholipids than neutral lipids (Table I). This difference becomes greater with time because the rate of synthesis of neutral lipids decreases after 10 min although that of phospholipids remains about the same. Saponi-

TABLE II

Fatty Acid Composition of Major Lipid Classes from Perfused Hamster Lungs[a]

	% of Total fatty acids[b]		
	PC	PE	TG
14:0	1.5 ± 0.02	1.7 ± 0.5	1.4 ± 0.03
16:(B)[c]	0.4 ± 0.02	6.5 ± 2.0	0.3 ± 0.02
16:0	53.2 ± 1.2	23.4 ± 1.8	25.8 ± 1.1
16:1	4.7 ± 0.2	3.8 ± 0.8	6.4 ± 0.4
18:0	10.0 ± 0.0	23.9 ± 1.1	5.0 ± 0.5
18:1	14.1 ± 0.7	26.1 ± 0.9	34.8 ± 0.7
18:2	6.7 ± 0.4	11.5 ± 1.2	24.1 ± 0.7
18:3	4.5 ± 0.5	tr	tr
20:4	4.8 ± 0.1	tr	2.2 ± 0.1
22:6		?	

[a]Fatty acid composition of the three major lipid classes isolated from hamster lung in which the simultaneous incorporations of (2-^{3}H) glycerol and (1-^{14}C) palmitate were examined. The results are expressed as mean percentage ± SEM of three experiments.

[b]PC = 3-sn-phosphatidylcholine, PE = 3-sn-phosphatidylethanolamine, TG = triacyl-sn-glycerol.

[c]Probably branched chain not definitely identified.

fication of lipids and isolation of fatty acids indicated that no more than 5-8% of the ^{3}H activity was incorporated into the fatty acid fraction. The highest incorporation of glycerol occurred in PC, a result which would appear contrary to that observed with subcellular fractions (5). The (2-^{3}H) glycerol: (1-^{14}C) palmitate incorporation ratio serves as an indication as to whether palmitate incorporation is occurring via synthetic versus exchange reactions and whether the various lipid fractions are derived from a common 3-sn-phosphatidic acid pool.

In separate experiments not reported here, the ratio of glycerol to palmitate incorporation was the same between 1,2-DG and 3-sn-phosphatidic acid (PA). Therefore, if the labeling of the isolated 1,2-DG represents the pattern of labeling of a common PA pool from which both the neutral and phospholipids are derived, then the (2-^{3}H) glycerol: (1-^{14}C) palmitate ratio should be the same in 1,2-DG and phospholipids. The triglyceride (TG), having an additional fatty acid (three instead of the two for 1,2-DG and phospholipids), should have a (2-^{3}H) glycerol: (1-^{14}C) palmitate ratio 1/3 smaller than that of 1,2-DG (0.2 vs. 0.3), as would be expected if they were derived from a common 3-sn-phosphatidic acid and fatty acid pool. The ratio of ^{3}H:^{14}C at 40 min is used because these numbers represent values obtained after equilibration of the perfusion media and the lung has occurred. Shorter perfusion periods may not permit complete equilibration. Both PC and 3-sn-phosphatidylethanolamine (PE), on the other hand, have a much higher (0.7 and 1.00) ^{3}H:^{14}C ratio and thus do not appear to utilize the same pool as the neutral lipids.

The fatty acid analysis (Table II) indicates that PC and PE are not derived from the same PA pool because they do not have the same fatty acid composition. The incorporation of glycerol paralleled that of palmitate into PC, resulting in an almost constant (2-^{3}H) glycerol: (1-^{14}C) palmitate ratio.

Formation of the Nitrogenous Base of PC

It was observed that in the experiments utilizing the bicarbonate buffer, a rapid increase in the pH of the perfusate occurred unless acid was periodically added to the perfusate reservoir. The loss of CO_2, both into the alveolar spaces and from the open end of the constant pressure column, invariably led to a decreasing $[H^+]$ of the perfusate. For experimental convenience, a phosphate buffer was employed for the following experiments, eliminating the need for titration of the perfusate to maintain physiological pH. In addition, use of phosphate buffer not saturated with a gaseous atmosphere allowed us to examine the effects of breathing enriched O_2 on PC synthesis, uncomplicated by problems associated with other dissolved gases. Nevertheless, because bicarbonate buffer is most commonly used for studying lipid synthesis, some experiments were done with bicarbonate for comparison (Tables III and IV). The results using bicarbonate (Table III) and phosphate buffer (Table IV) appear compatible to the incorporation of the nitrogenous base and their ratios.

From the data in Table III, the relative contribution of the two pathways may be estimated by following the simultaneous incorpora-

TABLE III

Simultaneous Incorporation of (CH_3-^3H) Methionine and (CH_3-^{14}C) Choline into 3-sn-Phosphatidylcholine by Perfused Hamster Lung[a]

Perfusion period (min)	nmol/0.5 g lung		
	(CH_3-^3H) Methionine	(CH_3-^{14}C) Choline	(CH_3-^3H) Methionine[b] / (CH_3-^{14}C) Choline
10	4.5 ± 0.2	6.0 ± 1.9	0.3 ± 0.2
30	18.4 ± 3.4	22.7 ± 7.7	0.9 ± 0.2
60	33.1 ± 2.6	37.0 ± 6.5	0.7 ± 0.1
90	59.0 ± 10.6	78.9 ± 14.5	0.8 ± 0.1

[a]Perfusion conditions are described in Materials and Methods. Bicarbonate buffer was used in the perfusion fluid containing 6% bovine serum albumin, 0.05 M glucose, and 2 mM nitrogenous base. The flow rate was 2.0-2.5 ml/min and the total perfusate volume 15 ml. Results are expressed as means of four experiments ± SEM.

[b]The ratio was calculated from the individual observations.

TABLE IV

Effect of the Composition of Inspired Air on Incorporation of (CH_3-^{14}C) Choline and (CH_3-^3H) Methionine into 3-sn-Phosphatidylcholine by the Pefrused Lung of Rats and Hamsters[a]

Animal species	Composition of inspired air	nmol/g rat lung or 0.5 g of hamster lung		
		(CH_3-^3H) Methionine	(CH_3-^{14}C) Choline	(CH_3-^3H) Methionine[b] / (CH_3-^{14}C) Choline
Hamster	Room air	20.1 ± 2.4	38.2 ± 4.7	0.5 ± 0.02
	95% O_2 + 5% CO_2[c]	15.9 ± 0.6	25.5 ± 1.8	0.6 ± 0.02
Rat	Room air	58.7 ± 2.8	116.9 ± 6.9	0.5 ± 0.01
	95% O_2 + 5% CO_2	38.1 ± 2.5	71.0 ± 7.1	0.6 ± 0.04

[a]Perfusion conditions are described in Materials and Methods. Perfusion period was 40 min. Phosphate buffer was used in the perfusion medium containing 6% bovine serum albumin, 0.05 M glucose, and 2 mM nitrogenous base. The flow rate for the rats was 4.0-4.5 ml/min, for the hamsters 2.0-2.5 ml/min, and the total perfusate base. Results are expressed as mean of three experiments ± SEM.

[b]The ratio was calculated from the individual observations.

[c]Only two experiments were conducted.

tion of choline and the methyl group of methionine into PC. The methylation pathway would appear to be of great importance, as it was responsible for from 40% to 50% of the total de novo synthesis of PC when bicarbonate was used as a buffer (Table III) and ca. 35% when phosphate was employed. The (CH_3-^3H) methionine:(CH_2-^3H) choline ratio after 10 min was nearly constant for all the perfusion periods studied (Table III).

Exposure of certain animal species to increased partial pressures of O_2 leads to a variety of pathological, histological, and biochemical alterations in the lung, including decreased dipalmitoyl-PC concentrations (17). It was therefore considered important to examine the effect of oxygen composition of respired air on the perfused lung. After 40 min of perfusion, the lungs respiring the O_2-enriched atmospheres showed a reduction of synthesis by both pathways (compared to those respiring room air) (Table IV). Although the number of animals per group is small, the tendency for decrease ($P<0.05$) in synthesis by both pathways in O_2-enriched atmospheres is shown in both hamsters and rat, implying some degree of significance in the data.

DISCUSSION

There are a number of steps involved in the de novo synthesis of the examined glycerides. The primary reactions are 1) the formation of PA by acylation of sn-glycerol 3-phosphate (derived from glycerol and adenosine triphosphate; and 2) the hydrolysis of PA by phosphatase to give 1,2-DG, which is a central intermediate for both phospholipid and triacyl-sn-glycerol (TG) biosynthesis. The 1,2-DG can then unite with either an acyl-CoA to give a TG or with the appropriate nitrogenous base to give a phospholipid.

Glycerol incorporation into lipids is an indication of de novo lipid synthesis, as glycerol

incorporation requires the formation of two ester bonds for 1,2-DG and three ester bonds for TG or phospholipids, making the initial incorporation by exchange reactions unlikely. Palmitate, on the other hand, can donate its carbons by known exchange reactions and, therefore, can label lipids both through exchange reactions and through true biosynthetic processes. The ratio of (2-^{3}H) glycerol to (1-^{14}C) palmitate incorporation can indicate the existence of a common precursor pool and provide an estimate of exchange reactions. The results presented here indicate that the 1,2-DG unit of PC and PE arises from a different source than the 1,2-DG of the neutral lipids. This difference in the 1,2-DG moiety between neutral and phospholipids was observed previously with rat lung slices (18) and has been indicated by an observation of differential rates for incorporation of palmitate into neutral and phospholipids (19).

Our results, suggesting that the rat and hamster lung tissue are capable of de novo PC synthesis, agree with the results obtained from newborn rabbit lung slices when the glycerol incorporation was studied (20), from the rat lung perfusion experiments when the incorporation of glucose and long chain fatty acids into lipids were examined (21), and those where the incorporation of glycerol and palmitate into lipids in vivo and in vitro by adult rabbit lung was studied (4). Studies with rat lung slices (7), on the other hand, indicated very little de novo synthesis of PC. This discrepancy could be due to different methods of tissue preparation, as it has been demonstrated that lung slices are extremely labile and easily lose certain of their biosynthetic capabilities: for example, the ability to synthesize fatty acids from glucose (22). It has also been shown that the ability of lung tissue to synthesize PC de novo is lost during subfractionation (5). In our studies, we include all the species of PC; it is therefore possible that our estimate does not represent an exact measurement of the surfactant dipalmitoyl-PC.

The high de novo synthesis of PC found in this study is accompanied by a moderately large biosynthesis of PE. This is in keeping with the importance of the methylation pathway in PC synthesis, which would require a large pool of PE for stepwise methylation to form PC. While there was greater de novo synthesis of PE than of TG, more palmitate was incorporated into the TG.

The ratio of (2-^{3}H) glycerol to (1-^{14}C) palmitate incorporation into PC was constant during the 40 min perfusion. This observation is in good agreement with the report of the half-life of lung PC (6). This report stated that: "The half-lives of radioactivity in the tritium-labled glycerol and in the ^{14}C-labeled fatty acid portions were not significantly different in any of the four lecithin subfractions." PC can be formed from the 1,2-DG by the incorporation of a molecule of phosphorylcholine through the cytidine monophosphate-phosphorylcholine pathway. A second pathway for the synthesis of PC is through the stepwise methylation of PE using the methyl groups of S-adenosylmethionine. These two mechanisms of PC synthesis are usually referred to as de novo synthesis. The ratio of incorporation of methionine to choline is taken as a measurement of the relative contribution of the two mechanisms. In addition, free choline can be incorporated into PC by exchange reactions.

The ratio of incorporation of (CH_3-^{3}H) methionine to (CH_3-^{14}C) choline ranged from 0.53 to 1.00. When the lung tissue was perfused with bicarbonate buffer, the contribution of the methylation pathway was greater than when it was perfused with phosphate buffer. The methionine:choline incorporation ratio indicates a significant contribution to PC synthesis by the methylation pathway, which agrees with our previous findings with lung subcellular fractions (12) and with Morgan's suggestion of the importance of the methylation pathway (11). Bjørnstad and Bremer (23) also reported significant incorporation via the methylation pathway after intravenous injections in rats of the same compounds used in this study. The magnitude of synthesis via the methylation pathway in this study contrasts with that in reports on rat lung slices, in which the (CH_3-^{14}C) methionine incorporation into PC was only 2.6% of the incorporation of (CH_3-^{14}C) choline (10). The degree of utilization of methionine and choline in our study also contrasts with results from in vivo experiments where, after intravenous administration of L-(CH_3-^{14}C) methionine and (CH_3-^{3}H) choline, 160-250 times more ^{3}H from choline than ^{14}C from methionine was found in lung PC (9).

We believe that the perfused lung preparations allowed a more reasonable estimate to be made of the partition of the two major pathways for the formation of the nitrogenous base of PC than either lung subcellular fractions, lung slices, or whole animal experiments. This estimate may be more representative because of both the structural integrity of the perfused lung, as judged by microscopy, and the elimination of the biosynthetic activity of other organs. Inhaling an O_2-enriched atmosphere for 40 min tends to inhibit the formation of the

nitrogenous base in our preparations. Previous experiments (24,25) found that a high in vitro PO_2 interferes with the last step in the methylation of PE to PC by N-methyl transferase isolated from dog lung. In the present experiments, we found both a reduction in the incorporation of (^3H-CH_3) methionine and of (^{14}C-CH_3) choline into PC. This apparent reduction in the de novo synthesis of PC at the level of the nitrogenous bases may be one cause of the reduced amounts of dipalmitoyl-PC found after inhalation of O_2-enriched atmospheres, which may result in the development of pulmonary distress (17,24). Also, this reduction could account for our difficulty in maintaining lung preparations respiring 95% O_2 for >45-50 min (due to edema), while similar preparations respiring room air last for 2-3 hr.

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Utilization of L-Serine in the In Vivo Biosynthesis of Glycerophospholipids by Rat Liver[1]

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ABSTRACT

The incorporation of L-serine-U-^{14}C, L-serine-3-^{14}C, and D,L-serine-1-^{14}C into the glycerophospholipids of rat liver in vivo was determined over a period of 3 min to 13 hr following intravenous injection. The radioactivity from these serines was transferred to variable extent into the glycerol, fatty acid, and nitrogenous base parts of all the glycerophospholipids and neutral lipids. The half-lives and turnover rates of phosphatidylserine calculated from the precursor-product specific activity curves obtained with L-serine-U-^{14}C were 14 min and 0.28 μmol/min/liver, respectively. The half-lives and turnover rates of phosphatidylserine as measured from the decay data of lipid serine from all markers averaged, respectively, 8.2 hr and 0.008 μmol/min/liver. The discrepancy between these turnover rates was attributed to an underestimation of degradation of phosphatidylserine due to its continued biosynthesis and/or an extensive reutilization of L-serine. By monitoring the formation of radioactive lipid ethanolamine, it was found that phosphatidylserine was decarboxylated at one-half the rate of lipid serine biosynthesis. It is suggested that as much as one-half of total phosphatidylserine may be degraded by other mechanisms, such as base exchange with choline, ethanolamine, and serine, as already demonstrated in vitro by other workers. The time course and nature of labeling of phosphatidylcholine was consistent with an extensive conversion of radioactive L-serine to 1-carbon fragments and a rapid methylation of phosphatidylethanolamine to phosphatidylcholine.

INTRODUCTION

Extensive in vitro studies have demonstrated (1,2) that phosphatidylserine (PS) is synthesized in the microsomes of rat liver via exchange of L-serine for ethanolamine in phosphatidylethanolamine (PE). The PS thus formed may be subsequently decarboxylated in the mitochondria (2) to reform PE, which upon reentering the microsomes may be reutilized for exchange with more L-serine or be methylated to form phosphatidylcholine (PC) (3,4). Studies in rat liver slices have shown (5) that the radioactivity from L-serine-U-^{14}C becomes distributed into tissue lipids, reflecting the summation of independent pathways of L-serine metabolism. A similar complexity appears to exist in vivo (3). An alternative pathway of biosynthesis of PS involving the exchange of L-serine for choline in PC has been demonstrated in mouse fibroblasts (6,7). Furthermore, there is evidence (8,9) that lipid serine is readily exchanged in vitro with free L-serine, as well as with free choline and ethanolamine.

On the basis of the early pathways of phospholipid metabolism in rat liver and the initial time course of distribution of radioactivity of L-serine-3-^{14}C among the glycerophospholipids, Wise and Elwyn (10) have calculated the turnover rates for lipid choline and ethanolamine to be more than 10 times faster than those reported from measurements with other tracers (11-13). The turnover of lipid serine was found to be ca. 100 times slower than that of lipid choline (10). As a background for further work on molecular species, we have reexamined the labeling of rat liver phosphatides from L-serine containing other radioactive carbons and have demonstrated that the rates of biosynthesis of lipid serine are comparable to those of other hepatic glycerophospholipids.

MATERIALS AND METHODS

Radioactive Tracers

L-serine-U-^{14}C (162 mCi/mM) and L-serine-3-^{14}C (48 mCi/mM) were purchased from Amersham-Searle (Arlington Heights, IL). DL-serine-1-^{14}C (8.2 mCi/mM) was obtained from New England Nuclear (Boston, MA). The radiochemical purity of these compounds was stated to be 97% or better. For injection into the experimental animals, the above compounds were dissolved in normal saline to give 3.7 x 10^6 to 10.2 x 10^6 dpm/ml.

Animals

The experimental animals were male Wistar

[1]Presented in part at the AOCS Fall Meeting in Ottawa, September 1972.

rats (275-325 g) and had been maintained on a Purina chow diet for at least 2 weeks prior to experimentation. Each rat was injected with 1 ml of the radioactive serine solution via the jugular vein under light diethyl ether anesthesia. The animals were allowed to recover and had access to food and water. At the time of sacrifice the animals were again anesthetized with diethyl ether and the livers removed and immediately extracted as described below.

Isolation of Lipid Classes

Total lipids were extracted according to Folch et al. (14). The lipid extracts were resolved by thin layer chromatography (TLC) (15) on Silica Gel H with chloroform:methanol:acetic acid:water, 25:15:4:2 (v/v/v/v) to give PE, PC, sphingomyelin, one fraction containing a mixture of PS and phosphatidylinositol (PI), and another fraction containing total neutral lipids and cardiolipin. The lipid fractions were located by spraying with 2,7-dichlorofluorescein and viewing the plates under ultraviolet light. The lipids were eluted from the gel with chloroform:methanol:acetic acid:water, 50:39:1:10 (v/v/v/v) (16). Pure PS was separated from PI by TLC in the solvent system chloroform:acetone:methanol:acetic acid:water, 50:20:10:10:5 (v/v/v/v/v) (17).

Determination of Distribution of Radioactivity

To obtain the distribution of radioactivity between the diacylglycerol and nitrogenous base moieties, the phospholipids were subjected to acetolysis according to Renkonen (18). The excess acetic anhydride was destroyed by reacting with methanol at ice temperature, and the reaction mixture evaporated to dryness. The residue was dissolved in chloroform: methanol, 2:1, and resolved by TLC in heptane:isopropyl ether:acetic acid, 60:40:3 (v/v/v) (19). The diacylglycerol acetates, and any unhydrolyzed phospholipids remaining at the origin, were eluted as above and the radioactivities determined along with that in an aliquot of the original phosphatide. In control experiments with glycerol-1-^{14}C PC, over 95% of the total radioactivity was recovered in the diacylglycerol acetates using the above routine.

Alternatively, the distribution of radioactivity was determined by digestion with phospholipase C (B cereus), and the completeness of digestion was verified by TLC (20).

To distinguish between the radioactivity in the fatty acid and glycerol moieties, the diacylglycerol acetates were transmethylated and the radioactivity determined in the lipid extract after partitioning the reaction mixture between petroleum spirit (bp 30-60 C) and water. Radioactivity and specific activity were determined in a liquid scintillation spectrometer as described (21). For comparison, all values were normalized to represent the radioactivity recovered when given a dose of 10 x 10^6 dpm.

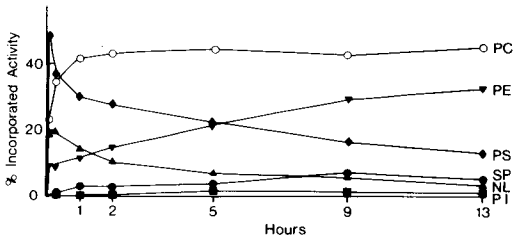

FIG. 1. Time-course of incorporation of L-serine-U-^{14}C into various lipids of rat liver in vivo. PC = phosphatidylcholine, PS = phosphatidylserine, PE = phosphatidylethanolamine, PI = phosphatidylinositol, NL = neutral lipid, SP = sphingomyelin. Injected dose: 10.2 x 10^6 dpm. Each point represents an average of 2-5 rats.

Determination of Free L-Serine

For this purpose, the livers were quickly frozen in liquid nitrogen and homogenized without prior thawing in 200 ml chloroform: methanol, 2:1. The homogenate was filtered through a Whatman No. 1 filter paper under mild suction. The filter cake was repeatedly washed by homogenization with 100 ml chloroform:methanol, 2:1, 100 ml distilled water, and 20 ml methanol, in this order. The chloroform: methanol filtrate was partitioned with 0.2 volume of 0.05 M NaCl. The lipid phase was treated as described above. The saline phase was pooled with the water and methanol filtrates, concentrated, and further deproteinated with 1% picric acid (22). Picric acid was removed, and aliquots of the free amino acid solution were separated (23) by a Beckman/Spinco Model 120 Amino Acid Analyzer. After passing through the colorimeter for mass determination, the eluate corresponding to serine was collected and aliquots counted in Aquasol (New England Nuclear). Quenching due to color in the eluate was corrected with standard radioactive serine and various amounts of the fraction corresponding to threonine which contained no radioactivity.

Methods of Calculation

Turnover rate was obtained by dividing the number of μmol of the product by the turnover time, which was obtained by the use of either the precursor-product specific activity curves or the decay in specific activity of the product (24).

TABLE I

Incorporation of L-Serine-U-^{14}C into Diacylglycerol,[a] Fatty Acid,[b] and Glycerol[c] Moieties of Rat Liver Glycerophospholipids

Time (min)	Diacylglycerols						Fatty acids		Glycerol	
	PS[d]		PE		PC		PC		PC	
	dpm	SA	dpm	SA	dpm	SA	dpm	SA	dpm	SA
3			448	5	334	2				
6			1,120	12	1,490	9				
10			1,120	12	1,490	9				
15	209	27	2,560	27	3,780	22	199	1	3,580	21
60	189	25	3,850	41	6,390	37	395	2	5,990	35
300	599	78	6,250	67	12,200	71	1,300	8	12,200	72
540	347	45	3,430	37	8,110	47	1,930	11	6,190	36
780	434	56			7,170	42				

[a]As estimated by the radioactivity recovered in the diacylglycerols liberated by phospholipase C or in the diacylglycerol acetates generated by acetolysis of the corresponding glycerophospholipids. Each estimate is an average of 2-5 rats.

[b]As measured by the radioactivity recovered in the methyl esters following transmethylation of the diacylglycerols. Each estimate is an average of two transmethylations.

[c]As estimated by subtracting the radioactivity in the fatty acids from the radioactivity in the total diacylglycerols.

[d]PS = phosphatidylserine, PE = phosphatidylethanolamine, PC = phosphatidylcholine, SA = specific activity, as dpm/μmol of phosphatide.

TABLE II

Incorporation of L-Serine-U-^{14}C into the Base Moieties[a] of Rat Liver Glycerophospholipids

Time (min)	Serine		Ethanolamine		Choline	
	dpm	SA[b]	dpm	SA	dpm	SA
3	5,540	719	580	6	1,470	9
6	8,030	1,040	1,670	18	3,810	22
10	16,500	2,140	1,550	17	8,000	47
15	20,400	2,650	2,030	22	16,400	96
60	26,200	3,400	6,120	65	30,100	176
300	27,100	3,520	15,900	170	43,300	253
540	15,400	2,000	22,900	245	30,700	180
780	14,900	1,940			44,200	258

[a]As estimated by subtracting the radioactivity in the neutral lipid moiety from that in the corresponding total glycerophospholipid. Each estimate is an average of 2-5 rats.

[b]SA = specific activity.

RESULTS AND DISCUSSION

Incorporation of L-Serine-U-^{14}C

Figure 1 gives the time-course of incorporation of L-serine-U-^{14}C into various lipids of rat liver following intravenous injection of the tracer. At 3 min, PS was the most extensively labeled lipid and accounted for nearly 50% of the total lipid radioactivity. With progressing time, the labeling fell to 35% of total at 15 min and to 13% of total at 13 hr. In contrast, the radioactivity of the PE increased steadily to ca. 33% of total at 13 hr. Except for the initial times, the bulk of radioactivity was found in the PC, which accounted for 38-45% of total. There was a rapid but transient incorporation of radioactivity in the neutral lipid fraction. It reached ca. 20% of total radioactivity at 15 min and declined thereafter to <4% by the end of the 13th hr. The percentage of radioactivity recovered over the period 3 min to 13 hr varied from 0.04 to 1.2% of the injected dose.

Table I shows the time-course of appearance of radioactivity in the neutral lipid moieties of the various glycerophospholipids following intravenous injection of L-serine-U-^{14}C. During the initial 15 min, ca. 50% of the total labeling of PE was due to radioactivity in the neutral lipid moiety. It decreased to ca. 12% of total after 9 hr. The neutral lipid moiety of PC accounted for 13-25% of the total radioactivity in this phospholipid throughout the experimental period. At 15 min, ca. 95% of the radioactivity in the diacylglycerol moiety of PC was

contributed by glycerol and 5% by fatty acids. With time, the proportion of radioactivity in fatty acids increased. The neutral lipid moiety of PS contained very little radioactivity at any time. The total amounts of radioactivity in the neutral lipid moieties of the glycerophospholipids were proportional to their mass distribution.

Table II gives the time-course of incorporation of radioactivity from L-serine-U-^{14}C into the nitrogenous bases of the three major glycerophospholipids of the rat liver. These values were obtained by subtracting the amount of radioactivity in the diacylglycerol moiety from that found in the corresponding total glycerophospholipid. It can now be seen that the specific activity of lipid serine throughout the experimental period was 5-100 times higher than that of lipid choline. Furthermore, the radioactivity in the choline moiety appeared to level off at about the 5th hr, while the ethanolamine moiety continued to increase in radioactivity even at 9 hr. This suggests that much of the radioactivity incorporated into the choline base was derived via methylation from the 1-carbon metabolite pool also labeled from the radioactive L-serine. At 15 min, the radioactivity of the choline moiety was ca. 8 times that of the ethanolamine moiety. The exact rate of methylation of lipid ethanolamine to lipid choline could be determined from the change of the specific activity of the 1-carbon pool (S-adenosylmethionine) and the specific activity of the methyl group of lipid choline at these times. The incorporation of L-serine-U-^{14}C into lipid serine increased rapidly up to ca. 60 min, after which time it began to decline.

Incorporation of L-Serine-3-^{14}C and D,L-Serine-1-^{14}C

The findings with L-serine-U-^{14}C were cross-checked with the data from L-serine-3-^{14}C and D,L-serine-1-^{14}C. Figure 2 gives the time-course of incorporation of L-serine-3-^{14}C into various lipids of rat liver following intravenous injection of the tracer. The percentage of total radioactivity recovered over the period of 15-540 min varied from 1.3 to 2.1%. The largest proportion of activity again was found in the PC, which accounted for 57-65% of the total at all times. PS was the next most heavily labeled lipid class and comprised 17% of the total labeling at 15 min. With progressing time the labeling fell to 11% of total at 300 min and 9% of total at 540 min. In contrast, the radioactivity of PE increased from ca. 5% at 15 min to 23% by the end of the experimental period. Again, there was a rapid but transient appearance of radioactivity in the neutral lipid fraction of the liver lipids.

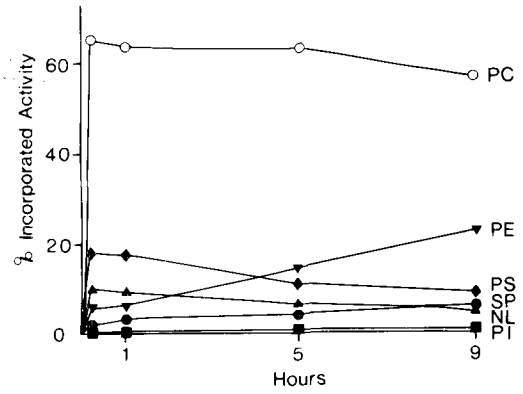

FIG. 2. Time-course of incorporation of L-serine-3-^{14}C into various lipids of rat liver in vivo. PC = phosphatidylcholine, PS = phosphatidylserine, PE = phosphatidylethanolamine, PI = phosphatidyinositol, NL = neutral lipid, SP = sphingomyelin. Injected dose: 3.7 x 10^6 dpm. Each point represents an average of 2 rats, except that at 15 min, which is from 1 rat.

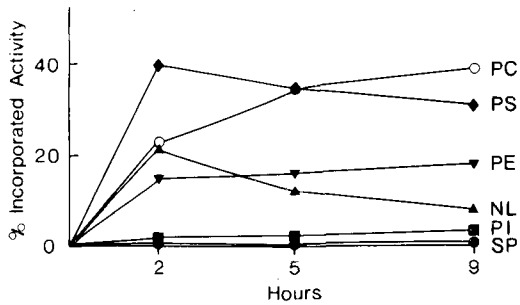

FIG. 3. Time-course of incorporation of D,L-serine-1-^{14}C into various lipids of rat liver in vivo. PC = phosphatidylcholine, PS = phosphatidylserine, PE = phosphatidylethanolamine, PI = phosphatidylinositol, NL = neutral lipid, SP = sphingomyelin. Injected dose: 5.8 x 10^6 dpm. Each point represents as average of 2 rats.

Figure 3 gives the distribution of radioactivity among the rat liver lipids after administration of D,L-serine-1-^{14}C. About 0.5% of the injected dose was recovered at each time of sampling. At early times, the bulk of the radioactivity was found in the PS, but PC also became labeled rapidly and surpassed the labeling of PS after the 5th hr. The amount of radioactivity detected in the PE was much lower.

Table III shows that the incorporation of radioactivity from L-serine-3-^{14}C and D,L-serine-1-^{14}C into the diacylglycerol moiety of the glycerophospholipids was quite similar to that seen for L-serine-U-^{14}C (Table I). However, the proportions of radioactivity found in the diacylglycerol portion of PC over the entire

TABLE III

Incorporation of L-Serine-3-^{14}C and D,L-Serine-1-^{14}C into the Diacylglycerol Moiety[a] of Rat Liver Glycerophospholipids[b]

Time (min)	PS		PE		PC	
	dpm	SA	dpm	SA	dpm	SA
L-serine-3-^{14}C						
15			3,010	32	2,000	12
60	64	8	3,500	37	5,340	31
300	108	14	2,100	22	4,920	29
540	232	30	3,590	38	7,110	42
D,L-serine-1-^{14}C						
120	49	6			9,710	57
300	67	9	5,570	60	11,500	67
540	244	32			11,800	69

[a]Estimated as described in Table I.

[b]PS = phosphatidylserine, PE = phosphatidylethanolamine, PC = phosphatidylcholine, SA = specific activity. Each value is an average from 2 rats, except that at 15 min, which is from 1 rat.

TABLE IV

Incorporation of L-Serine-3-^{14}C and D,L-Serine-1-^{14}C into the Base Moieties[a] of Rat Liver Glycerophospholipids

Time (min)	Serine		Ethanolamine		Choline	
	dpm	SA[b]	dpm	SA	dpm	SA
L-serine-3-^{14}C						
15	25,300	3,290	4,770	51	93,300	546
60	35,300	4,580	11,200	120	126,000	737
300	16,000	2,080	19,100	204	87,200	510
540	10,700	1,390	25,400	272	65,100	381
D,L-serine-1-^{14}C						
120	19,600	2,550			1,490	9
300	13,900	1,810	1,370	15	3,120	18
540	10,300	1,340			1,350	8

[a]Estimated as described in Table II. Each value is an average of 2 rats, except that at 15 min, which is from 1 rat.

[b]SA = Specific activity.

experimental period were much greater with D,L-serine-1-^{14}C than with L-serine-U-^{14}C (13-25%) or with L-serine-3-^{14}C (2-11%). At the same time, only 0-2% of total radioactivity in PS was contributed by the neutral lipid moiety. The relatively higher proportional contribution of radioactivity from D,L-serine-1-^{14}C to the neutral lipid fraction is due to the rapid loss and limited utilization of the radioactive carboxyl carbon for the biosynthesis of the nitrogenous bases.

Table IV gives the incorporation of radioactivity from L-serine-3-^{14}C and D,L-serine-1-^{14}C into the base moieties of rat liver phosphatides. It is seen that the labeling of lipid choline is 4 times higher from L-serine-3-^{14}C than from L-serine-U-^{14}C at 15 min and 2 times higher at 5 hr. This is a further indication that much more radioactivity was incorporated into lipid choline via the one carbon metabolite pool than via transfer of radioactive PE derived from decarboxylation of PS. The labeling of lipid ethanolamine from both L-serine-U-^{14}C and L-serine-3-^{14}C continued to rise slowly throughout the experimental period. On the basis of the relative labeling of the ethanolamine and choline bases, it was obvious that the contribution of radioactive L-serine-3-^{14}C to lipid choline via the ethanolamine moiety was much lower than that via the methylation pathway. The exact contribution, however, was not determined. Earlier work (3) with intraperitoneal injection of L-serine-3-^{14}C had given a value of 1/6th. With D,L-serine-1-^{14}C,

80-90% of the total radioactivity was recovered in the diacylglycerol moiety of the PC and PE samples analyzed. The maximum contribution of radioactivity from D,L-serine-1-^{14}C to the lipid choline and ethanolamine was 10-20% of total radioactivity in these phosphatides, and the absolute incorporation of radioactivity from D,L-serine-1-^{14}C (0.01-0.03% of injected dose) into the nitrogenous bases was much lower than that (0.2-0.9% of injected dose) from other radioactive serines at comparable times. Previously, Wilson et al. (25) found that D,L-serine-1-^{14}C gave rise to small amounts (0.004% of total radioactivity) of radioactive lipid ethanolamine as well as radioactive free ethanolamine and phosphorylethanolamine. Since the D,L-serine-1-^{14}C contained >95% of the total radioactivity in the carboxyl carbon, the bulk of radioactivity would be lost upon decarboxylation of the radioactive PS to PE. The radioactivity found in lipid choline and lipid ethanolamine may, therefore, be due to contributions of other minor radioactive serine species present as contaminants in the D,L-serine-1-^{14}C.

Turnover of Hepatic Glycerophospholipids

Figure 4 gives the radioactivity decay data for the serine phosphatides. A half-life of 8.1 hr was obtained from the decay of the specific activity of lipid L-serine-3-^{14}C. The half-lives of PS calculated from the decay data of radioactive lipid L-serine-1-^{14}C and L-serine-U-^{14}C were 7.5 and 8.9 hr, respectively. These times are about twice those (3.7 hr) calculated by Wise and Elwyn (10) from the specific activities of the precursors and products labeled from L-serine-3-^{14}C. Since this difference was clearly outside the range of experimental error and since both estimates were much lower than those obtained for other glycerophospholipids in more recent experiments (26-28), it was possible that both measurements were in error.

The turnover of the hepatic glycerophospholipids was reinvestigated by determining the specific activities of free serine, lipid serine, and lipid ethanolamine in the rat liver during the initial 3-18 min following intravenous injections of L-serine-U-^{14}C. Figure 5 shows that the specific activity of free serine peaks at ca. 6 min, as also observed by Wise and Elwyn (10). The specific activity of lipid serine continues to rise up to 18 min, also in agreement with the data of Wise and Elwyn (10). A calculation of the conversion of free serine to lipid serine (Table V) yielded an average value which was 10 times higher than that estimated by Wise and Elwyn (10). The discrepancy between the present and the previous values appears to

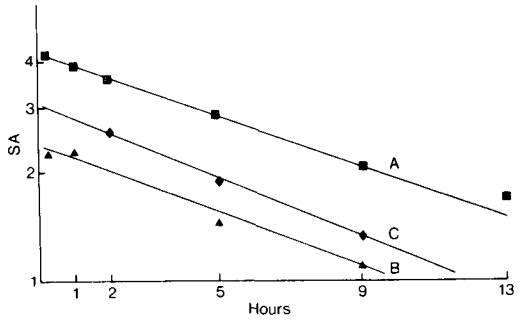

FIG. 4. Time-course of decay of specific radioactivity of lipid serine in rat liver in vivo following injection of different tracers. A = L-serine-U-^{14}C, B = L-serine-3-^{14}C; C = D,L-serine-1-^{14}C. Injected doses: as in Figures 1-3. The specific activity in curves A and B has been divided by the total radioactivity recovered from each liver. Each point is an average of 2 rats, except that for L-serine-3-^{14}C at 15 min, which is from 1 rat.

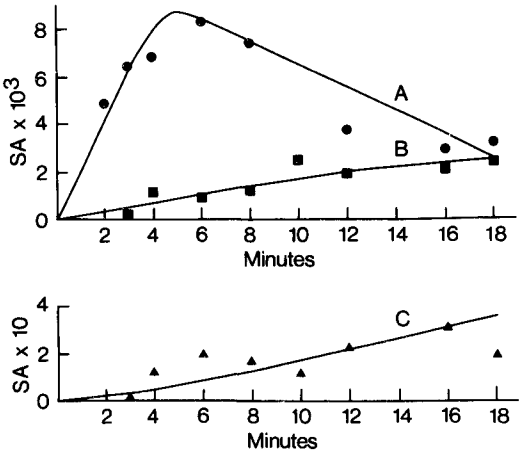

FIG. 5. Time-course of incorporation of L-serine-U-^{14}C into rat liver. A = free serine; B = lipid serine; C = lipid ethanolamine. Specific activity given as dpm/μmol. Each point is from 1 rat.

be due to the low specific activity of lipid serine (1-9% of that of free serine) in the 10-20 min time interval calculated by Wise and Elwyn (10). This is ca. 10 times lower than the values (22-76% of that of free serine) measured in the present experiments. This low specific activity of lipid serine in the study of Wise and Elwyn (10) apparently resulted from the use of the mass values of Collins and Shotlander (29) for hepatic lipid serine, which probably represented a more complete recovery of this lipid class than that achieved by themselves for the radioactivity of PS. Wise and Elwyn (10) reported difficulties in recovery of the lipid bases. In the present experiments, the mass and radio-

TABLE V

Turnover Rates[a] of Lipid-L-Serine-U-^{14}C

Time (min)	Transformation	
	Serine to lipid serine	Lipid serine to lipid ethanolamine
3-4	.23	.16
4-6	.19	.19
6-8	.23	.17
8-10	.21	.13
10-12	.25	.10
12-16	.30	.11
16-18	.57	.08
3-8	.22	.17
8-18	.33	.11
3-18	.28	.13

[a]As μmol/min/liver calculated with data from Figure 4 as described in Materials and Methods.

activity of PS were measured on similar or same preparations of PS. The new estimates of the rate of biosynthesis of PS of 0.28 μmol/min/liver compare closely to the rate (0.20-0.27) of biosynthesis of PC from choline-^{14}C (26-28) and to the rates (0.06-0.35) of biosynthesis of PE from ethanolamine-^{3}H or ethanolamine-^{14}C (12,27).

Table V also shows that the rate of lipid serine decarboxylation is ca. 0.13 μmol/min/liver, which accounts for ca. 50% of its rate of biosynthesis. Wise and Elwyn (10) had reported values of 0.024 and 0.030 μmol/min/liver, respectively, as the rates of synthesis and decarboxylation of PS, which implied that decarboxylation alone accounted for the degradation of PS. This latter rate of decarboxylation, however, was arrived at by attempting a best fit of experimental data assuming various rates of transfer of radioactivity from L-serine to lipid ethanolamine. This had been necessary because the rate of decarboxylation of PS calculated on the basis of lipid serine as the sole precursor of lipid ethanolamine had given a rate of degradation of 0.09 μmol/min/liver, which was ca. 4 times higher than their measured rate of synthesis of PS. The latter rate of decarboxylation of PS is within the range observed in the present experiments and could have been correct due to a constant ratio of the recovered lipid bases. Such a rate of degradation of PS would allow for other mechanisms of destruction of this phosphatide, such as exchange with free choline, ethanolamine, and unlabeled serine, as already demonstrated in vitro (8,9). Furthermore, this rate of decarboxylation of PS (0.13 μmol/min/liver) compares closely to the rate of biosynthesis of rat liver PE from ethanolamine-^{3}H and ethanolamine-^{14}C, which has been reported (12,27) to range from 0.06-0.35 μmol/min/liver. This suggests that the decarboxylation of lipid serine provides the main source of lipid ethanolamine as demonstrated previously by independent experiments (3,25).

Finally, it may be pointed out that the large discrepancy in the turnover rates of PS calculated on the basis of the decay data and the precursor-product specific radioactivities must be due to a continuous biosynthesis of radioactive PS in our experiments, because a true pulse label was not obtained, or to an extensive reutilization of L-serine. Under such conditions, the measured rates of lipid serine degradation would be lower than the true values.

In summary, these studies have demonstrated that the rate of biosynthesis of PS in the rat liver compares closely to the rates of biosynthesis of other glycerophospholipids in this tissue. However, decarboxylation to PE may account for only about one-half of the degradation of PS, the rest being possibly subject to base exchange with choline and ethanolamine. Further insight into the metabolism of PS and its conversion into other glycerophospholipids has been obtained by analyses of the molecular species of these phosphatides (Yeung and Kuksis, to be published).

ACKNOWLEDGMENTS

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Lipid Composition of Rat Sciatic Nerve

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ABSTRACT

In the course of our study on the lipids of the rat sciatic nerve, the analysis of the neutral lipids allowed us to detect and characterize cholesteryl esters present at a relatively high level (5%). Among the phospholipids, ethanolamine phosphoglyceride is the most abundant fraction and contains nearly all the plasmalogens (20% of total lipid phosphorus). The glycolipids consist of five different fractions; the cerebrosides with hydroxy fatty acids account for 38% of total glycolipids. Monogalactosyl diglyceride represents 7% of total glycolipids.

INTRODUCTION

Much is known about the histology and ultrastructure of the peripheral nervous system (PNS) (1-3), but numerous biochemical studies remain to be made, including studies of the lipids of PNS of numerous animals, using modern techniques. Recently, some reports (4-7) appeared dealing with the distribution of lipids and other compounds in the PNS of the cat, chickens, rabbit, and rat, both in normal nerve and during the Wallerian degeneration, a period where the normal biochemistry is deeply altered. For instance, the changes of the lipids occurring during this period have been recently reviewed (8).

We are interested in lipid changes in both proximal and distal stumps of the rat sciatic nerve during the Wallerian degeneration. But, before commencing a study of the lipids of the distal stump of the transected rat sciatic nerve as Wood and Dawson (7), we studied the distribution of the lipids in the normal tissue. The present paper deals with the neutral lipids, phospholipids, and glycolipids of peripheral nerve.

EXPERIMENTAL PROCEDURES

Reagents

All chemicals and solvents were of analytical grade. Di- and monopalmitin (both grade I) were purchased from Sigma (St. Louis, MO). Standards glycolipids (cerebrosides and sulfatides extracted from pig brain, monogalactosyl diglyceride from spinach leaves) were gifts. Cholesteryl stearate, tripalmitin, ascorbic acid, precoated plates (Catalog No. 5715), and Silica Gel G and H were purchased from Merck (Darmstadt, West Germany). Florisil 60-100 mesh came from Serlabo (Paris, France) and silicic acid Mallinkcrodt 100 mesh from Mallinckrodt Chemical Works (St. Louis, MO).

Plates

Precoated silica gel plates were used for fractionation and densitometry of neutral lipids and glycolipids. Silica Gel H coated plates, prepared according to the method of Neskovic and Kostic (9), were used for the study of phospholipids after thin layer chromatograph (TLC). Phosphorus was estimated after scraping the silica gel. Glycolipids were fractionated and isolated on similar plates. Silica Gel G coated plates, prepared with a 5% ammonium sulfate solution (10), were used for the fractionation of neutral lipids. For densitometry of neutral lipids, the precoated plates were first sprayed with the 5% ammonium sulfate solution (10) and dried. All the plates were activated at 100 C prior to use.

Sciatic Nerve

Throughout our study, we used adult rats of the Wistar strain of both sexes. After decapitation, the sciatic nerves were placed on dry ice, washed with cold saline, and lyophilized for 2 days; the dry tissue was stored in the cold (-20 C) on Actigel until used.

Extraction of the Lipids

Dried nerves (200 mg to 2 g) were cut into small pieces and lipids were extracted according to Suzuki (11). The lipid extract was washed (12) first with 0.2 vol of 0.88% KCl solution, then with the Folch theoretical upper phase in which water was replaced by the KCl solution (chloroform:metahnol:0.88% KCl solution, 3:45:47, v/v). After partition, the aqueous upper phases were pooled; these were used for the study of gangliosides (13). The lower phase was evaporated to dryness at 37 C in a rotating evaporator. The fatty residue was dissolved in a few milliliters of chloroform, and the solution was again evaporated. The residue was redissolved in a few milliliters of chloroform, and the solution was filtered through paper (Macherey Nagel and Co. Düren, West Germany) into a stoppered cylinder. Chloro-

TABLE I

Total Lipid Dry Weight, Cholesterol, and Lipid Phosphorus of the Sciatic Nerve of the Rat[a]

Total lipid dry weight	Total cholesterol	Lipid phosphorus	% Cholesterol/g dry weight / Total lipid/dry weight	% Phospholipids / Total lipid/dry weight
502 ± 49[b]	108 ± 8	9.5 ± 1.4	21.3 ± 3	48 ± 5
n = 12[c]	n = 13	n = 10	n = 13	n = 9

[a] Results are expressed in mg/g dry weight.

[b] The figures given are the mean values ± the standard error.

[c] n = number of estimations.

form was added to a known volume. This solution constitutes the total lipid extract (TLE), including the proteolipids.

Analytical Procedures

Total lipid dry weight was estimated after evaporation of an aliquot of TLE, at 37 C, to constant weight. Total cholesterol was estimated according to Mårtensson (14), and lipid phosphorus was determined by the method of Chen, Toribara, and Warner (15). Total plasmalogens were estimated according to Horrocks and Sun (16) on an aliquot of TLE. Scans were obtained with a Vernon integrator, with visible light and without filters.

Column Chromatography

The column (1 cm [inside diameter] x 16 cm) was prepared with prewashed silicic acid, according to Vance and Sweeley (17). The loading charge was ca. 30 μmol of lipid phosphorus. After adsorbtion on silicic acid, the lipids were eluted according to Vorbeck and Marinetti (18) in the following order: neutral lipids with 100 ml of chloroform, glycolipids with 40 ml of chloroform:acetone mixture (1:1, v/v), then with 80 ml of acetone. The phospholipids were eluted with 50 ml of chloroform:methanol (90:10, v/v), 50 ml of chloroform:metahnol (50:50, v/v) mixtures, and 100 ml of methanol. The eluates were evapoarated to dryness, taken up in a known volume of chloroform, and applied to the TLC.

Thin Layer Chromatography

Neutral lipids: The reference solutions were prepared by dissolving 10 mg of each sample (cholesterol, cholesteryl stearate, tri-, di-, and monopalmitin, stearic acid, and methyl palmitate) in 4 ml of chloroform. These solutions were chromatographed with aliquots of the neutral lipid fraction after column chromatography and TLE. The samples were streaked with a Hamilton microsyringe (100 μl), 1.5 cm from the bottom edge of the plate; each streak was 1 cm in length. The solvents systems used were derived from those of Freeman and West (19). The first solvent, benzene:diethyl ether: ethanol:acetic acid (50:40:2:0.2, v/v) was run up to 3 cm from the origin. After drying, the plate was put into the second solvent, hexane: diethyl ether (80:20, v/v), which was run to 15 cm from the origin. The third solvent, hexane, was used to bring the impurities to the solvent front. The spots were detected by exposure to iodine vapors (for scraping the silica gel), or after heating to 180 C for 30 min, for densitometry.

Glycolipids: Aliquots of a mixture of glycolipids standards, the enriched fraction of glycolipids (GL), and TLE were applied with a Hamilton microsyringe at 1.5 cm from the bottom edge of the plate and over 1 cm in length. Fractionation was achieved with the solvent system described by Nussbaum et al. (20): chloroform:methanol:20% aqueous ammonia (70:25:5, v/v). After migration, the plate was dried. The spots were detected with iodine vapors or alternatively by spraying the orcinol sulphuric acid reagent (21) and heating 10 min at 120 C until the spots appeared violet on a white background. For the densitometry, after heating, the plate was covered with a glass plate to prevent the fading of the staining.

Phospholipids: We applied on the bottom left corner of the plate an aliquot of TLE corresponding to 1 μmol of lipid phosphorus. Bidimensional TLC was carried out according to Nussbaum et al. (22) in two solvents systems: first direction (chloroform:methanol:20% aqueous ammonia, 70:25:5, v/v); second direction (chloroform:methanol:acetone:actic acid: water, 140:25:35:20:9, v/v). After drying, the plate was exposed to iodine vapors and the spots outlined. After sublimation of the iodine, the silica gel corresponding to each phospholipid was scraped and transferred to a test tube. The determination of phosphorus was achieved by the method of Chen et al. (15). Before reading, the silica gel was sedimented by centrifugation.

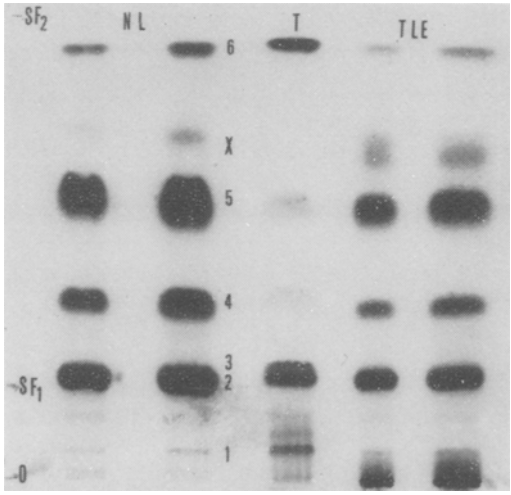

FIG. 1. Thin layer chromatography of the neutral lipid fraction (NL), total lipid extract (TLE), and standards (T). Numbers refer to: 1 = monoglycerides, 2 = cholesterol, 3 = diglycerides, 4 = free fatty acid, 5 = triglycerides, X = not identified, 6 = cholesteryl ester, 0 = origin, SF_1 = solvent front number 1, SF_2 = solvent front number 2. Detection after heating. For conditions, see Experimental Procedures section.

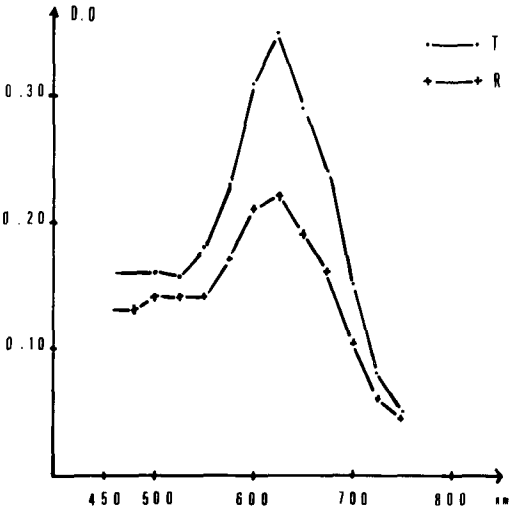

FIG. 2. Visible spectra after the Liebermann-Burchard reaction of pure cholesteryl stearate (T) and of the sample eluted after thin layer chromatography (R). For conditions, see Experimental Procedures section.

Plasmalogens: We followed the procedure of Horrocks and Sun (16) for the estimation of plasmalogens by bi-dimensional TLC. About 1 μmol of lipid phosphorus was applied. The two solvents systems used were: first direction, chloroform:methanol:20% aqueous ammonia (65:25:4, v/v); second direction, chloroform:methanol:acetone:acetic acid:water, (75:15:30:15:7, v/v). After the migration in the first direction, the plate was dried, placed face down for 5 min on a Pyrex tray containing concentrated HCl, and dried again in a stream of hot air. After the second solvent, the plate was dried and exposed to iodine vapors. The different spots were marked and the phosphorus content estimated according to Chen et al. (15).

RESULTS AND DISCUSSION

Expression of the Results

All results in this study refer to dry weight because the water content of the tissue was variable for the following reasons. First, during the collection of the nerves, there was a rapid exchange of the water content of the nerves with the surrounding atmosphere. Second, to remove hairs and droplets of blood it was necessary to wash the nerves with cold saline, and the remaining water content was unknown.

Individual factors, such as diet, hormonal state of the animal, as well as the length and thickness of each nerve, are important, but they were not taken into account in this study.

Composition of the Normal Rat Sciatic Nerve

Table I shows the composition of the lipids of the dry sciatic nerve, minus the glycolipid fraction (in preparation). Total cholesterol accounts for ca. 10% of the tissue dry weight and represents 21% of the total lipid dry weight. The value of 25% indicated by Pratt et al. (5) for the same ratio is close to ours. We note (Table I) that phospholipids account for about half of total lipid dry weight extracted from the tissue.

Neutral Lipids

Figure 1 shows a thin layer chromatogram of the neutral lipid fraction (NL), total lipid extract (TLE), and standards (T). In the neutral lipid fraction, we did not observe any spot corresponding to phospholipids, which would have remained at the origin (cf. the TLE fraction). In the chromatogram of the neutral lipids of the rat sciatic nerve, we observed sometimes a very faint band, between the origin and SF_1, which may be monoglycerides, fractionated by the first solvent from the remaining neutral lipids. At SF_1, there is a large spot which migrates as pure cholesterol and gives Liebermann Burchard reaction (14) after elution from Silica Gel G with chloroform. Above the cholesterol, occasionally there was a band which migrated at about the same height as the diglycerides

TABLE II

Distribution of the Neutral Lipids of the Dry Sciatic Nerve of the Rat after Thin Layer Chromatography of Total Lipid Extract[a]

Cholesteryl ester	Not identified	Triglycerides	Free fatty acids	Cholesterol + diglycerides
5.5 ± 0.8[b]	17.5 ± 4.5	21 ± 3.2	16.7 ± 1.6	39 ± 3.5
n = 18[c]	n = 18	n = 19	n = 16	n = 20

[a]Results are expressed in percentage of total neutral lipids.
[b]The figures are the mean values ± the standard error.
[c]n = number of estimations.

standard. As we were unable to clearly separate cholesterol from the diglyceride spot, in contrast to Freeman and West (19), we combined the two spots as one in our quantitative results. A compound of unknown composition "X" migrates just above the triglycerides, as one spot or sometimes two small; we do not know whether its presence is linked to the conditions of the storage of the sciatic nerve or is an artifact. We think that this substance is not a methylated fatty acid because the R_fs are different (0.76 for X, 0.86 for methyl palmitate). Preliminary results of the composition of the methylated fatty acids of this substance, measured by the gas phase chromatography, show a high content of palmitic acid, oleic acid, and, to a less extent, stearic acid.

At the same height as the cholesterol ester standard, there is a spot which, after elution from Silica Gel G, gave a positive Liebermann Burchard reaction (14) and has a similar visible spectrum as the cholesteryl stearate (Fig. 2). We have found and characterized cholesteryl esters which exist at a relatively high level (Table II). The presence of this compound in the sciatic nerve is controversial. Yates and Wherrett (6) and D'Hollander and Chevallier (23) found some cholesteryl esters in the normal rabbit and rat sciatic nerves. William et al. (24), Mezei et al. (25), and Berry et al. (4) estimated this compound, respectively, in the hen sciatic nerve, the chicken sciatic nerve before and after hatching, and in the cat and chicken sciatic nerves. Mokrasch (26) suggests that the occurrence of cholesteryl esters in myelin at a high level is a sign of a pathological state or of immature myelin. However, small amounts are encountered in mature myelin. Mannell (27) and Wood and Dawson (7) could not detect cholesteryl esters in the normal rat sciatic nerve but found it during Wallerian degeneration.

In Table II, we present the results concerning the distribution of the neutral lipids of the rat sciatic nerve after thin layer chromatography of TLE and densitometry. The values are distributed roughly into three classes. The highest value, including cholesterol and diglycerides, accounts for ca. 40% of the total neutral lipids, the triglycerides content for 21%. In addition to the high content of triglycerides, Berry et al. (4) found a low content of di- and monoglycerides in the normal sciatic nerve of the cat and the chicken and a low content of free fatty acids, compared to our values (16% of total neutral lipid content). The values of cholesteryl ester (5% of total neutral lipids) is high compared to other values in the literature (23-25).

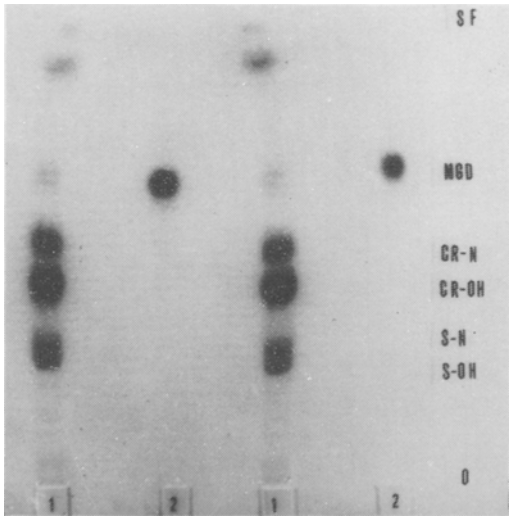

FIG. 3. Thin layer chromatography of the enriched glycolipids fraction (1) and monogalactosyl diglyceride (2). Orcinol sulphuric spray detection. For conditions, see Experimental Procedures section. SF = solvent front, MGD = monogalactosyl diglyceride, CR-N = cerebrosides with saturated fatty acids, CR-OH = cerebrosides with hydroxy fatty acids, S-N = sulfatides with saturated fatty acids, S-OH = sulfatides with hydroxy fatty acids.

Glycolipids

Figure 3 represents the pattern of the glycolipids of the rat sciatic nerve. At the same heights as the spot of pure monogalactosyl diglyceride (MGD), we observe a faint spot. We

TABLE III

Distribution of Individual Glycolipids of the Rat Sciatic Nerve after Thin Layer Chromatography of Glycolipid Fractions or Total Lipid Extract[a]

Monogalactosyl diglyceride (MGD)	Cerebrosides with saturated fatty acid (CR-N)	Cerebrosides with hydroxyl fatty acid (CR-OH)	Sulfatide with saturated fatty acids (S-N)	Sulfatide with hydroxyl fatty acid (S-OH)
7.2 ± 1.5[b]	28.4 ± 3.5	38.5 ± 3.5	15.5 ± 2.9	10.4 ± 2.2
n = 18[c]	n = 21	n = 20	n = 15	n = 18

[a]Results expressed in percentage of total glycolipids.
[b]The figures given are the mean values ± the standard error.
[c]Number of estimations.

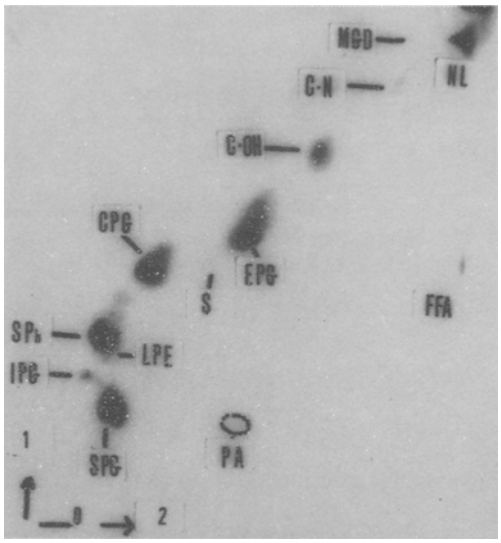

FIG. 4. Bi-dimensional thin layer chromatogram of an aliquot of total lipid extract. Iodine detection. For conditions, see Experimental Procedures section. MGD = monogalactosyl diglyceride, C-N = cerebrosides with saturated fatty acids, NL = neutral lipids fraction, C-OH = cerebrosides with hydroxy fatty acids, CPG = choline phosphoglycerides, EPG = ethanolamine phosphoglycerides, S = sulfatides, FFA = free fatty acids, Sph = sphingomyelin, LPE = lysophosphatidyl ethanolamine glycerides, IPG = inonitolphosphoglycerides, SPG = serine phosphoglycerides, PA = phosphatidic acid. 1 = first direction, 2 = second direction.

believe it is the same compound as that discovered by Steim and Benson (28), Norton and Brotz (29) in the brain, and by Neskovic et al. (30) in lipids of the rat brain. Inoue et al. (31) studied the distribution of this compound in the CNS and other tissues of the rat, and Yates and Wherrett (6) detected it in the sciatic nerve of the rabbit. Below this faint spot, according to Neskovic et al. (30), we observed two groups, each of two spots. The upper pair are well separated and correspond to cerebrosides with saturated fatty acids (CR-N) and hydroxy fatty acids (CR-OH). After staining with the orcinol sulphuric reagent, these spots appear deeply violet colored on a white background. Beneath are two spots, poorly resolved and faintly stained in purple. They correspond to sulfatides with saturated fatty acids (S-N) and hydroxy fatty acids (S-OH). The pattern of fractionation and the percentage of each glycolipid after densitometry were the same for the two fractions considered (TLE, GL). Table III represents the values of the percentages of individual glycolipids. CR-OH constitutes the most important fraction and accounts for 38% of the total glycolipids. This results seems to agree with those of Pratt et al. (5), who found

TABLE IV

Distribution of Sciatic Nerve Phospholipids, Including Proteolipids[a]

Fraction[b]	n	μmol lipid phosphorus g dry weight	Percent total lipid phosphorus	Pratt et al.[c] rat sciatic nerve (% total lipid phosphorus)	Evans & Finean[d] rat sciatic nerve myelin (% total lipid phosphorus)	Berry et al.[e] cat sciatic nerve (% total lipid phosphorus)	Sheltawy and Dawson[f] hen sciatic nerve (% total lipid phosphorus)
PA	14	3.9	1.60 ± 0.5[g]	---	---	---	1.3
Lyso-derivatives	12	6.4	3 ± 0.5	---	---	0.05	---
MPI	13	14	6.1 ± 1.1	19.9	19.0	21.22	11.1
SPG	14	30	14 ± 0.6	37.5	37.19	31.14	34.4
EPG	12	62	28.4 ± 2.1	25.7	23.96	21.90	14.6
EPG plasmalogens	20		21.3 ± 3.9	16.9	19.83	21.90	22.1
CPG	14	65	30.0 ± 1.30				
Sph (+ lyso EPG)	14	39	16.3 ± 1.3				

[a]Results expressed in μmol lipid phosphorus/g dry weight and in percent total lipid phosphorus.

[b]PA = phosphatidic acid, MPI = monophosphatidyl inositol, SPG = serine phosphoglycerides, EPG = ethanolamine phosphoglycerides, CPG, choline phosphoglycerides, Sph = sphingomyelin.

[c]Ref. 5.

[d]Ref. 32.

[e]Ref. 4.

[f]Ref. 33.

[g]The figures given are the mean values ± the standard error.

more polar cerebrosides (CR-OH) than non-polar cerebrosides (CR-N) in the normal rat sciatic nerve. The value for the CR-N fraction (28% of the total glycolipids) is roughly twice those of the values of S-N (15%) plus S-OH (10%). The MGD fraction represents 7% of the total glycolipids.

Phospholipids

Figure 4 shows the pattern of fractionation of the lipids of the rat sciatic nerve on a precoated plate. The use of the bi-dimensional TLC (22) allowed us to detect phosphatidic acid which is present at a very low level and, tentatively, some lysophosphatidyl choline near the phosphatidyl inositol spot (this spot is not on Figure 4). Lysophosphatidyl ethanolamine, which migrates with sphingomyelin, was estimated as one spot. We did not detect any cardiolipin, even if mitochondria were normally present in the Schwann cells (3) of the PNS. The estimation of plasmalogens according to Horrocks and Sun (16) allowed us to detect ethanolamine phosphoglycerides plasmalogens, some traces of serine, and choline phosphoglycerides plasmalogens. We did not estimate the latter two phospholipids.

We present in Table IV our data (left side of the table) concerning the individual phospholipids of the rat sciatic nerve. Our results are in reasonable agreement with the values in the literature presented on the right side of the table (4,5,32,33). We can divide the phospholipids of the rat sciatic nerve into three classes. The first contains the minor phospholipids, phosphatidic acid, choline lysoderivative, and monophosphatidyl inositol; all three account for 10% of total lipid phosphorus. The second class contains serine phosphoglycerides (14%) and sphingomyelin (+ lyso EPG), 16% of total lipid phosphorus. The highest values are those of choline phosphoglycerides (29%) and ethanolamine phosphoglycerides, including the values of plasmalogens (29%) and ca. 20% of the total lipid phosphorus.

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Quantitation of Phosphatidyl N-Methyl- and N,N-Dimethyl-aminoethanol in Rat Liver

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ABSTRACT

The contents of phosphatidyl N-methyl- and N,N-dimethylaminoethanol were determined in the liver of rats injected with (Me-C^{14}) methionine. Total phospholipids were extracted from aliquots of the liver and fractionated by two-dimensional thin layer chromatography after addition of carrier phosphatidyl-N-methyl- and N,N-dimethylaminoethanol. The radioactivity present in the two phosphatide spots was determined and used to calculate total disintegrations per min/100 g body wt. The remainder of the livers was pooled, and total phospholipids were isolated and subjected to acid hydrolysis. N-methyl- and N,N-dimethylaminoethanol were purified by thin layer chromatography, and their specific activity was determined after quantitation by gas liquid chromatography and radioactivity measurement. The liver contents of phosphatidyl N-methyl- and N,N-dimethylaminoethanol were determined by dividing disintegrations per min/100 g body wt by the specific activity of N-methyl- or N,N-dimethylaminoethanol.

INTRODUCTION

In a previous communication (1), a thin layer chromatographic (TLC) method was described allowing the separation of phosphatidyl N-methylaminoethanol (PMME) and of phosphatidyl N,N-dimethylaminoethanol (PDME), as well as of the other phospholipids, from rat tissues. Considerable amounts of both PMME and PDME were found to accumulate in liver and lungs of rats fed a diet supplemented with N-methylaminoethanol (MME). On the other hand, in rats fed a standard laboratory chow, neither PMME nor PDME could be quantitated since they were present in insufficient amounts. However, when (Me-^{14}C) methionine was injected into one of these rats and liver phospholipids were separated after addition of carrier PMME and PDME, considerable radioactivity was found in the spots corresponding to the two phosphatides. This finding undoubtedly reflected the fact that PMME and PDME, even though present in normal rat tissues only in trace amounts (2), are nonetheless very active metabolically as intermediates in the synthesis of lecithins via the stepwise methylation of cephalins (3). For this reason, it was decided to explore the possibility of quantitating PMME and PDME in the liver of rats.

TABLE I

Total and Specific Activity and Pool Size of PMME and PDME of Rat Liver after Injection of (Me-^{14}C) Methionine[a]

Pulse		Total activity (dpm/100 g body wt)		Specific activity (dpm/nmol)		Pool size (nmol/100 g body wt)	
		PMME	PDME	PMME	PDME	PMME	PDME
60 sec	1	28475	75225				
	2	56611	155493				
	3	90771	272566				
Mean		58619	167761	1072	1797	54.68	93.36
90 sec	1	100000	248748				
	2	91000	364197				
	3	78033	243763				
Mean		89677	285569	1380	3356	64.98	85.09
180 sec	1	98660	383035				
	2	181115	606825				
	3	50263	142213				
Mean		110013	377358	1844	4102	59.66	91.99

[a]PMME = phosphatidyl N-methylaminoethanol, PDME = phosphatidyl N,N-dimethylaminoethanol, dpm = disintegrations per min.

TABLE II

Total and Specific Activity and Pool Sizes of PC and PE in Rat Liver at Three Time Intervals after Injection of (Me-^{14}C) Methionine[a]

Pulse		Total activity (dpm/100 g body wt)		Specific activity (dpm/μmol)		Pool size (μmol/100 g body wt)	
		PE	PC	PE	PC	PE	PC
60 sec	1	2975	78226	52	877	57.07	89.19
	2	7044	153844	113	1533	62.56	100.35
	3	10595	283119	193	2851	55.31	99.31
Mean		6871	171729	119	1754	58.32	96.28
90 sec	1	3373	271538	82	3758	41.28	72.26
	2	3815	662835	149	7778	25.47	85.22
	3	14041	280292	250	3089	56.07	90.74
Mean		7076	404888	160	4875	40.94	82.74
180 sec	1	12188	1147660	177	9822	62.46	106.32
	2	23580	912017	260	6910	80.76	117.47
	3	6255	231529	93	2912	59.79	70.76
Mean		14007	763735	177	6548	67.67	98.18

[a]PC = phosphatidyl choline, PE = phosphatidyl ethanolamine, dpm = disintegrations per min.

A description of the approach followed in this study and of the results that were obtained is the object of this report.

MATERIALS AND METHODS

Nine male rats of the Sprague-Dawley strain (Sprague-Dawley, Inc., Madison, WI), weighing 81-104 g, were used. The animals were fed a choline supplemented diet (4) after an overnight fast and had access to the diet and to water ad libitum. After 24 hr, 10 μCi of (Me-^{14}C) methionine (specific activity 121 mC/mmol, obtained from New England Nuclear, Boston, MA) dissolved in 0.1 ml of saline was injected intraportally to the rats. The liver of three rats was taken at 60, 90, or 180 sec after injection of the label. The animals were kept under ether anesthesia from the time of laparotomy to removal of the liver.

The livers were weighed, and aliquots of ca. 0.5 g were immediately frozen in liquid N_2 and stored at -60 C. Total lipids were extracted (5) from these aliquots with a mixture of chloroform:methanol (2:1, v/v) and were separated by the two-dimensional TLC method reported previously (1) after addition of carrier PMME and PDME (General Biochemicals, Chargen Fall, OH). Phospholipid spots corresponding to cephalins (phosphatidyl ethanolamine [PE]), PMME, PDME, and lecithins (phosphatidyl choline [PC]) were scraped off the plates and eluted (6). Aliquots of the PE and PC eluates were used for lipid P determinations (7), and aliquots of all the eluates for radioactivity measurements.

The remainder of the livers of each time interval were pooled, and total lipids were extracted from each pool as indicated above. Total phospholipids were separated from neutral lipids by chromatography on silicic acid columns (8) and were then hydrolyzed with 6 N HCl for 3 hr at 100 C (2). The hydrolyzates were chromatographed on thin layer plates of Silica Gel H (Uniplates, Analtech, Newark, DE) using a solvent system of n-butanol:methanol: HCl-H_2O (50:50:10:10, v/v). Bands corresponding to reference standards of N-methylaminoethanol (MME) and N,N-dimethylaminoethanol (DME) (Eastman Organic Chemicals, Rochester, NY) were eluted with a mixture of methanol:acetic acid:H_2O (39:1:10, v/v) and were rechromatographed in the same manner. Rechromatography was needed to remove traces of ethanolamine from the MME band and of choline from the DME band obtained after the first separation. MME and DME were eluted as indicated above. The eluates were evaporated under a stream of N_2, and the residues were dissolved in methanolic KOH and neutralized as indicated by Lester and White (2). Aliquots were taken for radioactivity measurement and for quantitation by gas-liquid chromatography (2). Single peaks of MME and DME were obtained. Quantitation was made with the aid of an internal standard (2).

Radioactivities were measured with an Intertechnqiue Model SL30 liquid scintillation spectrometer equipped with a computer program for automatic correction of quenching. Samples of the phospholipids and of MME and DME were prepared for counting as previously reported (1).

TABLE III

Total Activity/100 g Body Wt as Percentage of the Injected Dose of Rat Liver Phosphatides at Three Time Intervals after Injection of (Me-^{14}C) Methionine[a]

Pulse		PE	PMME	PDME	PC
60 sec	1	0.014	0.134	0.354	0.369
	2	0.033	0.267	0.733	0.726
	3	0.050	0.420	1.285	1.336
Mean		0.032	0.276	0.791	0.810
90 sec	1	0.015	0.471	1.173	1.281
	2	0.017	0.429	1.718	3.13
	3	0.066	0.368	1.150	1.322
Mean		0.033	0.423	1.347	1.91
180 sec	1	0.057	0.465	1.807	5.414
	2	0.111	0.854	2.863	4.303
	3	0.030	0.237	0.671	1.092
Mean		0.066	0.519	1.780	3.603

[a]PE = phosphatidyl ethanolamine, PMME = phosphatidyl N-methylaminoethanol, PDME = phosphatidyl N,N-dimethylaminoethanol, PC = phosphatidyl choline.

The results were expressed and calculated as follows: PE and PC, μmol/100 g body wt, from lipid P determinations; total radioactivity in PMME and PDME, disintegrations per min (DPM)/100 g body wt, from the radioactivity obtained after two-dimensional TLC; PMME and PDME, μmol/100 g of body wt, from dividing the total radioactivity in PMME and PDME by the specific activity of MME and DME; specific activities, PE and PC, DPM/μmol lipid P, PMME and PDME, DPM/nmol MME or DME; total activities, DPM/μmol or DPM/nmol per liver, or as percentage of the injected dose.

RESULTS AND DISCUSSION

Table I shows the total radioactivity present in liver PMME and PDME 60, 90, and 180 sec after the injection of the label. There was some variation between animals in the same groups which we attribute mainly to difficulty in timing exactly the duration of the pulse. The radioactivity of both PMME and PDME increased with time, but at all three time intervals the radioactivity of PDME was ca. threefold greater than that in PMME. The specific activity of MME and DME, obtained as indicated under Materials and Methods after hydrolysis of the total phospholipids of three pooled livers, is also shown in Table I. As was the case with the total radioactivity, the specific activity of both MME and DME increased with time. At all three time intervals, the specific activity of DME was ca. twofold that of MME, reflecting the relative number of methyl groups present in the two phospholipid bases. Table I shows, finally, the liver content of PMME and PDME calculated as indicated under Materials and Methods. The content of PMME estimated at the three time intervals was 54.5, 65.0, and 60.0 nmol/100 g body wt, respectively, with an average of 59.8 nmol. The estimated content of PDME was ca. 50% greater than that of PMME and averaged 90.1 nmol/100 g body wt. Table II shows the total radioactivity, specific activity, and liver content of PE and PC. The total and specific activity of both PE and PC increased with time, but those of PE were almost negligible. Low incorporation of the methyl group of methionine into PE has been previously reported (3). The liver contents of PE and PC showed some variation between animals of the same group and averaged 55.6 and 92.4 μmol/100 g body wt, respectively. Table III shows the total radioactivity/100 g body wt as percent of the injected dose recovered into PE, PMME, PDME, and PC. At all three time intervals, the fraction incorporated into PE was indeed negligible and almost constant. The fraction of label incorporated into the three other phospholipids increased with time, that of PDME being greater than that of PMME and smaller than that of PC. The above results are consistent with PMME and PDME being intermediate in the methylation pathway from PE to PC and show that the liver content of the former two phospholipids is three orders of magnitude smaller than that of PE and PC, the major phosphatides of rat liver (1).

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Effects of Sex on Formation and Properties of Plasma Very Low Density Lipoprotein In Vivo[1]

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ABSTRACT

The concentration and composition of the very low density lipoprotein (VLDL) lipids and the behavior of the VLDL in a density gradient in the zonal ultracentrifuge were examined in plasma obtained from normal fed male and female rats before and after intravenous injection of Triton WR-1339. Concentration of lipids in plasma VLDL of female rats was about half that of male animals. Following injection with Triton WR-1339, the concentration of VLDL lipids was higher in female rats (triacylglycerol) or similar (phospholipid, cholesterol, and cholesteryl esters) in both sexes. Female rats secreted much more VLDL triacylglycerol into the plasma compartment than did the male animals under the same experimental conditions. No differences were observed in lipid composition of the VLDL or in the position of the VLDL in the zonal rotor after ultracentrifugation in a density gradient of the lipoprotein from plasma of normal male and female rats before treatment with the detergent. However, after treatment with Triton, a higher proportion of the VLDL particles isolated from plasma of female rats displayed a more rapid rate-zonal flotation in the ultracentrifuge than did the VLDL produced by the male. The VLDL secreted by female rats contained fewer moles of phospholipid and free sterol per mol triacylglycerol than did the VLDL secreted by male animals under identical experimental conditions. The molar ratio of free cholesterol:cholesteryl ester in the VLDL secreted after treatment with Triton increased in both male and female rats. Simultaneously, the content of arachidonic acid in phospholipid of VLDL increased with a concomitant decrease in cholesteryl ester. These changes in fatty acid composition suggest that the inhibitory effect of Triton on lecithin-cholesterol acyl transferase activity affects the exchange of lipids between VLDL and high density lipoprotein. It can be concluded from the data reported here that sex influences the concentration of plasma lipids in vivo and the output and properties of the VLDL.

INTRODUCTION

It was reported previously from this laboratory that the rate of output of triacylglycerol (TG) and the very low density lipoprotein (VLDL), and certain physicochemical properties of the VLDL secreted by isolated perfused livers from normal fed male and female rats, are sex dependent (2-5). It was observed that the output of TG as a component of the VLDL by perfused livers from female rats exceeded that of livers from male animals. Furthermore, the VLDL secreted in vitro by livers from female animals displayed a more rapid rate-zonal mobility in the ultracentrifuge and contained less phospholipid (PL) and cholesterol (C) per mol of TG than that produced by the male from equal quantities of oleate infused.

To correlate the observations obtained with perfused rat liver in vitro with regulatory mechanisms in the intact animal, the concentration and composition of the VLDL lipids and the behavior of the VLDL in a density gradient in the zonal ultracentrifuge were examined in plasma obtained from normal fed male and female rats before and following intravenous injection of Triton WR-1339. This detergent blocks the removal of VLDL from the plasma compartment (6,7), and the lipoprotein which accumulates can be considered to be representative of newly secreted VLDL (8). The data reported here indicate clearly that sex influences the concentration of plasma VLDL lipids in vivo and, as was observed in vitro, the output of plasma VLDL lipids, the rate-zonal mobility

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TABLE I

Concentration of VLDL Lipids in Plasma of Normal Fed Rats before and 2 hr after Intravenous Injection of Triton WR-1339

Group	VLDL lipid concentration (μmol/100 ml of plasma)[a]			
	TG	PL	C	CE
I. Before treatment with Triton				
A. Male (7)[b]	33.9 ± 3.5[c]	11.7 ± 1.1	5.5 ± 0.7	3.7 ± 0.2
B. Female (7)	18.6 ± 2.7	6.6 ± 0.6	3.6 ± 0.3	2.1 ± 0.1
II. Following injection of Triton				
C. Male (6)	219.7 ± 11.6	73.3 ± 3.5	49.4 ± 4.1	20.4 ± 2.2
D. Female (4)	314.6 ± 38.5	75.8 ± 6.1	49.2 ± 6.2	17.4 ± 0.9
Significance of differences (P values)				
A vs. B	0.005	0.001	0.01	0.001
C vs. D	0.05	NS	NS	NS
A vs. C	0.001	0.001	0.001	0.001
B vs. D	0.001	0.001	0.001	0.001

[a]VLDL = very low density lipoprotein, TG = triacylglycerol, PL = phospholipid, C = cholesterol, CE = cholesteryl esters.

[b]Figures in parentheses indicate number of observations in each group. Each observation was obtained on pooled plasma from at least four animals (see text).

[c]All values are means ± SE.

of the VLDL particles in the ultracentrifuge, and the molar ratio of TG to polar lipids. Furthermore, the relative proportions of C and cholesteryl esters (CE) and the percentage composition of the fatty acids in phospholipids and cholesteryl esters in the VLDL isolated from plasma of either sex before and after treatment with Triton WR-1339 lead us to suggest that lecithin-cholesterol acyltransferase (EC 2.3.1.43) (LCAT), which is inhibited by Triton WR-1339 (9-11), plays an important role in the dynamic equilibration among the different lipoprotein classes of specific lipid components.

EXPERIMENTAL METHODS

Chemicals

Chemicals used were reagent grade, and all solvents were redistilled from glass before use. Triton WR-1339 (p-iso-octylpolyoxyethylenphenol polymer) was purchased from Ruger Chemical Co. (New York, NY).

Animals and Experimental Design

Sprague-Dawley rats, purchased from the Holtzman Company (Madison, WI), were maintained on a balanced laboratory ratio (Purina Chow) and water ad libitum for at least 2 weeks before use. The rats weighed 250 ± 10 g (±SE) and were not fasted prior to sacrifice. One group of rats was injected via the femoral vein with 150 mg (0.75 ml of a 20% solution) of Triton WR-1339 in 0.9% NaCl. The control group received saline only. Blood was collected 2 hr later by aortic puncture into tubes containing 0.1 ml of 0.2 M ethylenediaminetetraacetic acid. Injections of Triton or saline, and blood collections, were carried out with the animals under light ether anesthesia.

Separation of VLDL

Plasma from several rats was pooled for isolation of the VLDL. Chylomicrons were removed from the plasma by centrifugation at 12,000 g for 20 min (12). The VLDL was isolated by zonal ultracentrifugation from 15 ml (Triton group) or 30 ml (control group) of chylomicron-free plasma (4).

Extraction and Analysis of Lipids

The fractions (tubes 1-18) obtained from the zonal rotor which contained the VLDL were combined, lyophilized, and extracted 3 times with 25 ml each of $CHCl_3$:CH_3OH, 2:1 (v/v). The extracts were washed, fractionated, and analyzed as reported elsewhere (5); phospholipids were separated with chloroform: methanol:acetic acid:water, 50:28:10:5 (v/v).

Calculations

The net production of VLDL lipid in male and female rats was calculated using the following equation: Production of VLDL lipid (μmol/g liver/hr) = $1/2\ [C_tV_t/LW_t\text{-}CV/LW]$, where C_t and C are μmol of VLDL lipid/ml of plasma from tritonized and nontritonized rats, respectively; V_t and V are ml plasma (calcula-

TABLE II

Output of Lipids in the VLDL in Normal Fed Rats Injected with Triton WR-1339[a]

Group	Output of VLDL lipids (μmol/g liver/hr)[b]			
	TG	PL	C	CE
Male (3)[c]	0.95 ± 0.09[d]	0.29 ± 0.02	0.19 ±0. 01	0.07 ± 0.01
Female (3)	1.84 ± 0.27	0.41 ± 0.06	0.26 ± 0.09	0.08 ± 0.01
Significance of differences (P values)				
	0.05	NS	NS	NS

[a]Output of VLDL lipids was calculated as descriabed in the text. Average body weight was 249.2 ± 8.1 g for the males and 254 ± 10.3 g for the female animals, respectively. Average liver weight was 10.7 ± 0.8 for the males and 9.5 ± 0.6 for the females.

[b]VLDL = very low density lipoprotein, TG = triacylglycerol, PL = phospholipids, C = cholesterol, CE = cholesteryl esters.

[c]Figures in parentheses indicate number of observations in each group. Each observation was obtained on pooled plasma from at least four animals (see text).

[d]All values are means ± SE.

ted at 4% of body weight) from tritonized and nontritonized rats, respectively; LW_t and LW = liver weight, g wet, of tritonized and nontritonized rats.

Three assumptions based on the literature (6,7,13,14) were made for these calculations: 1) Triton blocks almost completely the exit of TG from the plasma compartment. 2) The rate of increase of the plasma concentration of TG after Triton is linear as a function of time. In a single experiment, this linearity was confirmed under our experimental conditions. The rates of hepatic secretion of VLDL triacylglycerol in the male were observed to be 0.97 and 0.91 μmol/g liver/hr for the 1st and 2nd hr after Triton, respectively. Corresponding values for the female were 1.74 and 1.87. 3) The liver is the primary source of plasma VLDL.

The fatty acid composition of each lipid class (TG, PL, and CE) of VLDL secreted following injection of Triton was calculated from the total fatty acid output in each class and the output of individual fatty acids of the lipids. Both values were obtained using the above reported equation. In these calculations, the concentration of each individual fatty acid was determined from the total concentration of lipid fatty acids and its percent distribution obtained by gas-liquid chromatography.

The statistical significance of the differences between experimental groups was evaluated by means of a two-tailed table of Student's distribution for t. NS indicates a P value >0.05.

RESULTS

Plasma Concentration and Production of VLDL Lipids

The concentration of various lipids in the plasma VLDL of fed rats is sex dependent. Before treatment with Triton WR-1339, concentration of different VLDL lipids in the plasma of female rats was almost half that in plasma of male animals (Table I). Following injection with the detergent, the concentrations of all VLDL lipids increased in both sexes. The concentration of VLDL lipids was higher in the female (TG) or similar (PL, C, and CE) in both sexes. Clearly, as expressed in Table II, female rats secreted much more VLDL triacylglycerol into the plasma compartment than did the male animals under the same experimental conditions. Similar sex differences in the secretion rate of VLDL triacylglycerol have also been observed in rats fasted for 16 hr (I. Weinstein, F. Turner, and M. Heimberg, unpublished observations); in those experiments, output of VLDL triacylglycerol was 0.28 and 1.33 μmol/g liver/hr, for males and females, respectively.

No statistically significant increase was observed in the rate of secretion of PL and sterol of VLDL, although these values tended also to be higher in female rats compared to male animals. The lack of statistical significance for PL and C may be expected, since the particle of VLDL secreted by female rats contains less PL and C relative to TG than does the VLDL from male animals (Table III).

Zonal Pattern of the VLDL

No differences were observed after ultracentrifugation between the positions in the density gradient in the zonal rotor of the VLDL isolated from plasma of normal male or female rats (Fig. 1). After treatment with Triton, however, a higher proportion of the VLDL particles isolated from plasma of female rats displayed a more rapid rate-zonal mobility in the ultra-

Lipid Composition of Rat Plasma Very Low Density Lipoprotein

Group	Lipid class[a]				Molar ratio			
	TG	PL	C	CE	TG:PL	TG:C	PL:C	C:CE
I. VLDL before treatment with Triton								
A. Male (7)[b]	100	35.9 ± 2.6[c]	16.4 ± 0.7	11.5 ± 1.2	2.9 ± 0.2	6.2 ± 0.2	2.2 ± 0.1	1.5 ± 0.1
B. Female (7)	100	36.6 ± 1.8	20.5 ± 1.6	11.9 ± 1.4	2.8 ± 0.1	5.0 ± 0.4	1.8 ± 0.1	1.8 ± 0.1
II. VLDL secreted following injection of Triton								
C. Male (3)	100	31.1 ± 1.2	20.4 ± 1.3	7.1 ± 0.8	3.2 ± 0.1	4.9 ± 0.3	1.5 ± 0.1	2.9 ± 0.4
D. Female (3)	100	23.0 ± 1.2	14.6 ± 1.2	4.9 ± 0.6	4.4 ± 0.2	6.9 ± 0.5	1.6 ± 0.1	3.0 ± 0.1
Significance of differences (P values)								
A vs. B		NS	0.05	NS	NS	0.05	0.05	NS
C vs. D		0.01	0.05	NS	0.02	0.05	NS	NS
A vs. C		NS	0.05	0.05	NS	0.05	0.02	0.005
B vs. D		0.005	0.02	0.05	0.001	0.025	NS	0.001

[a]Values are moles of lipid relative to TG set = 100. TG = triglyceride, PL = phospholipids, C = cholesterol, CE = cholesteryl esters.

[b]Figures in parentheses indicate number of observations in each group. Each observation was obtained on pooled plasma from at least four animals (see text).

[c]All values are means ± SE.

centrifuge than did the VLDL produced by the male. The faster mobility is suggestive of larger particle size and/or lower density of the VLDL (4). Triton WR-1339 was reported to alter the electrophoretic mobility of lipoprotein (15) and to shift the distribution of protein among the different classes of plasma lipoproteins when these are isolated by conventional ultracentrifugal methods using angle-head rotors (16). However, no significant differences were observed in the zonal patterns of the VLDL after ultracentrifugation when 15 mg of Triton/ml of plasma were added in vitro (H.G. Wilcox, C. Soler-Argilaga, and M. Heimberg, unpublished observations).

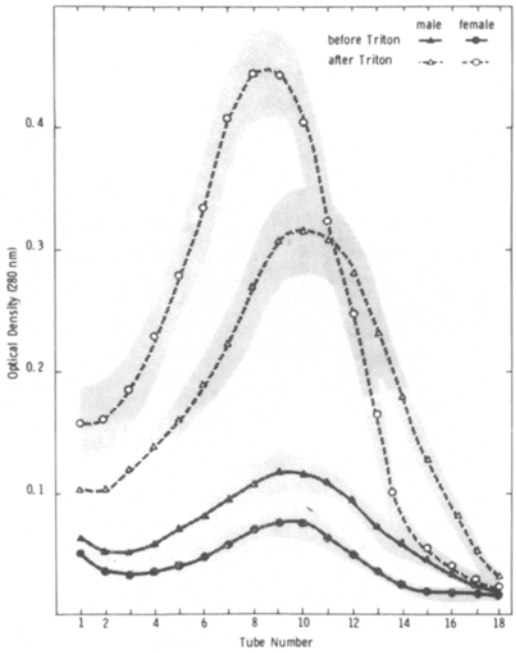

FIG. 1. Zonal ultracentrifugal patterns of the very low density lipoprotein (VLDL) isolated from rat plasma before and 2 hr after intravenous injection of Triton WR-1339. The procedure for zonal ultracentrifugation was similar to that reported previously (4). Gradient (d: 1.0 - 1.4,380 ml) was introduced behind 200 ml overlay of distilled water into the Ti-14 zonal rotor (Spinco) revolving at 3,000 rpm; the rotor was accelerated to 30,000 rpm, allowed to run at speed for 20 min, decelerated to 3,000 rpm, and the contents of the rotor were collected. Each fraction contained 25 ml. The direction of migration of the VLDL is from right to left. Shaded areas indicate ± 1 SE (n=4).

Lipid Composition of the VLDL

No significant differences were observed between composition of the plasma VLDL lipids, relative to TG, from untreated male or female rats, except for a slightly increased proportion of free cholesterol in the female (Table III, A vs. B). After injection of Triton, the VLDL secreted by female rats contained fewer mol of phospholipid and free sterol per mol TG than did the VLDL secreted by male animals under identical experimental conditions (C vs. D).

In rats of the same sex, the lipid composition of the VLDL isolated from plasma of untreated rats differed from that of the VLDL secreted after injection of Triton. The VLDL secreted by male rats treated with Triton WR-1339 contained similar quantities of PL, more C, and less CE relative to TG than did the VLDL from plasma of untreated animals (A vs. C). The VLDL secreted by female rats treated with the detergent contained relatively less PL, C, and CE per mol of TG than did the VLDL isolated from plasma of untreated female animals (B vs. D). These differences are amplified when certain molar ratios of VLDL lipids are

TABLE IV

Fatty Acid Composition of VLDL Triacylglycerol

Group	Fatty acid analyzed (% of total fatty acids)[a]			
	16:0	18:0	18:1	18:2
I. VLDL before treatment with Triton[b]				
A. Male (8)[c]	27.3 ± 1.0[d]	2.3 ± 0.3	32.7 ± 1.0	26.4 ± 1.2
B. Female (7)	28.3 ± 0.6	2.9 ± 0.3	31.2 ± 1.1	25.5 ± 1.2
II. VLDL secreted following injection of Triton[b]				
C. Male (3)	30.0 ± 1.8	1.7 ± 0.2	28.8 ± 1.2	28.0 ± 0.5
D. Female (3)	28.6 ± 0.5	2.4 ± 0.4	30.9 ± 0.8	27.2 ± 1.0

[a]The table gives major fatty acids only.

[b]No significant differences were observed when groups were compared (A vs. B; C vs. D; A vs. C and B vs. D). VLDL = very low density lipoprotein.

[c]Figures in parentheses indicate number of observations in each group. Each observation was obtained on pooled plasma from at least four animals (see text).

[d]All values are means ± SE.

TABLE V

Fatty Acid Composition of VLDL Phospholipids[a]

Group	Fatty acid analyzed (% of total fatty acid)				
	16:0	18:0	18:1	18:2	20:4
I. VLDL before treatment with Triton					
A. Male (8)[b]	23.8 ± 0.7[c]	17.5 ± 0.7	9.4 ± 0.6	26.8 ± 1.3	11.1 ± 0.6
B. Female (7)	19.4 ± 0.4	25.7 ± 1.5	8.9 ± 0.6	21.8 ± 1.2	13.8 ± 0.8
II. VLDL secreted following injection of Triton					
C. Male (3)	25.8 ± 1.1	12.1 ± 2.4	8.1 ± 1.0	26.3 ± 1.4	19.0 ± 1.1
D. Female (3)	17.2 ± 1.3	18.8 ± 1.9	4.5 ± 0.7	21.4 ± 1.4	28.1 ± 1.5
Significance of differences (P values)					
A vs. B	0.001	0.001	NS	0.02	0.025
C vs. D	0.01	0.05	0.05	NS	0.01
A vs. C	NS	0.02	NS	NS	0.001
B vs. D	NS	0.05	0.001	NS	0.001

[a]Data are as presented in Table IV. VLDL = very low density lipoprotein.

[b]Figures in parentheses indicate number of observations in each group. Each observation was obtained on pooled plasma from at least four animals (see text).

[c]All values are means ± SE.

TABLE VI

Fatty Acid Composition of VLDL Lecithin[a]

Group	Fatty acid analyzed (% of total fatty acid)				
	16:0	18:0	18:1	18:2	20:4
I. VLDL before treatment with Triton					
A. Male (3)[b]	23.5 ± 0.8[c]	17.9 ± 1.7	10.1 ± 0.6	27.9 ± 2.5	10.0 ± 0.9
B. Female (3)	16.7 ± 1.0	28.8 ± 1.7	7.3 ± 1.3	17.6 ± 1.5	16.9 ± 0.6
II. VLDL isolated following injection of Triton					
C. Male (3)	22.5 ± 0.5	19.5 ± 0.9	8.9 ± 0.5	23.2 ± 0.4	16.9 ± 1.0
D. Female (3)	15.7 ± 0.9	26.5 ± 1.5	5.6 ± 0.2	17.2 ± 0.6	22.1 ± 0.4
Significance of differences (P values)					
A vs. B	0.01	0.01	NS	0.025	0.005
C vs. D	0.001	0.02	0.005	0.001	0.005
A vs. C	NS	NS	NS	NS	0.01
B vs. D	NS	NS	NS	NS	0.005

[a]Data are as presented in Table IV, except for C and D, which represent directly the composition of very low density lipoprotein (VLDL) lecithin isolated after treatment with Triton.

[b]Figures in parentheses indicate number of observations in each group. Each observation was obtained on pooled plasma from at least four animals.

[c]All values are means ± SE.

examined. Thus, when one calculated the ratio C:CE, a higher proportion of cholesterol was observed to be in the free form in the VLDL particles following injection of Triton in animals of both sexes. This can be expected if the composition of the plasma VLDL after treatment of the animals with Triton WR-1339 is more typical of the noncatabolized lipoprotein. The VLDL secreted by the isolated perfused rat liver contains free cholesterol as the primary sterol (17).

Fatty Acid Composition of VLDL Lipids

The percentage composition of the major fatty acids of VLDL triacylglycerol was similar in both sexes before and after treatment with Triton (Table IV). The phospholipids of VLDL isolated from untreated female had more stearic (18:0) and arachidonic acids (20:4) and less palmitic (16:0) and linoleic acids (18:2) than did VLDL isolated from plasma of male animals (Table V, A vs. B). Similar relationships were observed in phospholipid of VLDL secreted by

TABLE VII

Fatty Acid Composition of VLDL Cholesteryl Esters[a]

Group	Fatty acid analyzed (% of total fatty acid)					
	16:0	16:1	18:0	18:1	18:2	20:4
I. VLDL before treatment with Triton						
A. Male (5)[b]	10.3 ± 0.4[c]	3.7 ± 0.2	1.5 ± 0.3	34.8 ± 3.4	22.9 ± 1.2	16.0 ± 2.1
B. Female (6)	11.6 ± 0.9	3.4 ± 0.3	3.2 ± 0.4	29.4 ± 2.8	19.7 ± 0.5	22.6 ± 2.0
II. VLDL secreted following injection of Triton						
C. Male (3)	14.2 ± 1.4	4.5 ± 0.2	1.7 ± 0.2	38.6 ± 2.5	22.9 ± 1.3	6.4 ± 0.1
D. Female (3)	13.4 ± 0.4	2.8 ± 0.2	2.9 ± 0.6	34.0 ± 3.7	22.0 ± 0.8	14.8 ± 2.5
Significance of differences (P values)						
A vs. B	NS	NS	0.025	NS	0.05	0.05
C vs. D	NS	0.01	NS	NS	NS	0.05
A vs. C	0.02	NS	NS	NS	NS	0.02
B vs. D	NS	NS	NS	NS	0.05	0.05

[a]Data are as presented in Table IV. VLDL = very low density lipoprotein.

[b]Figures in parentheses indicate number of observations in each group. Each observation was obtained on pooled plasma from at least four animals (see text).

[c]All values are means ± SE.

rats treated with Triton (C vs. D). Of particular interest is the large increase in 20:4 and simultaneous decrease in 18:0 in the VLDL phospholipids of either male (A vs. C) or female rats (B vs. D), respectively, after injection of Triton in comparison to the untreated control (Table V). An increase in 20:4 was also observed in VLDL lecithin after treatment with Triton (Table VI).

More 20:4 and 18:0 and relatively less linoleic acid (18:2) were detected in CE of plasma VLDL secreted by female rats in comparison to the male (Table VII, A vs. B). The content of 20:4 in CE of VLDL secreted after treatment with Triton remained higher in the female than in the male (C vs. D). Following injection of Triton WR-1339, a significant ($P<0.05$) decrease in the content of 20:4 in the CE of the secreted VLDL was observed in the male (A vs. C) and female (B vs. D), respectively.

DISCUSSION

Although several investigators have reported on the concentrations and/or composition of plasma lipoproteins in the male rat (12,18-21), no systematic comparison has been made of effects of sex on the composition and properties of the VLDL. It is evident from the work reported here that important sex differences exist not only in the concentration and composition of the VLDL isolated from normal plasma of fed rats, but also in the output and properties of newly synthesized particles of VLDL released into the circulation. The VLDL present in the plasma of the rats treated with Triton WR-1339 probably is more representative of the composition and properties of the lipoprotein as secreted by the liver, whereas the VLDL present under normal conditions in vivo may be partially catabolized. It was reported earlier, in agreement with data reported in this manuscript, that the concentration of TG in plasma of fed female rats is similar to or less than that of male animals (2,22). The present data on fatty acid composition of VLDL phospholipid and cholesteryl esters agree also with those of Lyman et al. (23), who reported that the proportions of stearic and arachidonic acids in PL and CE of plasma from female rats exceeded those of male animals.

It is clear from the current data, in agreement with observations made with the perfused rat liver in vitro (2-5) or in vivo (2,6,22), that output of VLDL triacylglycerol and other lipids in vivo in female rats exceeded that of male animals. On the basis of data obtained with the perfused liver, it is probable that the higher rates of secretion by the livers from females in vivo in comparison to the males is determined by the need to secrete TG, stimulated by exogenous free fatty acid (FFA); in these experiments in vivo, no significant differences in the concentration of FFA were observed. It was observed, however, in recent experiments (3) with the perfused rat liver that the uptake of oleate/g liver by livers from female rats exceeded that of the male when equal quantities of fatty acid were infused. Moreover, a greater proportion of oleate was esterified to TG by

livers from female rats and less to PL and oxidation products than by livers from male animals (3). The increased ability to synthesize TG from FFA must be an important stimulant to the elevated secretion of VLDL by the female.

Since plasma concentrations of VLDL triacylglycerol in female rats are less than those in male animals but hepatic output of TG is greater in the female, it can be presumed that extra-hepatic uptake and utilization of VLDL triacylglycerol is also more rapid in the female than in the male. The loss of radioactivity from the blood of male and female rats injected with VLDL labeled in the fatty acid moiety was investigated recently (24). A more rapid rate of disappearance of radioactivity from the plasma, suggestive of more rapid utilization of triacylglycerol fatty acids, was observed in female animals in comparison to males.

In agreement with data obtained with the isolated perfused liver infused with small amounts of oleic acid in vitro (3), the VLDL secreted by the female treated with Triton in vivo had a faster rate-zonal mobility in the ultracentrifuge and contained fewer mol PL and C per mol TG than did VLDL secreted into the plasma of the male. These differences may result in part from the enhanced capacity of livers from female rats to synthesize TG (3). It is of particular interest that when comparisons were made under conditions of equal output of TG by the isolated liver (i.e., unequal rates of infusion and uptake of substrate FFA), the rate-zonal mobility in the ultracentrifuge and the lipid composition of the VLDL secreted by livers from either male or female animals were similar when the output of TG reached values of ca. 1.0 μmol/g liver/hr (5). The apparent lack of sex differences in rate-zonal mobility and lipid composition of the VLDL from plasma of rats not treated with Triton may only be coincidental and may result from balanced rates of formation and utilization of the VLDL.

The data presented here on differences in the lipid class and fatty acid composition between VLDL isolated from plasma of untreated rats and VLDL accumulated after injection of Triton suggest that inhibition of LCAT is a factor in the hyperlipoproteinemia induced by Triton WR-1339. The inhibitory effect of Triton WR-1339 on activity of LCAT (9-11), which may result from a direct action of the detergent on the enzyme (C. Soler-Argilaga, R.L. Russell, and M. Heimberg, unpublished observations) supports this hypothesis. LCAT catalyzes the reaction of lecithin with free cholesterol of the high density lipoprotein (HDL); the resultant CE are transferred in part to the VLDL and low density lipoprotein (LDL), which, of themselves, have not been considered to be good substrates for the LCAT reaction (25-27). It can be proposed from the data of this paper that treatment with Triton diminishes transfer of free cholesterol and lecithin (enriched with 20:4) from VLDL to HDL, where they can be substrates for LCAT. The transfer of lecithin from VLDL to HDL, which is thought to occur when LCAT is active (28), may be specific for lecithins enriched with tetraenoic fatty acid and may be modulated by LCAT. Of related interest are the observations of a preferential esterification of 20:4 and free cholesterol under the influence of LCAT (29,30).

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Protective Effect of 3-Hydroxy-3-Methylglutaric Acid[1] in Alcohol-induced Lipemia

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ABSTRACT

Oral administration of 1 g of 3-hydroxy-3-methylglutaric acid (HMG) before the ingestion of whiskey and a fatty meal markedly reduced the elevation of serum triglycerides, β-lipoproteins, phospholipids, and cholesterol in man. In rats receiving an ethanol and corn oil mixture, HMG also inhibited the increase in postprandial serum and liver lipids. A comparative study of HMG and nicotinic acid in rats showed that, therapeutically, 50 mg MHG/kg body weight is equivalent to 200 mg nicotinic acid/kg body weight in offering almost total protection against lipemic effects of ethanol.

INTRODUCTION

The ingestion of alcoholic drinks before a meal is known to enhance the postprandial rise of plasma triglycerides in man (1-6). A similar effect has been observed in rats receiving ethanol and corn oil (7,8). This lipemic effect is more marked when alcohol is taken prior to ingestion of food (4). Nicotinic acid has been shown to markedly reduce the usual alcohol-induced enhancement of alimentary lipemia. In rats, it inhabits the increase of postprandial rise in plasma triglycerides associated with ingestion of corn oil-alcohol mixture, and lowers the incorporation of C^{14}-labeled dietary fat into plasma triglycerides (9). Taking into account the potential hypolipemic properties of 3-hydroxy-3-methylglutaric acid (HMG) in rats (10,11) and rabbits (12,13) and the effectiveness of this compound in the treatment of familial hypercholesterolemia (14), it was decided, therefore, to investigate the effectiveness of HMG in preventing the alcohol-induced postprandial rise of lipids in man. A comparative study of HMG and nicotinic acid was also performed in rats receiving corn oil-alcohol mixture.

MATERIALS AND METHODS

Human Studies

With slight modifications, the method used was essentially that of Barboriak and Meade (4). After an overnight fast, nine blood donors aged 35-60 years received 120 ml whiskey (85.6 proof, 42.8% ethanol) which was consumed within a 15 min period. The subject then ate a standarized mixed meal consisting of eggs, butter, toast, and tea. The total amount of ingested fat was 65 g. HMG (Calbiochem, San Diego, CA) in total dose of 1 g was given orally in four capsules before the consumption of the alcohol and the fatty meal. For control values, the test without HMG was repeated within 15 days on the same subjects. Venous blood samples were taken before the ingestion of the whiskey and 4 and 8 hr after the consumption of the meal, and serum was obtained. Serum cholesterol (15), phospholipid (16), triglyceride (17), β-lipoprotein (18), and alcohol (19), levels were measured. In the text, the initial value refers to zero hr value obtained on blood samples taken before the ingestion of whiskey.

Animal Studies

Thirty male albino adult rats of the Indian Veterinary Research Institute, (Izatnagar) strain weighing ca. 175 g each were divided into five groups. After an overnight fast, three treated groups received 5 g ethanol and 5 g corn oil/kg body weight by intubation and were pretreated intraperitoneally with 25 and 50 mg HMG and 200 mg nicitinic acid/kg body weight in 1 ml saline, respectively. One-half of the dose of the compound was administered 10 min and the other 60 min, before administration of the alcohol-corn oil mixture. The hypertriglyceridemic control group received the alcohol-corn oil mixture. The basal group receiving 5 g corn oil/kg body weight, and glucose in isocaloric amounts with ethanol was included in the study to show the combined hyperlipemic effect of corn oil-alcohol. These two groups received intraperitoneal injection of 1 ml saline. Sixteen hours after the meal, animals were anesthetized with ether, livers immediately removed, washed with saline, blotted, weighed, and suspended in a mixture of chloroform:methanol (2:1, v/v) for lipid extraction (20). Serum and liver lipids except cholesterol (21) were estimated as described above.

[1] U.S. Patent 3,629,449 (Dec. 21, 1971) on "Process of Combatting Hypercholesterolemia."

Statistical significance was calculated by Student's *t*-test. For P values, in human studies, paired comparison between HMG treated and nontreated values at indicated time intervals was done.

RESULTS

Human Studies

The subjects were not alcohol addicts. The average serum alcohol levels measured 4 and 8 hr after the meal were 74 ± 8 and 23 ± 3 mg/100 ml, respectively, for the series without HMG and 77 ± 12 and 23 ± 4 mg/100 ml for the series with HMG. In both series, Individual serum triglyceride, phospholipid, and β-lipoprotein levels correlated with serum alcohol levels. As shown earlier (4), no definite correlation existed between individual serum alcohol and cholesterol levels. The correlation between elevated serum alcohol and triglyceride levels is in agreement with earlier findings (22). The percent change in alcohol-induced postprandial lipemic response in man is reduced on oral administration of HMG (Fig. 1). Four and 8 hr after the meal, the average serum triglyceride levels in the series without HMG treatment, respectively, increased to 417 ± 24 (mean ± standard error) and 171 ± 13 mg/100 ml over the initial value of 227 ± 12 mg/100 ml. The corresponding rise in the HMG treated series amounted to 376 ± 19 and 59 ± 12 mg/100 ml over the initial value of 236 ± 14 mg/100 ml ($P < 0.05$ for 8 hr). A slight increase of 29 ± 2 mg/100 ml in the cholesterol level over the initial value of 299 ± 14 mg/100 ml was observed 8 hr after the meal in the series without HMG treatment. After 4 and 8 hr of HMG treatment, the serum cholesterol level was significantly decreased, respectively, by 90 ± 14 ($P<0.01$) and 106 ± 18 mg/100 ml ($P<0.05$) over the initial value of 328 ± 14 mg/100 ml. In agreement with earlier findings, alcohol ingestion induced hyperlipoproteinemia (23,24). Four and 8 hr after the meal in non-HMG treated series, the serum β-lipoproteins, respectively, increased by 211 ± 14 and 119 ± 15 mg/100 ml over the initial value of 161 ± 6 mg/100 ml. In the corresponding HMG treated series, the serum β-lipoproteins, over the same time interval, respectively, increased only 63 ± 5 and 48 ± 6 mg/100 ml from the initial value of 160 ± 9 mg/100 ml ($P<0.05$ for 8 hr). Although in paired comparisons phospholipids showed an insignificant decrease on HMG treatment, it was noted that 4 and 8 hr after the meal, serum phospholipid values in the series without HMG increased by 246 ± 23 and 132 ± 20 mg/100 ml over the initial value of 465 ± 35 mg/100 ml. The corresponding increase in HMG treated series was only 71 ± 6 mg at 4 hr, but none at all at 8 hr over the initial value of 571 ± 35 mg/100 ml. Such comparison makes the phospholipids data more meaningful. The cholesterol to phospholipid or triglyceride or serum β-lipoprotein ratio decreased 4 and 8 hr after HMG administration.

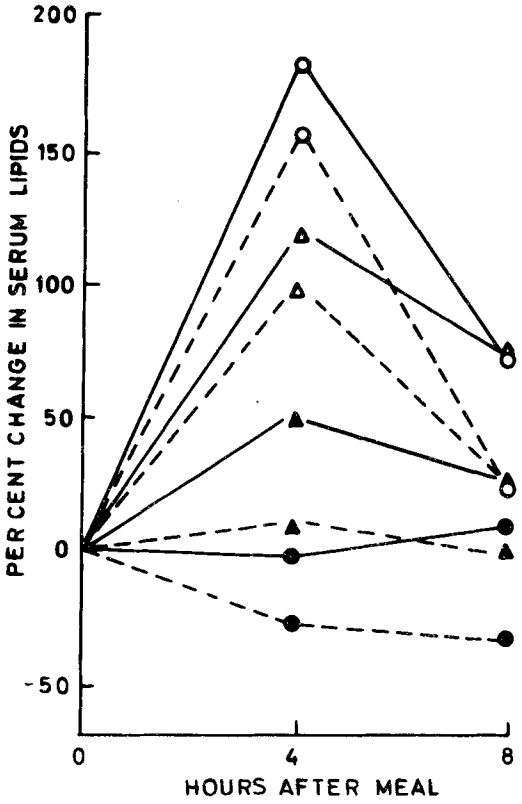

FIG. 1. Effect of 3-hydroxy-3-methylglutaric acid (HMG) on alcohol-induced postprandial lipemia in man. ○ △ ▲ ● represent the percent change: [(value at indicated hr - zero hr value)/value at zero hr] x 100, respectively in serum triglyceride, β-lipoprotein, phospholipid, and cholesterol levels. Solid lines represent values for control subjects, whereas broken lines represent HMG treated values.

Animal Studies

Data presented in Table I indicate that 16 hr after the administration of the corn oil-ethanol mixture, all the lipid parameters in the control group increased. The maximum increase was observed in triglyceride content. HMG treatment at two various doses reduced all lipid parameters of serum and liver except the liver phospholipids. In agreement with earlier

TABLE I

Effect of HMG and Nicotinic Acid on Serum and Liver Lipids of Alcohol-induced Lipemic Rats[a]

			Treated groups		
	Basal group	Control group	25 mg HMG/kg	50 mg HMG/kg	200 mg Nicotinic acid/kg
Serum lipids (mg/100 ml)					
Cholesterol	61 ± 5	77 ± 5	71 ± 6 (8)[b]	57 ± 4[c] (26)	58 ± 5[c] (25)
Phospholipids	133 ± 8	161 ± 12	153 ± 4 (5)	133 ± 9[d] (17)	120 ± 10[c] (26)
Triglycerides	57 ± 6	116 ± 8	76 ± 7[c] (35)	55 ± 8[c] (53)	42 ± 4[c] (64)
Liver lipids (mg/100 mg)					
Cholesterol	338 ± 35	370 ± 30	330 ± 23 (11)	284 ± 13[d] (23)	253 ± 13[d] (32)
Phospholipids	3950 ± 250	4060 ± 310	4080 ± 250	4040 ± 210	4030 ± 210
Triglycerides	1057 ± 190	2198 ± 290	1571 ± 150[e] (29)	1306 ± 109[f] (41)	1036 ± 64[f] (53)

[a]Mean ± standard error expressed for six rats. HMG = 3-hydroxy-3-methylglutaric acid.

[b]Values in parentheses indicate percent reduction with respect to hypertriglyceridemic control group.

[c-f]Significantly different from control; $c_p<0.001$, $d_p<0.02$, $e_p<0.05$, $f_p<0.01$.

findings (9), pretreatment with nicotinic acid significantly ($P<0.001$) reduced the ethanol-induced lipemia. Nicotinic acid at the dose of 200 mg/kg body weight reduced the serum lipid values below that of the control animal. Similar observations were made for hepatic cholesterol and triglycerides. HMG at the dose of 50 mg/kg brought a significant ($P<0.001$) reduction of the same magnitude as 200 mg nicotinic acid/kg body weight and offered almost total protection against the lipemic effect of alcohol.

DISCUSSION

The results show that HMG can effectively counteract the enhanced lipemic response to an alcohol dietary fat combination in both men and rats. In man, the response was observed with 1 g of HMG per subject. In rats, pretreatment with a dose of 50 mg HMG/kg body weight offered almost total protection against the lipemic effect of alcohol. The lipemic response of alcoholics to the dietary fat does not differ from that of nonalcoholics (25). As the subjects were nonalcoholics, the findings in the present study may not have relevance to the chronic situations of the alcoholics.

Besides contributing additional calories, alcohol is known to accentuate hypertriglyceridemia by stimulating fatty acid release from the adipose tissue, increasing very low density lipoprotein (VLDL) synthesis in the liver, and retarding removal of VLDL and chylomicrons from the circulation (24). The increased NADH:NAD ratio generated by oxidation of ethanol could possibly result in increased hepatic triglyceride synthesis and decreased fatty acid oxidation. Although it is premature to suggest the possible mechanism by which HMG interferes with the observed modification of alcohol-induced enhancement of alimentary lipemia, it is tempting to suggest that interference of HMG in any of the above metabolic processes could lead to the observed effects. As the decrease in serum lipids was not accompanied by a rise in liver lipids, the possibility of HMG inhibiting the release of lipoproteins can be excluded. It has already been established that HMG inhibits cholesterol synthesis (26,27) and may interfere at some stage of fatty acid synthesis similar to ethyl p-chlorophenoxyisobutyric acid and tetralylphenoxyisobutyric acid (28). The decrease in serum and liver triglycerides caused by HMG administration suggests that, like nicotinic acid (9), HMG may inhibit the mobilization of free fatty acids from endogenous lipid stores. Furthermore, like nicotinic acid, HMG significantly ($P<0.001$) reduced the ethanol-induced enhancement of lipemia and offered almost total protection against the lipemic effect of ethanol.

These and earlier studies (10-14) suggest that HMG may have great potential as a hypolipemic drug, being effective not only in animals but almost in human beings. As HMG is a natural metabolite (29), it is well tolerated and does not produce any side effects.

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Sensitive Fluorometric Method for Tissue Tocopherol Analysis

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ABSTRACT

A sensitive, highly reproducible method for tissue tocopherol analysis that combines saponification in the presence of large amounts of ascorbic acid to remove interfering substances, extraction of the nonsaponifiable lipids with hexane, and fluorometric measurement of the tocopherol is presented. The nonsaponifiable lipid phase contained only one fluorochrome in the 290 nm excitation and 330 nm emission range, and it was identified as tocopherol by thin layer and column chromatography. Column chromatography of the hexane extract of a saponified, ^{14}C-tocopherol-spiked microsomal fraction showed that no measurable oxidation to tocopheryl-quinone had occurred. The fluorometric method for tocopherol analysis was applied to homogenates and subcellular fractions from rat liver, kidney, lung, and heart and red blood cells. The heavy mitochondrial and microsomal fractions had the highest subcellular concentrations of tocopherol.

INTRODUCTION

Tocopherol analysis in tissues and subcellular fractions is a rather complicated procedure and is the subject of several reviews (1-3). The general procedure for analysis of cellular fractions must include lipid extraction and the removal of interfering substances as well as the determination of tocopherol.

The majority of interfering substances is normally removed by saponification prior to extraction. However, tocopherols are extremely sensitive to oxidation during alkaline saponification, and rather complex methods have been devised to compensate for this problem (4). Kayden et al. (5) prevent tocopherol oxidation by addition of very large amounts of antioxidants, either pyrogallol or ascorbic acid, prior to saponification. However, substantial amounts of interfering substances still remain after saponification and extraction. Therefore, most investigators have employed various procedures, such as thin layer chromatography (TLC) (5,6), column chromatography (7,8), gas-liquid chromatography (GLC) (4,9-11), or high pressure liquid chromatography (12,13) to separate tocopherol from these interfering substances prior to analysis.

Much of this involved general procedure has been dictated by the lack of specificity of the commonly used spectrophotometric methods for the detection of tocopherols. These methods (14-16) are based on the reduction of Fe^{+3} and the use of a colorimetric reagent to measure the Fe^{+2} produced. A more specific and sensitive method (17-20) is based on the native fluorescence of tocopherol, but problems with interfering substances have limited its usefulness to plasma (17-20) and adipose tissue (21).

This report details a new method for tocopherol analysis that combines the specificity and sensitivity of the fluorometric method with the saponification technique devised by Kayden et al. (5), and demonstrates its usefulness for the analysis of tocopherol in homogenates, subcellular fractions, and red blood cells (RBC).

EXPERIMENTAL PROCEDURE

Materials

α-Tocopherol was obtained from Nutritional Biochemicals Corp. (Cleveland, OH) and ^{14}C-α-tocopheryl succinate from Distillation Products Industries (Rochester, NY). Glass-distilled hexane and ethyl ether obtained from Burdick and Jackson Laboratories (Muskegon, MI) were of fluorometric quality without further treatment. Absolute ethanol and glass-distilled water were used. All other materials were prepared from reagent grade chemicals.

Preparation of Tissue Subcellular Fractions

Rat liver microsomal fractions were prepared by the method of Glende and Recknagel (22) from male Sprague-Dawley rats that had been starved for 24 hr prior to sacrifice. The final microsomal pellets were resuspended to a final volume of 2 ml/g liver tissue in 50 mM Tris-maleate buffer, pH 6.5, that contained 155 mM NaCl.

The subcellular fractions from rat liver, lung,

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heart, and kidney were prepared according to the method of de Duve et al. (23). The organs were excised, weighed, and homogenized in 5 volumes of isotonic KCl with a glass-Teflon homogenizer. After filtration through cheesecloth, the nuclear-cell debris, heavy mitochondrial, light mitochondrial-lysosomal, microsomal, and soluble fractions were obtained by centrifugation. All pellets were washed once prior to final resuspension in isotonic KCl.

RBC were added to aqueous 1% EDTA, disodium salt, mixed, and washed with an isotonic phosphate-buffered NaCl solution (pH 7.4) as described by Kayden et al. (5). The RBC were made up to a final hematocrit of 50%.

Protein concentrations of the various subcellular fractions, homogenates, and RBC were determined by the method of Miller (24).

Instrumentation

Fluorescence was measured with an Aminco-Bowman spectrophotofluorometer linked to a ratio photometer. Normally, a slit combination of 3-1-3 mm (excitation-emission-photomultiplier turret slit) was used. Fluorescence excitation and emission spectra were recorded on an X-Y recorder. A quinine sulfate standard (1 μg/ml in 0.1 N H_2SO_4) was used to check wavelength standardization and to adjust the ratio photometer to make correction for instrumental sensitivity. The instrument was continually adjusted to give an arbitrary fluorescence intensity of 400 for the quinine sulfate standard.

Radioactivity measurements were made with a Beckman LS-100 liquid scintillation counter. Samples eluted from chromatography columns with hexane-ethyl ether combinations were dried in scintillation vials and redissolved in toluene phosphor that contained 5 g of PPO (2,5-diphenyloxazole) and 0.3 g of POPOP (1,4-bis-[2-(5-phenyloxazolyl)]-benzene) per liter of toluene. Samples were counted for 10 min at >95% efficiency.

Standard Fluorometric Tocopherol Analysis

The usual saponification mixture contained 0.5 ml of 25% ascorbic acid (stored at 4 C), 1.0 ml of absolute ethanol, and 1.5 ml of subcellular suspension, homogenate, or red blood cells. The mixtures were preincubated at 70 C for 5 min in glass-stoppered 15 ml centrifuge tubes. Following addition of 1.0 ml of 10 N KOH, the mixtures were saponified for 30 min at 70 C. After cooling, 4.0 ml of hexane was added and the saponified mixtures were extracted by mixing for 1 min on a vortex mixer. The extracted mixtures were centrifuged in a clinical centrifuge for 5 min, and a portion of the hexane phase was removed for fluorometric measurement at 286 nm excitation and 330 nm emission. For all external standards and blanks, water was substituted for the tissue suspensions. External standards were prepared by addition of 1.0 ml of ethanol that contained 2.5 μg α-tocopherol to the 1.5 ml water and 0.5 ml 25% ascorbic acid mixture. Tocopherol concentrations were calculated from comparison of the sample fluorescence intensity with that of the external standard after correction had been made for the fluorescence intensity of the blank. Tocopherol recoveries were estimated from internal standards of 2.5 μg tocopherol added to 1.5 ml of subcellular suspension.

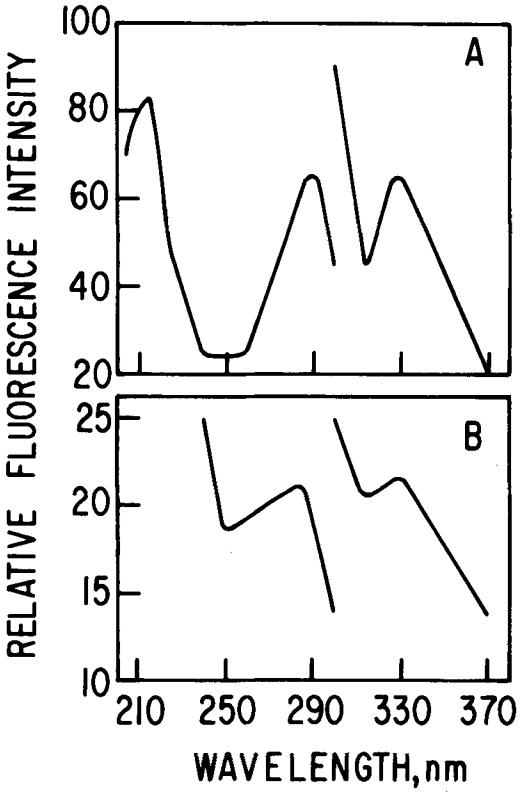

FIG. 1. Fluorescence spectra of (A) tocopherol (5 μg/ml) in hexane and (B) a hexane extract of saponified rat liver microsomal fraction prepared as described in Experimental Procedures. Fluorescence intensities are expressed relative to quinine sulfate standards.

Elimination of Vitamin A Interference

With some subcellular suspensions, particularly those from liver, the presence of relatively large amounts of vitamin A caused fluorescence

TABLE I

Solvent Effects on Relative Fluorescence Intensity and Excitation and Emission Maxima for α-Tocopherol

Solvent	Relative fluorescence intensity[a]	Excitation maximum (nm)	Emission maximum (nm)
Hexane	20.7	290	330
Cyclohexane	20.7	295	328
Ethyl ether	34.8	290	332
Methanol	27.0	288	335
Dioxane	32.6	292	335

[a]Corrected for background fluorescence as described in Experimental Procedure.

TABLE II

Effect of KOH Concentration on the Extractable Fluorescence and Tocopherol Recoveries from Rat Liver Microsomal Fractions

KOH concentration[a]	Relative fluorescence intensity[b]	Tocopherol recovery[c]
0	49	79
0.025	30	71
0.125	8	54
0.250	16	45
1.25	14	89
2.50	12	90

[a]Final normality of KOH in saponification mixture.

[b]Corrected for background fluorescence as described in Experimental Procedure.

[c]Percent recovery calculated from internal standards as described in Experimental Procedure.

quenching problems. This vitamin A interference was eliminated by the H_2SO_4 procedure of Fox and Mueller (25). The hexane phase was pipetted into a second centrifuge tube, 0.6 ml of 60% H_2SO_4 was added, and the samples were mixed vigorously for 30 sec with a vortex mixer. After centrifugation in a clinical centrifuge, the fluorescence of the hexane phase was measured.

Chromatography of Tocopherol

The nonsaponifiable lipids of the hexane phase were separated by chromatography on a neutral alumina (Brockmann, grade III) column by the procedure of Chow et al. (26). Samples were applied in hexane and eluted by hexane mixtures that contained increasing proportions of ethyl ether, which increased the polarity of the eluting solvent. Fractions were collected at 1 min intervals and monitored for fluorescence and radioactivity.

TLC of tocopherol was performed on silica gel (Quantagram Q1) plates with chloroform as the developing solvent. Spots were visualized by spraying the plates with iron-dipyridyl reagent (27). The R_f for α-tocopherol in this system was 0.57.

RESULTS

Fluorescence Spectra of α-Tocopherol

The excitation and emission spectra of α-tocopherol in hexane are shown in Figure 1A. Excitation peaks at 215 nm and 290 nm were noted, with one emission peak at 330 nm. Some background fluorescence, arising mainly from ethanol impurities, was always found. Fluorescence measurements were always corrected for this background fluorescence. Figure 1B shows the fluorescence spectra of the hexane extract of rat liver microsomes. The excitation and emission peaks are 286 nm and 330 nm, respectively. The slight shift in the excitation peak is probably caused by quenching of the incident light by vitamin A. The usefulness of the 215 nm excitation peak was limited by severe quenching problems at this wavelength.

Dependence of Fluorescence Spectra and Intensity on Solvent

As shown in Table I, α-tocopherol in solvents more polar than hexane, such as ethyl ether, methanol, and dioxane, exhibited greater fluorescence intensity at 290 nm excitation and 330 nm emission. The various solutions of α-tocopherol also exhibited slightly different

fluorescence spectra depending on the nature of the solvent. Hexane was selected as the solvent of choice based upon its immiscibility with water, its lipid extraction capabilities, its low background fluorescence and Raman scatter peak, and its stability. Ethyl ether was eliminated as the extracting solvent because of its high volatility and resultant quantification problems.

Optimal Saponification Conditions

A variety of saponification techniques were explored with rat liver microsomes. A variation of the method of Kayden et al. (5) proved to be the optimal procedure.

With 10 *N* KOH addition in the presence of ethanol and ascorbic acid, a 10 min saponification time at 70 C was sufficient for 1.5 ml of microsome suspension (ca. 12 mg protein). Increasing saponification time to 45 min had no further effect on extraction of tocopherol. Using the 30 min saponification period, the temperature of saponification had no influence on tocopherol extractability, with 0, 27, 37, 50, and 70 C being essentially equivalent.

Table II shows the effect of KOH concentration on saponification at 70 C for 30 min. In the absence of KOH or at low concentrations of KOH, interfering fluorescent substances were extracted into the hexane phase. Tyrosine was tentatively identified as a major contaminant by TLC on silica gel plates with butanol:acetic acid:water (4:1:1). The 0.125 *N* KOH treatment eliminated tyrosine, but tocopherol was incompletely extracted, perhaps due to incomplete digestion of the microsomal membrane. The tocopherol recovery was optimized with addition of 1 ml of 10 *N*KOH (final concentration, 2.5 *N*).

The inclusion of a final concentration of 25% ethanol in the saponification mixture was also important. Absence of ethanol resulted in extraction of considerable nontocopherol fluorescence. Also, the hexane phase was quite hazy in the absence of ethanol. A minimum final concentration of 12.5% ethanol was required for optimal saponification and separation of phases during extraction. Ethanol presumably acts as a protein precipitant, but n-butanol or 10% trichloroacetic acid were ineffective as replacements. It was noted also that use of absolute ethanol resulted in slightly lower background fluorescence than use of 95% ethanol.

The presence of an antioxidant in the saponification mixture also was necessary. Without antioxidant, considerable oxidation of α-tocopherol occurred during saponification. Both ascorbic acid and pyrogallol were effective antioxidants. However, pyrogallol was converted to pyrogallin during the saponification treatment, and the pyrogallin was extracted into the hexane phase to a certain extent, increasing the background fluorescence and limiting the sensitivity of the assay. Therefore, ascorbic acid was chosen as the optimal antioxidant. The optimal concentration was 0.5 ml of 25% ascorbic acid (3.1%) in each saponification mixture. Higher concentrations gave equivalent tocopherol recoveries, while lower concentrations of ascorbate gave progressively poorer recoveries.

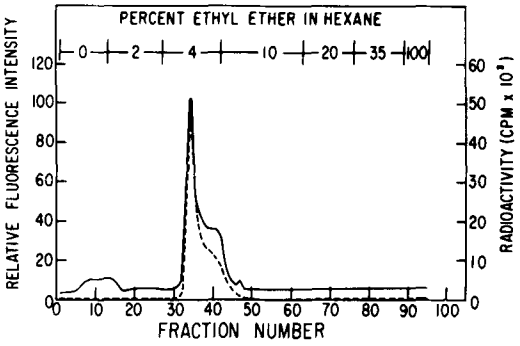

FIG. 2. Neutral alumina column chromatography of a hexane extract prepared from saponified rat liver microsomal fraction to which 0.13 μCi of α-tocopheryl succinate had been added prior to saponification. Fractions were collected at 1.0 min intervals, and the relative fluorescence intensity (——) and radioactivity (- - -) of each fraction were measured.

Optimal Extraction Conditions

A stirring time of 1 min using a vortex mixer was required for complete extraction of tocopherol from rat liver microsomes. Longer extraction times had no effect on the amount of tocopherol recovered. Successive hexane extractions also failed to extract any further tocopherol, which suggests that the partition coefficient between the hexane phase and the water-ethanol phase is quite favorable for tocopherol extraction.

A hexane volume of 4.0 ml was chosen for routine extraction. The volume of hexane can be decreased, with resultant improvement in assay sensitivity but, unfortunately, with magnification of fluorescence quenching when used with rat liver microsomes.

Coincidence between Extractable Fluorescence and α-Tocopherol

Figure 2 shows the results of an experiment in which 0.13 μCi of ^{14}C-α-tocopheryl succinate was added to a liver microsomal fraction just prior to saponification. Following saponification and extraction, 72 ml of combined

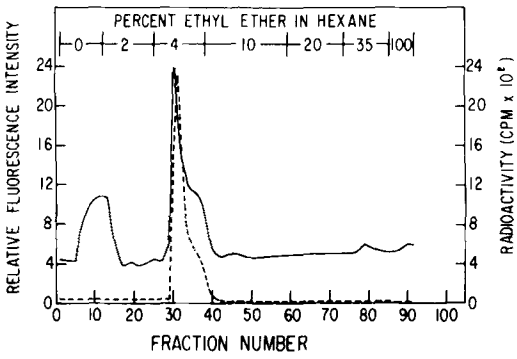

FIG. 3. Neutral alumina column chromatography of the hexane extract prepared from saponified rat liver microsomal fraction that was isolated from rats given 1.3 μCi each of α-tocopheryl succinate intragastrically 16 hr prior to sacrifice. Fractions were eluted with various ethyl ether-hexane combinations and were collected at 1.0 min intervals. The relative fluorescence intensity (——) and radioactivity (- - -) of each fraction were monitored.

hexane extract was subjected to alumina column chromatography. Only one fluorochrome was eluted, and the radioactivity was coincident with this fluorochrome. This experiment confirms that only one fluorochrome, identifiable as tocopherol, was present in the microsomal hexane extract. Whereas saponification of α-tocopherol standard in the absence of microsomes resulted in extraction of a tocopherol fraction that eluted from the alumina column with 10% ethyl ether in hexane, corroborating the findings of Chow et al. (26), the tocopherol extracted from the microsomal fraction was eluted with 4% ethyl ether in hexane. Apparently, the addition of microsomal fraction to the saponification mixture alters the elution pattern of α-tocopherol. The tocopherol fraction from saponified microsomes that eluted with 4% ethyl ether in hexane was shown by TLC to be chemically unaltered tocopherol.

In a further experiment, three rats were given 1.3 μCi each of ^{14}C-α-tocopheryl succinate by stomach tube. Sixteen hours later the rats were sacrificed, livers excised, and the microsomal fraction isolated. Only 0.18% of the radioactivity was recovered in the microsomal fraction, but after saponification and extraction, 93% of this radioactivity was recovered in the 4% ethyl ether in hexane fraction from alumina chromatography (Fig. 3). Again, the radioactivity coincided with the elution of tocopherol as measured fluorometrically. The initial fluorescent compound that eluted with the hexane wash (Fig. 3) was due to ethanol impurities and corresponded in magnitude to the background fluorescence always present in blanks.

Oxidation of α-Tocopherol during Saponification

The primary oxidation product of α-tocopherol, α-tocopherylquinone, is reported to elute from a neutral alumina column with 35% ethyl ether in hexane (26). However, saponification of ^{14}C-α-tocopheryl succinate in the presence and absence of microsomes yielded no measurable radioactivity in the 35% ethyl ether in hexane fraction (Fig. 2 and 3). This finding suggests that no significant oxidation of tocopherol occurred during the saponification and extraction process. Concentration of the hexane phase by evaporation prior to application on the alumina column resulted frequently in considerable oxidation of tocopherol to a nonfluorescent compound, presumably tocopherylquinone. Therefore, concentration of the hexane phase was avoided.

Linearity, Sensitivity, and Precision of the Fluorometric Tocopherol Assay

The relative fluorescence intensity of the hexane phase was linear with rat liver microsome concentration from 0 to 2.0 ml of microsomes. A correlation coefficient of 0.997 was found with duplicates run at each concentration. Some deviation from linearity was seen with higher concentrations of microsomes, presumably due to fluorescence quenching.

Under the optimal assay conditions, the fluorometric tocopherol determination was sensitive in the range of 0.10-50 μg α-tocopherol/ml (0.25-125 nmol/ml). The correlation coefficient for the standard curve was 0.998, with duplicates for each tocopherol concentration. The usual standard curve conversion factor was 12.5-13.0 relative fluorescence units per μg α-tocopherol. The assay can be made sensitive to 0.05 μg α-tocopherol/ml by use of the slit combination 5-5-5 mm rather than the usual 3-1-3 mm.

The precision of the fluorometric method was determined by analysis of 10 replicates of a single microsome preparation that contained 0.72 ± 0.018 (SD) μg of tocopherol per 1.5 ml of microsome suspension. The coefficient of variation was 2.5%.

Destruction of Vitamin A Interference

With some subcellular fractions from rat liver, there was quenching by vitamin A absorption of the incident light. Vitamin A was identified by its characteristic 325 nm excitation and 470 nm emission fluorescence spectra. Quenching was so severe in rat liver homogenate, nuclear, heavy mitochondrial, and soluble frac-

tions that measurements of α-tocopherol content were impossible. Destruction of the vitamin A by the H_2SO_4 method of Fox and Mueller (25) eliminated most of the quenching problems. The relative fluorescence intensity of external tocopherol standards was not affected by the H_2SO_4 treatment, which suggests that this treatment can remove vitamin A interference with no deleterious effects on α-tocopherol. Tocopherol recoveries from rat liver microsomes calculated from internal standards improved from 75-80% to 92-100% with H_2SO_4 treatment. This finding suggests that some of the quenching problems observed with concentrated microsome samples were likely due to vitamin A.

Ubiquinone Interference

Ubiquinols are commonly encountered interfering substances in the conventional spectrophotometric measurement of α-tocopherol due to their reducing potential (28). The major cyclized products of the ubiquinones, the ubichromenols, are formed by alkali treatment and are structurally related to tocopherols and might be expected to be fluorescent. To test for this potential interference, 5 mg of ubiquinone-50 was added to a microsomal saponification mixture. Ubiquinone-50 was fluorescent with 356 nm excitation and 416 nm emission. After saponification, no ubiquinone or ubichromenol fluorescence was detected in the hexane extract. Moreover, no fluorescence that would interfere with tocopherol analysis was detected under any conditions, including saponification in the absence of ascorbate.

Tocopherol Content of Homogenates, Subcellular Fractions, and Red Blood Cells

The tocopherol content of subcellular fractions from rat liver, lung, kidney, and heart is given in Table III. In all tissues, the heavy mitochondrial, light mitochondrial-lysosomal, and microsomal fractions had the highest concentration (μg/mg protein) of tocopherol; in particular, the lung heavy mitochondrial and microsomal fractions had high tocopherol concentrations. The soluble fractions contained very little tocopherol. Tocopherol recoveries based on internal standards ranged from 80 to 100% for most subcellular fractions. The liver subcellular fractions, treated with H_2SO_4 to destroy vitamin A interference, had tocopherol recoveries that ranged from 61% for soluble fraction to 92% for microsomal fraction. Some interference that is not destroyed by H_2SO_4 treatment seems to remain in the rat liver homogenate and soluble fractions. While the liver and kidney fractions had lower tocopherol concentration on a μg/mg protein basis, the tissue concentration on a wet weight basis showed little difference. Calculation of the tissue tocopherol levels on a μg/g wet weight of tissue basis from homogenate concentrations gave the following values: liver, 6.5 μg/g; kidney, 7.3 μg/g; lung, 8.2 μg/g; and heart, 7.2 μg/g. The subcellular distribution of tocopherol was distinctly different from the protein distribution as shown by the relative tocopherol contents in Table III. The tocopherol content of RBC was 9 ng/mg protein or 1.6 μg/ml of packed cells.

Storage Stability of Tocopherol

Rat liver microsomal fractions could be stored frozen for several weeks with no detectable loss of tocopherol. Freezing and thawing also had no effect on tocopherol content of liver microsomal suspensions. Homogenization of liver and resuspension of the microsomal fraction in isotonic 50 mM Tris-maleate buffer, pH 6.5, that contained 1% ascorbate neither improved tocopherol yields nor storage stability.

DISCUSSION

The coupling of a fluorometric determination of tocopherol with a saponification step for the removal of interfering substances seems to provide unique advantages for tocopherol analysis in a variety of tissue homogenates and subcellular fractions. The principal advantage of the described method for tocopherol analysis is the elimination of cumbersome and quantitatively inefficient chromatographic procedures, following saponification, for further separation of tocopherol from interfering substances. The fluorometric measurement of tocopherol is considerably more specific than the Emmerie-Engel reaction (14) if done after saponification and solvent extraction. Vitamin A was the only component of the nonsaponifiable lipid phase that interfered with the fluorometric tocopherol measurement. Fluorescence quenching by vitamin A was eliminated with a rather simple acid treatment of the hexane extract. Ubiquinols, which impede measurement of tocopherol in the nonsaponifiable lipid phase by spectrophotometric methods (28), have no deleterious effect on this fluorometric tocopherol determination. The inclusion of a saponification step extends the usefulness of the fluorometric assay, which, thus far, has been limited to plasma (17-20) and adipose tissue (21). Minimal tocopherol oxidation occurs during saponification in the presence of large amounts of antioxidant, as originally reported by Kayden et al. (5). However, as

TABLE III

Tocopherol Content of Various Tissue Homogenates and Subcellular Fractions

Tissue	Fraction	Tocopherol concentration (μg/mg protein)	Tocopherol concentration (μg/ml)	Total tocopherol[a]	Relative tocopherol concentration[b]	Tocopherol recovery[c]
Liver						
	Homogenate	0.04	1.3	104	1.0	70
	Nuclear	0.03	0.9	34	0.8	84
	Heavy mitochondrial	0.27	1.7	31	7.4	81
	Light mitochondrial	0.12	0.9	19	3.3	78
	Microsomal	0.08	0.8	18	2.2	92
	Soluble	0.002	0.1	2	0.1	61
Heart						
	Homogenate	0.12	1.4	58	1.0	90
	Nuclear	0.25	1.5	28	2.1	80
	Heavy mitochondrial	0.26	0.4	4	2.1	89
	Light mitochondrial	0.29	0.5	4	2.4	92
	Microsomal	0.37	1.1	9	3.1	101
	Soluble	0.02	0.2	5	0.2	94
Lung						
	Homogenate	0.14	1.7	99	1.0	95
	Nuclear	0.18	0.8	24	1.3	104
	Heavy mitochondrial	0.60	0.8	12	4.5	115
	Light mitochondrial	0.26	0.9	13	1.9	104
	Microsomal	0.56	1.4	12	3.9	99
	Soluble	0.04	0.3	13	0.3	103
Kidney						
	Homogenate	0.05	1.4	119	1.0	96
	Nuclear	0.08	1.1	41	1.5	96
	Heavy mitochondrial	0.07	1.2	25	1.4	92
	Light mitochondrial	0.11	0.8	16	2.1	96
	Microsomal	0.11	0.9	15	2.2	96
	Soluble	0.02	0.3	16	0.4	102

[a]Micrograms of tocopherol present in total fraction.

[b]Percent of total tocopherol divided by percent of total protein.

[c]Calculated from internal standards as described in Experimental Procedure.

opposed to some chromatographic methods (4,8-13), this fluorometric determination measures total tocopherols and tocotrienols.

Previously, the analysis of tissue tocopherol levels has been particularly cumbersome. Kayden et al. (5) used a similar saponification and extraction procedure but found that an additional TLC step was required prior to spectrophotometric analysis. Thompson et al. (29) also utilized a similar saponification treatment in the tocopherol analysis of various foods and tissues. However, that method also required a lengthy Soxhlet extraction and column chromatographic procedure prior to fluorometric measurement of tocopherol. The method of Edwin et al. (8) for analysis of rat liver tocopherol requires a lengthy Soxhlet extraction and several chromatographic steps to remove reducing contaminants prior to spectrophotometric analysis. Edwin et al. (8) emphasized that earlier analytical methods for tissue tocopherol gave misleading results. For GLC analysis, Bieri et al. (4) devised a time-consuming method to avoid tocopherol oxidation problems by conversion of tocopherol to tocopherylquinone prior to measurement. The pretreatment of samples for GLC required concentration of a hexane extract at 60 C, which may pose considerable oxidation risk. Also, prior removal of cholesterol is necessary for GLC analysis of tocopherols (9-11).

Information regarding the tocopherol content of subcellular fractions is lacking. Crane et al. (30) reported that the electron transport particles of beef heart contain 0.45 μg tocopherol per mg protein, which would agree with the values reported in Table III. Several studies on the distribution of labeled tocopherol among rat liver subcellular fractions (31,32) suggest that the bulk of the tocopherol occurs in the mitochondrial and microsomal fractions. Direct fluorometric analysis of the subcellular fractions confirmed this (Table III). Expression of tocopherol content in terms of μg/mg protein as used in Table III is somewhat misleading. Evarts and Bieri (33) have shown that relationships between tocopherol and polyunsaturated fatty acids are likely to be more important that protein-tocopherol relationships. The relative tocopherol content, percent of total tocopherol divided by percent of total protein, shows that tocopherol distribution among the various subcellular fractions is distinctly different from the protein distribution. However, these calculated values clearly show that certain subcellular fractions, particularly liver heavy mitochondrial and lung heavy mitochondrial and microsomal fractions, have higher concentrations of tocopherol relative to other fractions. The relatively high levels of tocopherol in the lung heavy mitochondrial and microsomal fractions may be important with regard to the higher oxygen tension in lung tissue and a resultant need for more antioxidant protection.

The reported fluorometric tocopherol assay also provides advantages over other methods in terms of sensitivity and reproducibility. Under the best conditions (16), the lower sensitivity limit for the commonly used spectrophotometric method is 1 μg/ml. A sensitivity limit of 0.05 μg/ml is routinely achieved with the fluorometric tocopherol determination (17,18). Detection following GLC is equally sensitive (4), while the lower sensitivity limit for the conventional 280 nm detectors for high pressure liquid chromatography is 20 μg (12). A special fluorescence detector coupled with high pressure liquid chromatography had a sensitivity limit of 0.01 μg (13). It should be noted that GLC and high pressure liquid chromatography have distinct advantages for the separation of the various tocopherol isomers (9-13).

Based on this evaluation, the fluorometric assay of tissue tocopherol would appear to be simpler, more specific, and more sensitive than other conventional methods for tocopherol analysis. Since the major oxidized product of α-tocopherol, α-tocopherylquinone, is non-fluorescent, this method should be useful in monitoring tocopherol oxidation in tissues. This method should also prove useful in the analysis of the effects of various dietary conditions on the tocopherol content of subcellular fractions.

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Low and Moderate Concentrations of Lysobisphosphatidic Acid in Brain and Liver of Patients Affected by Some Storage Diseases

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ABSTRACT

The relative amount of lysobisphosphatidic acid (LBPA), known also as bis(monoacylglyceryl)phosphate, among the total phospholipids was analyzed in post mortem samples of brain and liver of patients affected by four storage diseases. In spite of the extensive accumulation of storage lysosomes, none of the samples revealed a highly elevated LBPA content comparable to that found in the liver in Niemann-Pick disease and in the liver in lipidosis induced by 4,4'-diethylaminoethoxyhexestrol. We conclude that, although LBPA is often present in high concentration in lysosomes of many types of cells, it is not always a major component of these organelles.

INTRODUCTION

Lysobisphosphatidic acid (LBPA), also known as bis(monoacylglyceryl)phosphate, is a structurally unique phospholipid (1) which is a minor constituent of a number of animal tissues (2-5). This lipid is found enriched in purified Triton-containing lysosomes of rat liver (6), in paraffin oil-containing lysosomes of rabbit alveolar macrophages (7), and in the crude lysosomal fraction of cultured BHK cells (8). LBPA also has been detected in isolated ceroids and lipofuscins (9). Its concentration in other membranous organelles apart from lysosomes is small (10-13).

It would thus seem that LBPA is consistently present in the lysosomes of mammalian cells. This would also explain the high levels of LBPA in certain lipid storage diseases known to cause an increase in the number and size of the lysosomes in one or several tissues (14). Such observations include Niemann-Pick disease (15-18), the experimental lipidosis caused by 4,4'-diethylaminoethoxyhexestrol (19-21), and an experimental lysosomal hypertrophy in cultured BHK cells (13). Even the purified storage lysosomes of Niemann-Pick liver contain a high level of LBPA (16).

The present report, however, describes results obtained from analyses of brain and liver samples affected by several different storage diseases which show no or only a moderate increase of LBPA. The diseases examined were infantile neuronal ceroid-lipofuscinosis (INCL) (22-24), Spielmeyer-Sjögren type of neuronal ceroid-lipofuscinosis (SNCL) (9,25), aspartylglycosaminuria (AGU) (26-29), and G_{M2}-gangliosidosis (30-32). These diseases are characterized by pronounced accumulation of storage lysosomes in brain; in AGU the liver cells are also strongly affected. The present results are in accordance with the findings of Rouser et al. (15), who did not detect an increase of LBPA in tissues of patients suffering from other lipidoses like Gauchers disease, Tay-Sachs disease, metachromatic leucodystrophy, and juvenile amaurotic idiocy.

MATERIALS AND METHODS

The tissue samples were obtained within a day post mortem; that of the patient with G_{M2}-gangliosidosis was taken within 2 days. The samples were stored at -20 C. The thawed brain samples were dissected, and white matter and gray matter were analyzed separately. The morphological descriptions of the analyzed tissues of the patients with INCL, AGU, and G_{M2}-gangliosidosis have been reported elsewhere (22,23,29,32). The histological and ultrastructural features of the cases of SNCL corresponded to the earlier descriptions of the disease (9,25).

Lipids were extracted from the tissue samples according to Kates (33). Two-dimensional thin layer chromatography (2D-TLC) of the lipid extracts was performed as described elsewhere (5). Phospholipids were made visible on the chromatograms with a molybdenum blue spray reagent modified from that of Dittmer and Lester (34) as follows. Solution A was prepared by dissolving 10 g MoO_3 to 250 ml of 12.5 M H_2SO_4 by heating. Solution B was obtained by suspending 4.45 g Mo-powder into 125 ml of solution A; the suspension was boiled for 15 min, cooled, and decanted. The final reagent was prepared just prior to its use by mixing solution A, solution B, water, and ethanol in volume ratios of 1:1:4:6. Addition of ethanol to the orginal reagent served to enhance the adsorption of the spray on the chromatographic plate. LBPA was identified by its characteristic mobility (5) (Fig. 1).

The phosphorus contents of the phospholipid spots visualized by the molybdate spray or

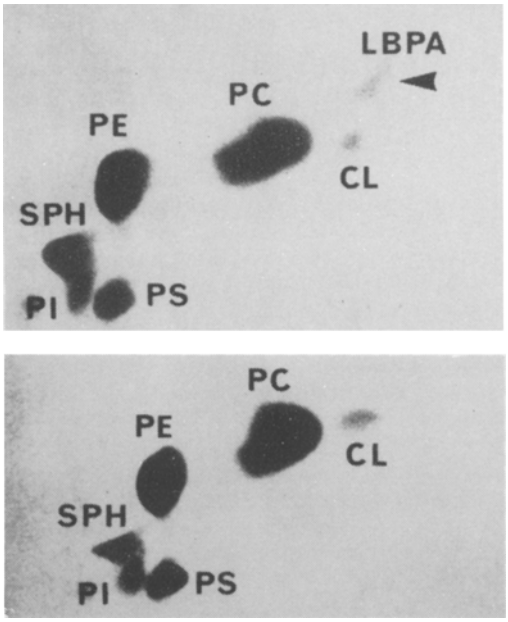

FIG. 1. Two-dimensional thin layer chromatograms of the gray matter lipids in (a) infantile neuronal ceroid-lipofuscinosis and (b) control brain stained with the molybdenum blue spray. LBPA is shown by the arrow in (a). LBPA = lysobisphosphatidic acid, CL = cardiolipin, PC = phosphatidyl choline, PE = phosphatidyl ethanolamine, SPH = sphingomyelin, PI = phosphatidyl inositol, PS = phosphatidyl serine.

by iodine vapor were determined by the method of Bartlett (35). A low-background lot of precoated thin layer plates (Silica Gel 60 F_{254}, E. Merck A.G., Darmstadt, West Germany) was used throughout, and blank areas were assayed on every plate. Detection limit was defined as a sample phosphorus of twice the blank value (which usually was ca. 0.05-0.1 μg). The net phosphorus content of the LBPA spot was expressed as percent of the total phosphorus in the lipid extract.

RESULTS

The separation of LBPA from other tissue phospholipids is achieved by 2D-TLC (5,15). No other phospholipid is known to co-migrate with LBPA in our chromatographic system. However, the cerebrosides, which are prominent in brain, partially overlapped the LBPA spot. To reduce the area of the spot collected for phosphorus analysis, we employed a specific molybdate stain to detect LBPA among the overlapping nonphospholipids. The concomitant reduction in the blank area considerably lowered the detection limit. The procedure was tested for possible interference caused by the molybdate reagent as follows. Aliquots of a reference brain lipid extract were separated by 2D-TLC and stained either with iodine vapor (5) or with the molybdate spray. The phosphorus contents of the different spots were almost identical in both experiments (Table I), indicating that the specific molybdate spray could be safely used before the phosphorus analysis. Dawson (36) has reached the same conclusion in the analysis of water soluble phosphate-esters by paper chromatography.

Brain Samples

The analysis of the brain samples of patients

TABLE I

Comparison of Iodine Vapor and Molybdenum Blue Stain as Visualizing Agents in Quantitative Phospholipid Analysis by Two-Dimensional Thin Layer Chromatography[a]

Components of the lipid extract	Phosphorus contents of the spots (μg)	
	Staining with iodine	Staining with molybdenum
Phosphatidylcholine	7.86 ± 0.11	7.83 ± 0.07
Phosphatidylethanolamine	5.14 ± 0.06	5.15 ± 0.08
Sphingomyelin	2.48 ± 0.08	2.44 ± 0.01
Phosphatidylserine	2.12 ± 0.20	2.15 ± 0.03
Phosphatidylinositol	0.64 ± 0.04	0.66 ± 0.04
Lysophosphatidylcholine	0.44 ± 0.07	0.42 ± 0.03
Cardiolipin	0.19[b]	0.18 ± 0.02
Phosphatidic acid	0.15 ± 0.04	0.15 ± 0.02
Lysobisphosphatidic acid	0.10 ± 0.03	0.06 ± 0.02
Blank spot	0.10 ± 0.04	0.09 ± 0.04

[a]Eight aliquots of equal size of a brain lipid extract were chromatographed two-dimensionally on thin layer plates. Four of the chromatograms were stained with the molybdenum blue stain and four with iodine vapor. The lipid spots were then analyzed for their phosphorus content. The results are mean ± SD. The blank value has not been subtracted from the figures. None of the differences were statistically significant.

[b]Only two analyses.

TABLE II

Content of Lysobisphosphatidic Acid (Percent of Lipid Phosphorus) in Brain and Liver Affected by Different Storage Diseases[a]

Disease	Patient	Sex	Age	Brain white matter	Brain gray matter	Liver
Infantile neuronal ceroid-lipofuscinosis (INCL)	M.I.	♂	9	0.94	0.84	0.64
	T.S.	♀	8	0.50	1.48	
	V.H.	♀	5	0.51	1.09	
	T.A.	♂	10			0.36
G_{M2}-gangliosidosis	A-C.L.	♀	3	1.36	0.93	
Spielmeyer-Sjögren type of neuronal ceroid-lipofuscinosis (SNCL)	B.N.	♀	20	ND[b]	ND	
	I.O.	♀	16	ND	ND	
	A.L.	♀	13			ND
Aspartylglycosaminuria (AGU)	A.V.	♂	27	ND	ND	0.62
	L.O.	♀	29			0.95
Normal values (16-19)				ND	ND	0.6±0.3

[a]The results are mean values of two independent thin layer chromatographic analyses. All deviations from the means were < 10%.

[b]Not detected. The detection limit was < 0.20% of lipid-P in all determinations.

with INCL and G_{M2}-gangliosidosis revealed a moderate increase in LBPA content both in the white and in the gray matter as compared to normal values (16-19) (Table II). The white matter of the INCL samples consisted mainly of astrocytes and some macrophages; axons and myelin are almost completely lost in the late stage of the disease (22,23). Hypertrophic astrocytes, known to contain membranous cytoplasmic bodies and other less characteristic inclusions (30-32), were also the main cellular constituents in the white matter sample of the patient with G_{M2}-gangliosidosis. The gray matter of the INCL samples contained numerous characteristic storage bodies in macrophages, in hypertrophic astrocytes, and also in the very few remaining neurons (Fig. 2). In G_{M2}-gangliosidosis, the cortical neurons or the proximal segments of their axons are distended by great amounts of membranous cytoplasmic bodies containing the stored ganglioside and showing acid phosphatase activity (30-32). This condition is also associated with severe cortical astrocytic hyperplasia and hypertrophy at the later stages of the disease.

None of the brain extracts from the SNCL and AGU patients contained detectable amounts of LBPA (Table II). The ultrastructural and histochemical examination of the samples revealed pronounced accumulation of storage material, but, unlike INCL and G_{M2}-gangliosidosis, no marked loss of myelin. In the gray matter of the AGU patient, the neurons were ballooned and rich in electron-lucent vacuoles and electron-dense granular bodies (29). In SNCL, the distended cortical neurons contain large amounts of autofluorescent lipopigment bodies with a characteristic ultrastructure consisting largely of fingerprint-like profiles (9,25). Similar bodies accumulate also in the astrocytes.

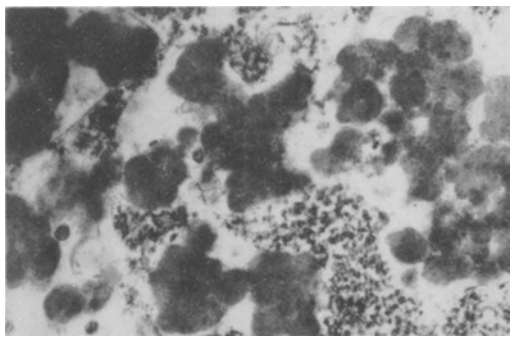

FIG. 2. Characteristic conglomerations of electron-dense globular bodies with finely granular ultrastructure in infantile neuronal ceroid-lipofuscinosis (nerve cell). Electron micrograph of an autopsy specimen, x 40,000.

Liver Samples

The LBPA concentration in the livers of INCL, SNCL, and AGU patients was within the normal range (Table II). The INCL sample was essentially normal and contained hardly any storage lysosomes. Also, in SNCL, the lipopigments are less prominent in the viscera than in brain (9). Therefore, it was no surprise that these livers did not contain elevated amounts of LBPA. However, the AGU livers showed extreme accumulation of storage vacuoles (Figs. 3-4) (29), which were obviously devoid of LBPA.

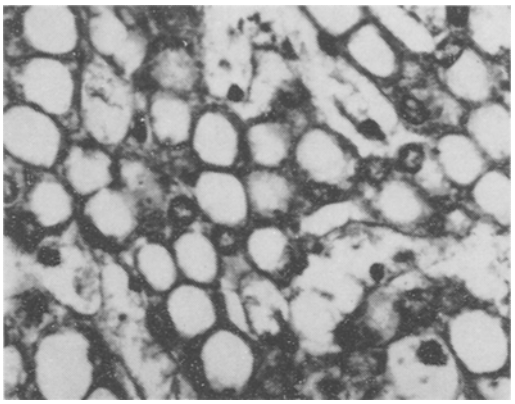

FIG. 3. Liver in aspartylglycosaminuria. Note the severe generalized hepatocellular vacuolation. Light micrograph of an autopsy specimen. Paraffin section stained with hematoxylineosin, x 790.

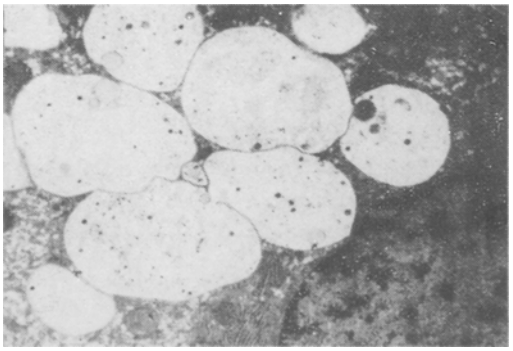

FIG. 4. A hepatocyte in aspartylglycosaminuria. The cytoplasm contains an aggregate of large, membrane-bound electron-lucent vacuoles. Nucleus in the lower right corner. Electron micrograph of biopsy specimen, x 20,000.

DISCUSSION

LBPA is known to be a lysosomal lipid (6-13), and most of the diseased tissues analyzed in the present study were rich in lysosomes. In the brain white matter samples of INCL and G_{M2}-gangliosidosis, the lysosome-derived structures of astrocytes are the most probable source of LBPA. In the gray matter of INCL and G_{M2}-gangliosidosis, the major fraction of LBPA may have resided in the astrocytes, but the data do not exclude its occurrence also in the neurons or macrophages and their storage bodies. However, the amount of LBPA in all these samples was much smaller than could be expected from the amount of lysosomes in the cells. In SNCL and AGU, no elevation of LBPA content was found in spite of extensive accumulation of storage bodies. The highest concentrations of LBPA found among total phosphatides of the tissues affected by the four different storage diseases were in the order of 1%; the concentration range found in healthy tissues and cells is 0.1–1.6% (2-5, 8,16-19).

In the white and gray matter of INCL and G_{M2}-gangliosidosis, where a moderate increase of the relative amount of LBPA was observed, it is possible that the elevation is due to the loss of myelin and enrichment of astrocytes rather than to the enrichment of storage bodies within the astrocytes.

The present observations as well as the data of Rouser et al. (15) are in striking contrast to several findings that show high concentrations of LBPA in both lysosome-rich tissues and isolated lysosomes. These include the liver of patients of Niemann-Pick disease, another storage disease, in which the livers may contain up to 11% of LBPA (15-18); the lipidosis caused by 4,4'-diethylaminoethoxyhexestrol, where the LBPA-content may rise up to 29% among liver phospholipids (19-21); the experimental lysosomal hypertrophy in cultured BHK cells (13); the isolated lysosomes of rat liver (6) and rabbit alveolar macrophages (7); and the crude lysosomal fraction of cultured BHK cells (8).

The contrast between these data and our present finding is probably not explainable by changes caused by post mortem autolysis in our samples. Brain lipids are fairly resistant to autolysis (37), and in the liver there appears to be little selectivity between the autolysis rates of the major phospholipid classes (15). Moreover, LBPA appears to be degraded slightly more slowly than the other phospholipids in isolated Triton-filled lysosomes of rat liver (38-39). Accordingly, we believe that, even if considerable autolysis of phospholipids had taken place in our samples the present analyses would show too high LBPA values rather than too low.

Consequently, it appears that the lysosomal storage conditions fall into two categories, the first involving little or no accumulation of LBPA and the second involving a large increase of this lipid. The existence of LBPA-rich and LBPA-poor storage bodies has also been demonstrated by Kamoshita et al. (16). They found that the storage bodies purified from the liver of a Niemann-Pick patient contained ca. 7% of LBPA, while similar bodies from the brain of the same patient were devoid of it. Mason et al. (7) have likewise found large amounts of LBPA in phagolysosomes of rabbit alveolar macrophages but not in those of guinea pig polymorphonuclear leucocytes.

It is not known at present what distinguishes

the LBPA-rich from the LBPA-poor lysosomes. Three possible explanations can be ruled out on the basis of present knowledge. (i) A direct genetic effect is ruled out by the increase of LBPA in experimentally induced lysosomal hypertrophies (13,19-21). (ii) The enzyme defect of the storage condition and the primary storage material per se is not the only factor regulating the LBPA concentration in the lysosomes. Lysosomes from Niemann-Pick liver and brain both contain large amounts of sphingomyelin, but LBPA is present only in liver (16). (iii) The possibility of rigid tissue dependence is ruled out by the observation that AGU liver does not contain large amount of LBPA, whereas Niemann-Pick liver does. There seems to be, however, some tissue specificity; brain is always low in LBPA, whereas liver and other visceral tissues sometimes contain remarkable amounts of this lipid.

The stereochemical structure of LBPA from most natural sources, including human brain and liver, is not known, but LBPA isolated from BHK cells is a derivative of 1-*sn*-glycerophosphoryl-1'-*sn*-glycerol (1). If this uncommon structure is also found in other natural LBPAs, it is clear that simple acylation-deacylation reactions (40) of phosphatidyl glycerol, a derivative of 3-*sn*-glycerophosphoryl-1'-*sn*-glycerol, cannot produce the lipid. We have suggested earlier that LBPA could be formed in lysosomes actively engaged in the degradation of lipids (1). This hypothesis offers one possible explanation for the accumulation of LBPA in only some—not in all—types of lysosomes: the LBPA-rich lysosomes may be engaged in lipid degradation, whereas the LBPA-poor lysosomes may not.

ACKNOWLEDGMENTS

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Cardiopathogenicity of Rapeseed Oils and Oil Blends Differing in Erucic, Linoleic, and Linolenic Acid Content

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ABSTRACT

Male Wistar rats were fed semipurified diets containing 20% fat for 25 weeks. Ten different oils or oil blends were employed, including rapeseed oils, simulated rapeseed-type oils, and modified rapeseed-type oils. Safflower, soybean, and hydrogenated coconut oils served as control oils. Histopathological examination of the cardiac tissue was conducted at the end of the study and an incidence-severity rating assigned to the lesions induced by each fat. Oils containing high levels of erucic acid (26-30%) induced the most severe cardiac necrosis, irrespective of the source of erucic acid (rapeseed oil or nasturtium oil). Increasing the linoleic: linolenic acid ratio of the high erucic oils to that of soybean oil failed to reduce necrosis, but the absence of linolenic acid from a high erucic acid oil blend resulted in a markedly reduced lesion incidence-severity rating, comparable to those obtained for low erucic acid rapeseed oil and soybean oil which were similar. Lowest lesion incidence was obtained with safflower oil and hydrogenated coconut oil. We have postulated that linolenic acid plays a role in the etiology of cardiac necrosis observed when rats are fed diets containing low erucic acid rapeseed oils.

INTRODUCTION

Several workers have demonstrated that inclusion of rapeseed oil in the diet of the rat for extended periods results in the development of lesions in the cardiac tissue (1-4). These lesions consist of focal necrotic degeneration of the muscle fiber and infiltration by mononucleocytes. Earlier studies involved those varieties of rapeseed oil in which erucic acid was a major component, accounting for 25-40% of the total fatty acids. This acid was implicated as a causative agent of the myocardial lesions (1-4). However, Rocquelin and Cluzan (2) found that the newer varieties of rapeseed oil, containing relatively low levels of erucic acid (1-2%), also induced cardiac lesions in the rat when incorporated in the diet. This finding was subsequently confirmed in a number of laboratories (5-7).

At the present time, the pathogenic agent in the low erucic rapeseed oils (LEAR) has not been identified. It is possible that even the low concentrations of erucic acid in these oil varieties are capable of lesion induction during prolonged feeding, but this appears improbable. A minor nontriglyceride component present in *Brassica* species could be the pathogenic factor, and this possibility is under active investigation in a number of laboratories in Canada. A third possibility is that the cardiopathogenicity of low erucic acid rapeseed oils is associated with their fatty acid balance. Rapeseed oils contain a relatively high proportion of linolenic acid in relation to linoleic acid. In view of the competition between the metabolic pathways of linoleate and linolenate, it is conceivable that a pseudo-essential fatty acid (EFA) deficiency is induced in LEAR-fed animals which may predispose them to cardiac lesions. Evidence has been presented for a marginal EFA-deficiency in the rat based on fatty acid analysis of testicular lipids (8). In this paper, this hypothesis was tested by feeding rats oils and oil blends of differing erucic acid contents and with different ratios of linoleic to linolenic acid. Cardiac tissue was examined for incidence and severity of lesions.

MATERIALS AND METHODS

Male Wistar Rats (Woodlyn Farms, Guelph, Ontario) weighing 40-70 g were randomly distributed among 10 experimental groups and housed individually in stainless steel cages with wire mesh floors. The animals were maintained on a 12 hr light-12 hr dark photoperiod and received food and water ad libitum for 25 weeks; they were weighed periodically throughout the experiment. The experimental diet contained, by weight, 20% vitamin-free casein, 37% dextrose, 18.5% cellulose, 3.5% modified Williams-Briggs salt mix, 1% vitamin mix (9), and 20% fat.

Ten different oils or oil blends were employed. Safflower, soybean, and hydrogenated coconut oils served as the low linoleic, linolenic-containing, and EFA-deficient control oils, respectively. *Brassica campestris* (32.6% 22:1) was the high erucic acid rapeseed oil (HEAR).

A simulated high erucic oil (SIMHEAO) was prepared using nasturtium oil (73% 22:1) as the source of erucic acid. "Soybean-high erucic acid oil (*Brassica*)" (SBHEAO(B)) and "soybean-high erucic acid oil (non-*Brassica*)" (SBHEAO (NB)) were formulated so that their erucic acid contents were similar to HEAR but their linoleate:linoleante ratios were similar to soybean oil. *Brassica napus* (40.0% 22:1) and nasturtium oil were the respective sources of erucic acid for these two oil blends. A "low linolenic-high erucic acid oil" (LLHEAO) was blended using nasturtium oil to supply erucic acid. *Brassica napus* var. 'Tower' was the LEAR, and a simulated low erucic oil (SIMLEAO) was prepared from non-*Brassica* oils. The oils employed in the diets were safflower oil (Teklad Mills, Madison, WI), soybean oil (Procter and Gamble Co. Ltd., Hamilton, Ontario), hydrogenated coconut oil (Canada Packers, Co. Ltd., Toronto, Ontario), *B. napus* var. 'Target' and *B. napus* var. 'Tower' (Co-operative Vegetable Oil Ltd., Altona, Manitoba), olive oil (Primo Importing & Distributing Co. Ltd., Toronto, Ontario), linseed oil (Toronto Elevators, Toronto, Ontario), and nasturtium oil. Nasturtium oil was obtained by hexane extraction of pulverized nasturtium seed, *Tropaeolum majus* (Northrup, King & Co., Minneapolis, MN) and was employed without further refining. The fatty acid compositions of the oils and oil blends and the formulations of the latter are presented in Table I.

The heart was sliced longitudinally through the middle. One-half of the organ was again sliced longitudinally through the middle. Three surfaces of the heart were thus exposed. The three segments of the organ were fixed in 10% neutral buffered formalin, imbedded in paraplast, and five micron sections prepared. The first 15 sections were discarded and the next three sections retained; this process was repeated twice more. Thus, three sections were obtained at each of three different depths in each of the three portions of the organ (27 sections per heart). The sections were stained with hematoxylin and eosin for routine histological examination.

RESULTS

The mean weight gains for rats in each dietary group are presented in Table II. There was no significant difference in the weight gains of rats in the two control groups fed safflower and soybean oils, but these animals significantly ($P<0.05$) outgained those in all other dietary groups. Although rats fed LEAR gained more weight than those fed HEAR or SBHEAO(B), differences among the *Brassica* groups were not significant. In spite of the similarity in fatty acid composition, the non-*Brassica* oil blends, SBHEAO(NB) and SIMHEAO, resulted in significantly ($P<0.05$) lower weight gains than their *Brassica* oil counterparts, SBHEAO(B) and HEAR. The presence of nasturtium oil in the non-*Brassica* blends appeared to depress growth. Very long chain monoenoic acids (20:1 and 22:1) constitute over 90% by wt of the total acids in this oil, and conceivably the absorption of the triglycerides containing these acids is poor in blends containing nasturtium oil. Nasturtium oil has a high sulfur content (J.D. Jones, personal communication), presumable derived from glucosinolates in the seed, and this could also account for the growth-depressing characteristics of this oil.

Somewhat surprisingly, the mixture of olive, safflower, and linseed oils in SIMLEAO did not support growth as well as the *Brassica* oil. No explanation can be offered for this phenomenon. Indeed, the SIMLEAO had a higher 18:2/18:3 ratio and a higher saturated:unsaturated fatty acid ratio than LEAR; both of these characteristics have been invoked to explain poorer performance in animals fed *Brassica* oils by other workers (6). Growth of animals fed the LLHEAO diet was also poor in comparison with that of all other rats except for those receiving the EFA-deficient diet. This blend again contained nasturtium oil, but it promoted significantly ($P<0.05$) lower weight gains than the other nasturtium oil-containing mixtures. It is difficult to believe that the absence of 6-7% 18:3 from this blend would affect the availability of the other fatty acids in the oil, but there does not appear to be any other explanation to account for the observed data.

Feed intakes were not recorded for the entire study, but they were monitored for 1 week after the animals had been on experiment for 3 weeks. Feed intakes ranged from 14.2 to 16.4 g per day during that period, but there appeared to be no correlation between food intake and overall weight gain.

Several different types of lesion were delineated in the histological examination of the heart. All classes of lesion were observed in all dietary groups on experiment. Microvascular alteration, consisting of edematous swelling and loosening of small and minute blood vessels, was observed in many rats (Fig. 1). These vessles exhibited many vacuoles of various sizes. Stenosis of the lumina or necrobiosis of smooth muscle cells of the walls was sometimes noted in the vessels.

Mild accumulation of small lipid droplets between the myofibrils and granular degenera-

TABLE I

Constitution and Fatty Acid Composition of Dietary Oils[a,b]

Oil	Safflower	Soybean	Hydrogenated coconut	HEAR	SIMHEAO	SBHEAO(B)	SBHEAO(NB)	LLHEAO	LEAR	SIMLEAO
Composition					Nasturtium 40% Olive 30% Safflower 18% Linseed 12%	*Brassica napus* 65% Safflower 34% Linseed 1%	Safflower 50% Nasturtium 40% Linseed 10%	Nasturtium 45% Safflower 45% Olive 10%		Olive 65% Safflower 22% Linseed 13%
Fatty acid										
8:0			8.1							
10:0			9.0							
12:0			52.0							
14:0			19.6							
16:0	6.6	11.2	12.0	4.4	4.8	4.4	4.8	4.6	4.3	8.1
18:0	2.4	4.1	8.3	1.4	1.7	1.5	1.5	1.4	1.8	2.7
18:1	12.5	25.4		28.6	28.4	17.1	10.0	16.0	57.8	53.8
18:2	77.4	50.7		17.9	19.0	36.1	40.8	42.1	22.9	26.4
18:3	0.3	8.4		7.9	8.3	5.9	6.5		9.0	7.9
20:1				11.1	7.5	8.5	8.0	7.1	1.8	0.3
22:1				28.8	29.9	26.0	28.5	28.7	0.8	
18:2/18:3	258.0	6.0		2.3	2.3	6.1	6.3		2.5	2.5

[a]Minor components have been omitted from the table.

[b]HEAR = high erucic acid rapeseed oil (*Brassica campestris*), SIMHEAO = simulated high erucic acid oil, SBHEAO(B) = soybean-high erucic acid oil (*Brassica*), SBHEAO(NB) = soybean- high erucic acid oil (non-*Brassica*), LLHEAO = low linolenic-high erucic acid oil, LEAR = low erucic acid oil var. 'Tower', SIMLEAO = simulated low erucic acid oil.

TABLE III

Incidence and Severity of Myocardial Lesions in Rats Fed Different Dietary Fats[a,b]

Dietary fat	Total rats in group	Microvascular alteration: Severity +++	++	+	Rating % Max	Fresh myocardial necrosis: Severity +++	++	+	Rating % Max	Relatively old myocardial necrosis: Severity +++	++	+	Rating % Max
HEAR	14		9	1	45		10	1	50	2	11	1	69
SIMHEAO	15		7	4	40	1	9	5	58		10	2	49
SBHEAO(B)	15		2	4	18	1	8	3	49	1	10	4	60
SBHEAO(NB)	15		3	8	31		14		62	1	11	2	60
LEAR	14		4	1	21		7	1	36		7	1	36
LLHEAO	14		2	5	21	1	2	4	21	1	4	5	38
Soybean	14		2	2	14		5		24		5	2	29
SIMLEAO	14		1	2	10		1	1	7		1	6	19
Coconut	14		2	1	12		2	2	14			4	10
Safflower	15		1		4			1	2			5	11

[a]+, very mild lesion, severity rating 1; ++, mild lesion, severity rating 2: +++, moderately severe lesion, severity rating 3. Overall rating = Σ (number of animals x severity rating) x 100% / (total number of animals) x (maximum severity rating).

[b]HEAR = high erucic acid rapeseed oil (*Brassica campestris*), SIMHEAO = simulated high erucic acid oil, SBHEAO(B) = soybean-high erucic acid oil (*Brassica*), SBHEAO(NB) = soybean-high erucic acid oil (non-*Brassica*), LLHEAO = low linolenic-high erucic acid oil, LEAR = low erucic acid oil var. 'Tower', SIMLEAO = simulated low erucic acid oil.

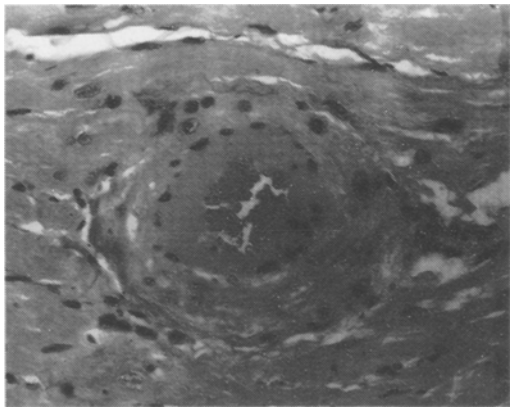

FIG. 1. Microvascular alteration. Wall of the small artery shows edematous loosening and vacuolation. Note swelling of the smooth muscle and endothelial cells. Diet: low erucic acid rapeseed oil. Hematoxylin and eosin, x 1024.

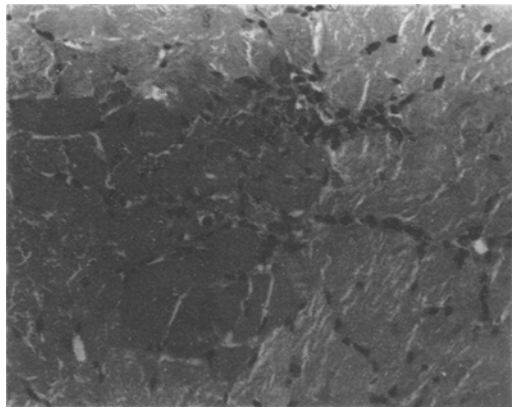

FIG. 2. Focal myocardial degeneration. Deeply eosinophilic muscle fibers contain small vacuoles; some of these fibers are infiltrated with mesenchymal cells. Diet: Soybean-high erucic acid oil. Hematoxylin and eosin, x 640.

tion of myocardial fibers were commonly seen in all dietary groups (Fig. 2). The area showing interstitial and/or perivascular edema was observed in various degrees in the different groups. Mast cells and pigmented macrophages were occasionally scattered in the edematous interstitium of the myocardium. Foci of myocardial degeneration exhibiting deeply eosinophilic fibers with large vacuoles were occasionally observed in this area.

Various degrees of focal myocardial necrosis were recorded in all dietary groups. The lesions were frequently found in the myocardium close to the epicardium of both ventricular walls; they were usually accompanied by congested minute blood vessels and/or extravasation of erythrocytes. In fresh myocardial necrotic lesions (Fig. 3), the muscle fibers frequently showed vacuolation, hyaline degeneration, basophilic degeneration, and sarcolysis. Necrotic muscle fibers were often phagocytosed by macrophages. Foci of relatively old myocardial necrosis showed remarkable mesenchymal cell proliferation with some fibroblastic differentiation (Fig. 4).

In Table III, the incidence and severity of the three major classes of lesion are recorded

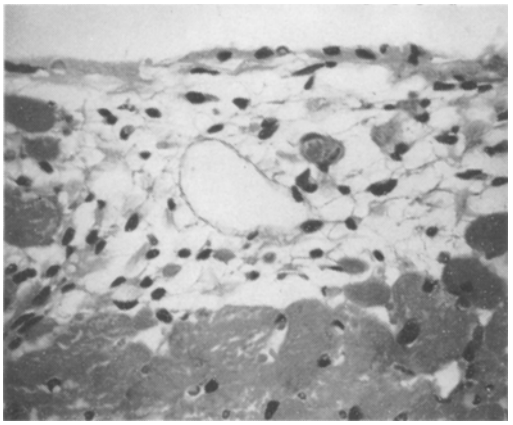

FIG. 3. Focus of fresh myocardial necrosis. Dilated minute blood vessels and residual fibers are present in the lesion. Diet: low erucic acid rapeseed oil. Hematoxylin and eosin, x 1024.

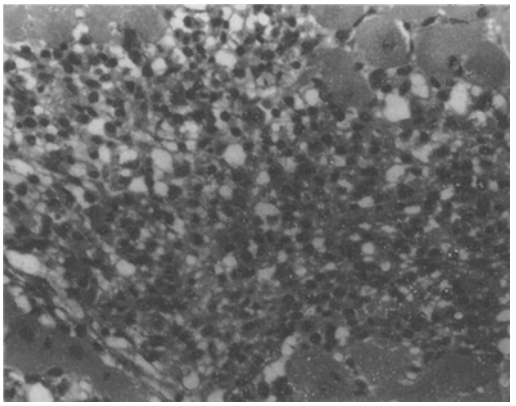

FIG. 4. Focus of relatively old myocardial necrosis. The area is replaced by proliferated mesenchymal cells. Diet: low linolenic-high erucic acid oil. Hematoxylin and eosin, x 640.

for each dietary group. These lesions are designated as microvascular alteration (Fig. 1), fresh myocardial necrosis (Fig. 3), and relatively old myocardial necrosis (Fig. 4). Hearts exhibiting extensive lesions or many small lesions in one longitudinal section of both ventricular walls were rated as moderately severe(+++). The occurrence of several small foci in the heart was recorded as a mild (++) involvement, whereas small and few lesions in the tissue resulted in a very mild (+) rating. To facilitate comparison among the various dietary groups, numerical values were assigned to each lesion rating as follows: +++, 3; ++, 2; and +, 1. The product of the incidence and severity of the lesion was summed for each dietary group and expressed as a percentage of the maximum possible

TABLE II

Total Weight Gain of Experimental Animals[a,b]

Dietary group		Mean weight gain (g)
Safflower oil	(15)	503 ± 9.6[a]
Soybean oil	(14)	488 ± 10.8[a]
Hydrogenated coconut oil	(14)	291 ± 8.5[f]
HEAR	(14)	417 ± 10.4[b]
SIMHEAO	(15)	394 ± 11.7[cd]
SBHEAO(B)	(15)	433 ± 11.6[b]
SBHEAO(NB)	(15)	383 ± 9.9[d]
LLHEAO	(14)	334 ± 9.7[e]
LEAR	(14)	443 ± 13.9[b]
SIMLEAO	(14)	395 ± 7.9[cd]

[a]Mean ± SEM. Means followed by the same superscript are not significantly different ($P<0.05$) analysis of variance and Duncan's multiple range test). Figure in parenthesis represents number of animals.

[b]HEAR = high erucic acid rapeseed oil (*Brassica campestris*), SIMHEAO = simulated high erucic acid oil, SBHEAO(B) = soybean-high erucic acid oil (*Brassica*), SBHEAO(NB) = soybean-high erucic acid oil (non-*Brassica*), LLHEAO = low linolenic-high erucic acid oil, LEAR = low erucic acid oil var. 'Tower', SIMLEAO = simulated low erucic acid oil.

incidence and severity (total animals x 3 for each lesion).

As is evident from Table III, we obtained a wide range of cardiac involvement in rats fed the 10 different dietary fats. Generally speaking, the rankings of the oils for incidence and severity were similar for all three lesions recorded in Table III, but there were some exceptions to this general observation. The high erucic oils which also contained linolenic acid resulted in the most severe cardiac lesions. All animals in these four groups (HEAR, SIMHEAO, SBHEAO(B), and SBHEAO(NB)) exhibited cardiac lesions. On the basis of the most commonly reported rapeseed oil-induced cardiac lesions, designated as fresh and relatively old myocardial necrosis in this paper, it is difficult to differentiate among these four oils with respect to the incidence-severity ratings. Adjustment of the linoleic:linolenic acid ratio of the dietary fat to that of soybean oil failed to alleviate the cardiopathogenicity of the high erucic oils on the basis of these two lesions (cf HEAR vs. SBHEAO(B) and SIMHEAO vs. SBHEAO(NB)). However, in the case of the microvascular alteration in the heart, this was not so. In rats fed the SBHEAO-type oils, the severity-incidence rating was lower than in the high erucic acid type oils; for the *Brassica* oils, this difference was substantial (HEAR vs. SBHEAO(B)).

A second group of oils ranked below the high erucic-high linolenic acid containing oils but still exhibited cardiopathogenicity. These

were LEAR, LLHEAO, and soybean oil. The results obtained with LLHEAO were surprisingly similar to those observed in the LEAR group. Microvascular alteration and foci of relatively old myocardial necrosis were of similar incidence and/or severity in these two groups, and the incidence-severity rating of fresh myocardial lesions was lower for the LLHEAO group. In terms of the latter type of lesion, rats in this group were similar to the soybean oil control snimals. Soybean oil fed-rats ranked at the bottom end of this group in microvascular alteration and relatively old myocardial necrosis ratings.

The three remaining dietary fats–SIMLEAO, hydrogenated coconut oil, and safflower oil–were least injurious to rat cardiac tissue. Rats fed safflower oil exhibited very mild infiltration with occasional incidences of myocardial necrosis and microvascular alteration. SIMLEAO and hydrogenated coconut oil behaved in a similar manner but had more adverse effects on the tissue than the safflower oil. For the most part, the lesions were very mild in nature, however.

DISCUSSION

The fat-induced cardiac lesions observed in the current study were basically those reported by other workers in this field (1-7). One exception was the microvascular alteration which has not previously been described in the literature on rapeseed oil-related cardiac pathogenicity. The occurrence of this lesion in heart tissues from rats fed rapeseed oil has been observed previously in similar studies at this institution and possibly represents the early phases of cardiac pathogenesis (10).

Indication of cardiac necrosis is not restricted to oils of the *Brassica* genus. In the present study, diets containing substantial proportions of nasturtium oil induced severe cardiac necrosis. Undoubtedly the high erucic acid content of this oil is a major factor in lesion induction. Abdelatif and Vles (11) reported that an equicaloric mixture of trierucin and sunflower oil produced cardiac lesions in rats. The origin of the trierucin was not reported, but conceivably the erucic acid was derived from rapeseed oil and may have contained minor components from that oil. Mustard oil, which is also high in erucic acid, will induce cardiac lesions in the monkey (12).

It is very apparent from the results obtained with animals fed hydrogenated coconut oil that a deficiency of essential fatty acids was not involved in the etiology of cardiac necrosis in the rat. High triene:tetraene fatty acid ratios and lack of arachidonic acid in a number of tissues (McCutcheon, unpublished data) indicated that these rats were indeed EFA-deficient, and yet lesion incidence was very low in this group, in contrast with the high erucic-high linolenic acid groups.

The possibility that a linoleic-linolenic acid imbalance facilitated lesion formation in rat cardiac tissue was only partially supported by the data obtained in this study. Increasing the linoleic:linolenic acid ratio of the high erucic acid oils did reduce the incidence-severity rating of microvascular alteration in animals consuming them. This was true for both the rapeseed and nasturtium based oils. However, modification of the linoleate:linolenate ratio did not affect the incidence-severity rating of the high erucic acid oils with respect to the more commonly reported myocardial necrosis. It is possible that the linoleate:linolenate ratio may influence the initiation of myocardial lesions since microvascular alteration could be one of the factors contributing to myocardial necrosis. However, the presence of the more advanced necrotic lesions in the cardiac tissue may be distorting the picture to some extent, and a much shorter experimental period would be preferable for providing more definitive information concerning the correlation of linoleate: linolenate ratio and early degenerative changes in the myocardium.

As was noted above, modification of the high erucic oils to yield a similar linoleate: linolenate ratio to soybean oil failed to alleviate myocardial necrosis. In contrast, elimination of linolenic acid from a high erucic acid oil markedly lowered the incidence-severity score for that oil (cf. LLHEAO, Table III). Indeed, this particular oil ranked with LEAR and soybean oil in its ability to induce myocardial necrosis. All three oils were less toxic than the high erucic-high linolenic acid oils but more toxic than the safflower and coconut control oils.

It is possible that the lower toxicity of LLHEAO in comparison to the high erucic-high linolenic acid oils is due to a lower availability of erucic acid to rats fed the former oil. Weight gains were significantly ($P<0.05$) lower in rats fed LLHEAO, and this did not appear to be due to differences in food intake (cf. Table II) but may indicate lower availability of this fat. Since the matabolizable energies of the oils were not determined, this cannot be confirmed. However, fatty acid analyses of several tissues from rats fed the various dietary fats revealed that all of the high erucic acid oils, including LLHEAO, resulted in similar accumulations of erucic acid in the total tissue lipids (McCutcheon, unpublished data). Apparently, dietary erucic acid

was readily available to rats fed LLHEAO, and it is doubtful that the lower incidence-severity rating for myocardial necrosis can be attributed to imparied absorption of this acid.

On the basis of the data obtained, the presence of linolenic acid in an oil may be considered as a contributing factor in the etiology of myocardial necrosis. There is evidence for and against this concept. Beare-Rogers et al. (7) reported that partial hydrogenation of high or low erucic acid rapeseed oils reduced the incidence of cardiac lesions. In their experiments, hydrogenation resulted in a substantial decrease in the linolenic acid content of the oils whereas the docosenoic acid content was only slightly affected. They postulated the presence in rapeseed oils of a pathogenic factor other than erucic acid which was removed or destroyed by hydrogenation. Their results with hydrogenated high erucic oils parallel those obtained with LLHEAO in the present study. Since the LLHEAO contained no *Brassica* oil, the second toxic factor is not restricted to that species. It could very well be linolenic acid. Similar results have been obtained in hydrogenation studies in this laboratory (Slinger et al., unpublished data).

In a very comprehensive study, Kramer et al. (13) investigated the cardiopathogenicity of several fractions obtained by absorption chromatography or molecular distillation of a low erucic acid rapeseed oil. All fractions, including a highly purified triglyceride fraction, proved to be toxic. Most of the fractions obtained represented enrichments of nontriglyceride components in a triglyceride carrier, the latter constituting the bulk of the material. The linolenic acid contents of the fractions were similar to that of the intact oil. The authors concluded that if some factor other than erucic acid were responsible for the toxicity of the oil, it was not a minor component, such as brassicasterol, but rather a characteristic of the triglyceride fatty acid distribution. Low saturated:unsaturated fatty acid ratio, low linoleic:linolenic acid ratio, and the high linolenic acid content of rapeseed oils were offered as possible contributing factors to rapeseed oil cardiopathogenicity. As has been pointed out, increasing the 18:2/18:3 ratio failed to decrease the incidence of necrotic lesions in rats fed the high erucic diets in the present study. There was no correlation between the saturated:unsaturated fatty acid ratio or the saturated:monoenoic acid ratio and the cardiopathogenicity of the oils.

In considering linolenic acid as a potential cardiopathogenic factor in the rat, it is interesting to note that most oils that have been employed as control oils in previous studies on rapeseed oil, and which have induced lesions in relatively few animals, contain little or no linolenic acid. They include, in addition to the safflower oil employed in the current study, peanut oil, olive oil, and a lard:corn oil mixture (3:1). Soybean oil, on the other hand, has given mixed results. Beare-Rogers et al. (7) obtained a lower incidence of lesions with soybean oil than with low erucic rapeseed oils, although it was inferior to the lard:corn oil mixture. Vogtmann et al. (14), using relatively few animals, found no difference between soybean oil and low erucic rapeseed oils.

In contrast to the evidence implicating linolenic acid in the cardiopathogenicity of rapeseed oils are the results obtained with the simulated low erucic oil in the present study. This oil rated lower than the other high linolenic oils on the incidence-severity scale for the three classes of lesions, although the total numbers of animals exhibiting cardiac lesions were similar for this group, LEAR, and soybean oil. The SIMLEAO was devoid of erucic acid, which may account for its lower pathogenicity vis-a-vis LEAR but not vis-a-vis soybean oil. This anomaly remains to be investigated. It should also be noted that some investigators have found no unusual cardiopathogenicity associated with rat diets containing linseed oil (J.L. Beare-Rogers, personal communication). This also conflicts with the results obtained in the present investigation.

On the basis of results obtained in this study, we are proceeding with an investigation of the role of linolenic acid in the cardiopathogenesis of rapeseed oil. There does appear to be a relationship between the total linolenic acid content of the oil and its ability to induce myocardial necrosis in the rat. It does not appear at present to involve the balance of linoleic and linolenic acid in the oil. Any involvement of linolenic acid in this pathological process may not necessarily be ascribed to abnormal metabolic processes associated with this acid. In view of the propensity of linolenic acid for autoxidation, it is conceivable that destruction of nutrients in the diet or gut as a result of this latter process may contribute to the cardiac lesions in the rat. These possibilities are under active investigation.

ACKNOWLEDGMENTS

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Study of Bound Phospholipase Activities of Fungal Mycelia Using an Organic Solvent System

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ABSTRACT

It has been possible to demonstrate and characterize high phospholipase activities in mycelia of *Rhizopus arrhizus* and *Mucor javanicus* by use of a system in which substrates were dissolved in diisopropyl ether. Such activities were associated with bound enzymes and would have been difficult to detect using aqueous assay systems. In both cases, phosphatidylcholine hydrolysis was by phospholipase A_1 (EC 3.1.1.32) activity followed by the action of lysophospholipase (EC 3.1.1.5). Phospholipase D (EC 3.1.4.4) activity was also detected. The methods used appear to be of general applicability for the detection and study of insoluble phospholipases.

INTRODUCTION

During a general program of investigation into the action of naturally insoluble enzymes acting on substrates dissolved in organic solvents, it was observed that certain fungal mycelia had a high phospholipase activity. Most existing methods for the assay of phospholipase have used systems in which the substrate was dispersed in aqueous media (1,2), sometimes with addition of ether or other activating substances (3-7). It became obvious that phospholipase activities of mycelia were often considerable in organic solvent systems while being undetectable in aqueous systems, and, to study these insoluble phospholipases, it was necessary to devise an assay based on nonaqueous systems. The work reported here describes the applicability of such a system to an investigation of the mycelia of *Rhizopus arrhizus* and *Mucor javanicus*.

In the assay system described, the levels of substrate required for optimal enzyme activity were so high that the routine use of pure substrate was impracticable, and methods have been designed to take this into account.

Recent observations on other microbial phospholipases have suggested that insoluble or membrane-bound phospholipases may be of widespread occurrence (8-13). Studies on such enzymes have involved their solubilization with possible modification of enzymic properties. This suggests that direct observations on the bound enzymes would be of particular interest.

MATERIALS AND METHODS

Production of Mycelial Preparations

Cultures of *R. arrhizus* (CMI 83711) and *M. javanicus* (CMI 25330) were obtained from the Commonwealth Mycological Institute (Surrey, England). The culture medium employed in the cultivation of both organisms was based on that of Fukomoto et al. (14) and consisted of corn-steep liquor (5%), olive oil (2%), KH_2PO_4 (0.2%), KCl (0.05%), $NaNO_3$ (0.05%), and $MgSO_4$ $7H_2O$ (0.05%), all w/v. The medium had an initial pH of 4.6. The corn-steep liquor and the olive oil were commercial grade and obtained locally. Other chemicals were "Analar" grade, supplied by B.D.H. Chemicals Ltd., Poole, England.

Erlenmeyer flasks (2 liters), each containing 400 ml of the sterile culture medium, were inoculated with equal volumes of the appropriate spore suspension in water. Incubation of the inoculated medium was carried out using an "Orbital Incubator Shaker" (Gallenkamp, England) at 26 C and at a shaking speed of 250 rpm. At the end of 5 days, the mycelia were harvested by filtration, washed several times with distilled water, and freeze dried. The mycelia were then defatted with diethyl ether using a Soxhlet apparatus, residual ether removed under vacuum, and the mycelial material ground until the particles passed through a No. 16 (0.05 in.) mesh. The material was stored over P_2O_5 until used. At the time of use, the moisture level of the mycelia was of the order of 8%, as measured by drying at 105 C to constant weight.

Preparation of Crude Pancreatic Phospholipase A_2

Fresh pig pancreas was homogenized in water (200 g/100 ml) at 4-6 C. The homogenate was freeze dried, defatted, and stored as described for the mycelial preparations.

Substrate

Egg lecithin (B.D.H. Chemicals Ltd.) was partially purified by acetone precipitation from

an ethereal solution followed by washing in acetone which was then removed under nitrogen. The freshly purified lecithin was dissolved in dry solvent (normally diisopropyl ether) to give the required concentration (10% w/v unless otherwise stated).

Phopholipase Assay

Into 25 ml conical screw-cap flasks (Sovirel Laboratory Glassware, Levallois-Perret, France) were placed weighed quantities of mycelial preparations (normally in the range 40-100 mg), and to each was added a predetermined amount of water (usually 80 μl), care being taken that the mycelial preparation was not wetted. An aliquot of the lecithin solution (normally 2.0 ml) was then pipetted into each flask and shaken to ensure good mixing. The sealed flasks were then incubated with shaking in a water bath (Mickel Laboratory Engineering Co., Surrey, England) at the required temperature (50 C in most experiments) for a period in the range 0.3 to 48 hr. At the end of the appropriate reaction period, each flask was cooled and 4 ml of chloroform:methanol, 2:1 (v/v), added and mixed. Following centrifugation, the supernatant fluid was recovered and the mycelial material washed twice with 4 ml volumes of the chloroform:methanol mixture. The combined solvent extracts were reduced in volume under a stream of nitrogen and made up to 10.0 ml in a volumetric flask with chloroform:methanol. The appropriate flasks and controls were prepared in a similar manner.

Phospholipase activity was assayed by titration of liberated fatty acids, using suitable controls, and, after separation of components by thin layer chromatography (TLC), by measurement of phosphatidylcholine (PC) destroyed and lysophosphatidylcholine (LPC) formed by estimation of phosphorus as described below.

Fatty Acid Value

A 2.5 ml aliquot of the chloroform:methanol extract was added to 10 ml ethanol and titrated against 0.05 M NaOH in 90% methanol (15) using thymolphthalein as indicator.

Thin Layer Chromatography

A 10 μl sample of the extract was applied to a freshly activated TLC plate coated with a 0.25 mm thick layer of "Kieselgel G" (E. Merk, Darmstadt, West Germany) and the lipids separated in chloroform:methanol:30% ammonia solution:water, 60:35:5:2.5 (v/v/v/v) (16). The separated components were detected by spraying with Zinzadze reagent (17).

When fatty acid analyses were to be performed, components were located by spraying with 2,6-dichlorofluorescein and viewing under shortwave UV light prior to removal for gas-liquid chromatography (GLC) analysis.

Phosphorus Estimation

Following TLC, appropriate areas were scraped off, transferred to test tubes, and phosphorus estimated by the method of Morrison (18). Blanks were measured by removal and analysis of equal areas of adsorbent from the plate. The silica was removed by centrifugation prior to spectrophorometric measurement at 822 nm.

Gas-Liquid Chromatography

Methyl esters were prepared by the method of Morrison and Smith (19) and separated using a Pye Series 104 Chromatograph (W.G. Pye and Co., Ltd., (Cambridge, England) which utilized a 6 ft x 1/8 in. (internal diameter) stainless steel column packed with preconditioned 15% EGGS-X on Gas-Chrom. Q(100-120 mesh) (Applied Science Laboratories, State College, PA) maintained at 190 C. Argon at a flow rate of 40 ml/min was used as the carried gas, and the eluted esters were detected using a hydrogen flame ionization detector. Samples of 1.0-1.5 μl of the ester solution were injected. $C_{17:0}$ fatty acid was employed as an internal standard. Peak areas were obtained from the product of the retention times and respective peak heights. From these values the amount of each fatty acid present could be determined.

Extraction of Water-Soluble Products

Following extraction of lipids the mycelial pad was dried in a stream of nitrogen and extracted several times with small volumes of water which were combined. The components of the reaction mixture which were soluble in chloroform:methanol were separated and analyzed in the usual manner. The components in the aqueous fraction were separated by paper (Whatman, Grade 1) chromatography, the developing solution being n-propanol:30% ammonia:water, 60:30:10 v/v/v (1). Separated components were visualized employing shortwave UV light, modified Hanes-Isherwood reagent (20) for phosphorus compounds or ninhydrin for nitrogen compounds. The appropriate areas (including blanks) were cut out and the amount of phosphorus in each component estimated as already described.

Disintegration of Cell Walls

By use of a rotary bead mill ("Dynomill' Type K.D.L., W.A. Bachofen, Basle, Switzer-

TABLE I

Relative Phospholipase Activity of Mycelial Preparations in Various Solvents

Solvent	Relative phospholipase activity	
	Rhizopus arrhizus	*Mucor javanicus*
Diisopropyl ether	100	100
Diethyl ether	125	139
Heptane	30	29
Carbon tetrachloride	0	27
Cyclohexane	0	18

land), mycelia of *M. javanicus* were disintegrated under the following conditions: 4% w/v suspension passed at a rate of 1 liter/hr at a chamber temperature of 8-10 C, chamber volume 0.61, bead volume 0.51, bead size 0.2-0.5 mm, disc speed (peripheral) = 10 m/sec.

Microscopic examination showed that virtually complete cell wall rupture had occurred.

The soluble and insoluble fractions were separated by centrifugation (1 hr at 30,000 g), the insoluble fraction washed by decantation with distilled water and centrifugation. Both fractions were freeze dried and stored over P_2O_5.

RESULTS

Selection of Solvent

Using mycelial preparations of *R. arrhizus* and *M. javanicus* as enzyme sources, the suitability of various solvents for use in assay systems was examined by comparison of the rates of fatty acid liberation obtained. In all cases, the substrate concentration was 10% (w/v) and the reactions were carried out under identical conditions for 1 hr at 25 C. Although no detailed studies were carried out, it can be seen from Table I that the acitivity obtained with diethyl and diisopropyl ether was at least 3-4 times greater than in any of the other solvents. Since diisopropyl ether had a higher boiling point than diethyl ether, it was employed in all further studies.

Hydrolysis Reaction in Aqueous Systems

An aqueous emulsion of lecithin at a concentration of 2% w/v was sonicated until a clear dispersion was obtained. Following incubation for 17 hr with 50 mg mycelial preparation at 37 C, no fatty acid liberation could be detected using either *R. arrhizus* or *M. javanicus* preparations. By contrast, employing a lecithin solution of a similar concentration in diisopropyl ether and the same reaction conditions, the fatty acid liberated amounted to 90 and 71 μm with *R. arrhizus* and *M. javanicus*, respectively. Another aqueous system incorporating Ca^{++} ions and borax buffer (21) was also examined, but the degree of hydrolysis did not exceed 6% following prolonged incubation, compared with conversions of over 40% obtained with the organic solvent system.

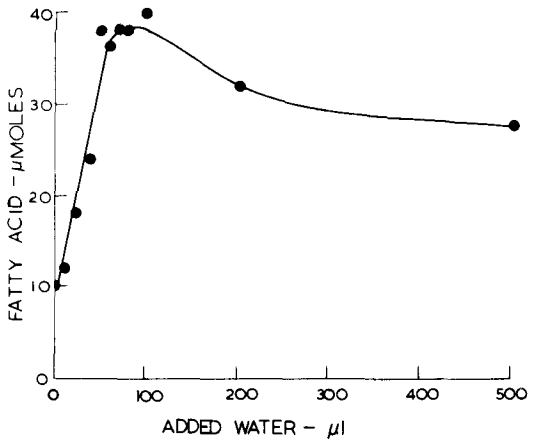

FIG. 1. Effect of quantity of added water on phospholipase activity of mycelia of *mucor javanicus*. The reaction mixture consisted of 2 ml 10% (w/v) lecithin in diisopropyl ether and 60 mg mycelia. Activity is reported as titratable fatty acid liberated over a 2 hr period.

Effect of the Amount of Water

It was established in initial experiments that the same degree of hydrolysis was observed irrespective of whether water was added directly to the system or an identical amount was introduced by humidification of the mycelial preparations to a predetermined weight over saturated salt solutions. The former, simpler method was consequently adopted. The effects of different amounts of added water on rates of lecithin lydrolysis by *M. javanicus* preparations were examined (Fig. 1). It will be seed that hydrolysis still occurs in the absence of added water, presumably due to

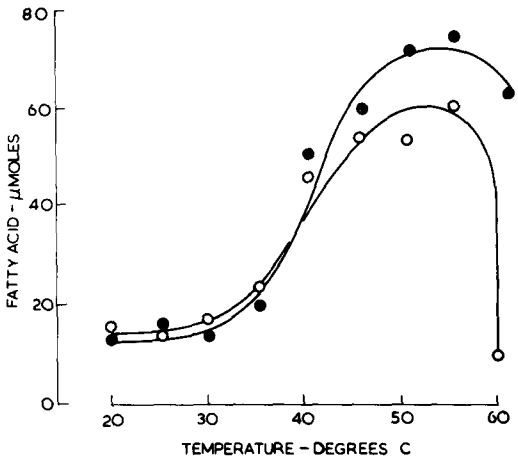

FIG. 2. Effect of temperature on phospholipase activity of mycelia. The reaction mixture consisted of 2 ml 10% (w/v) lecithin in diisopropyl ether, 60 μl water, and 60 mg mycelia of *Rhizopus arrhizus* (○) or of *Mucor javanicus* (●). Activity is reported as titratable fatty acid liberated over a 1 hr period.

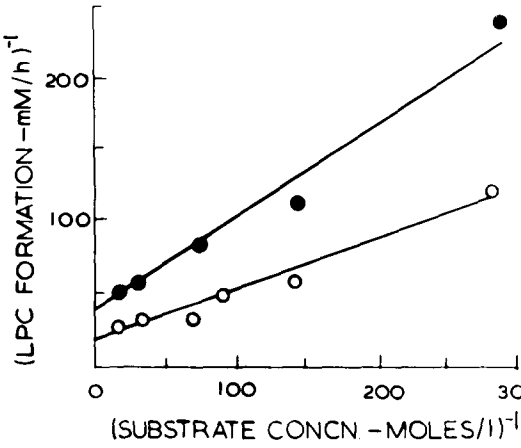

FIG. 3. Effect of substrate concentration on initial rate of phosphatidylcholine hydrolysis by mycelia of *Rhizopus arrhizus* (●) or *Mucor javanicus* (○). The reaction mixture consisted of 2.5 ml of lecithin in diisopropyl ether, 100 μl water, and 150 mg mycelia. Activity was estimated by the amount of lysophosphatidylcholine formed during a 1 hr period.

the moisture retained by the mycelia. Similar results were obtained with *R. arrhizus*. The diminution in activity with greater amounts of added water is probably due to "clumping" of the mycelia and the consequent inaccessibility of the substrate to the enzyme.

Effect of Temperature

As can be seen in Figure 2, there is little change in reaction velocity between temperatures of 20 C and 30 C compared to 35 C and 40 C, the curve being atypical of those obtained for most enzyme reactions. Changes in the solubility of lysolecithin produced, accessibility of substrate to enzyme, and distribution of water between phases may be variables contributing to this observation. In subsequent experiments, the temperature of 50 C was used since it was close to the optimal temperature of enzyme activity but substantially below the boiling point (68-69 C) of diisopropyl ether.

Effect of Substrate Concentration

When using a solid phase crude enzyme acting on a substrate which is not necessarily in molecular solution, it is obviously not possible to describe reaction kinetics in other than empirical terms. It was nevertheless of interest to examine the applicability of Michaelis-Menten kinetics as have other workers using phospholipase in emulsion systems (2,7,10).

The effects of varying the lecithin concentration on the rate of formation of LPC catalyzed by mycelial preparations of *R. arrhizus* and *M. javanicus* are shown in Figure 3.

The apparent Km values obtained were of the order of 16 mM for *R. arrhizus* and 20 mM for *M. javanicus*. Whether this similarity for the two sources is indicative of similarity of the enzymes involved or alternatively due to physical characteristics of the system is not known at present.

It was confirmed that at the amounts of mycelia employed in this study there was a correlation between weight of mycelia and reaction velocity when a fixed lecithin concentration of 10% w/v was used. It was, however, noted that when < 50 mg of mycelia were used the observed activity was in some cases less than anticipated.

Nature of the Hydrolysis Reaction

Since the hydrolysis of lecithin was carried out using a crude mycelial preparation which might contain more than one type of enzymic activity, it was necessary to examine the nature of the reaction in more detail over longer periods. Amounts of LPC, residual PC, and fatty acid liberated were all measured at intervals over a period of 46 hr. Results are shown in Figures 4 and 5 for *R. arrhizus* and *M. javanicus*, respectively. It can be seen that for both organisms the initial, rapid accumulation of LPC corresponds closely with the destruction of PC. This is followed by a decrease in the concentration of the lyso-derivative, the destruction of the original substrate continuing throughout the reaction period. Thus, there

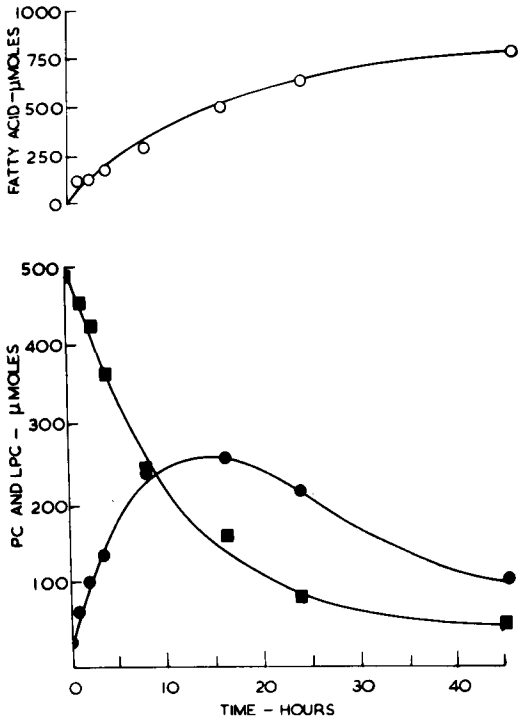

FIG. 4. Change in concentrations with time of phosphatidylcholine (PC) lysophosphatidylcholine (LPC), and liberated fatty acids during the hydrolysis of PC by mycelia of *Rhizopus arrhizus*. The reaction mixture consisted of 2.5 ml of 20% (w/v) lecithin in diisopropyl ether, 100 μl water, and 150 mg of mycelia. PC (■), LPC (●), and fatty acid (○).

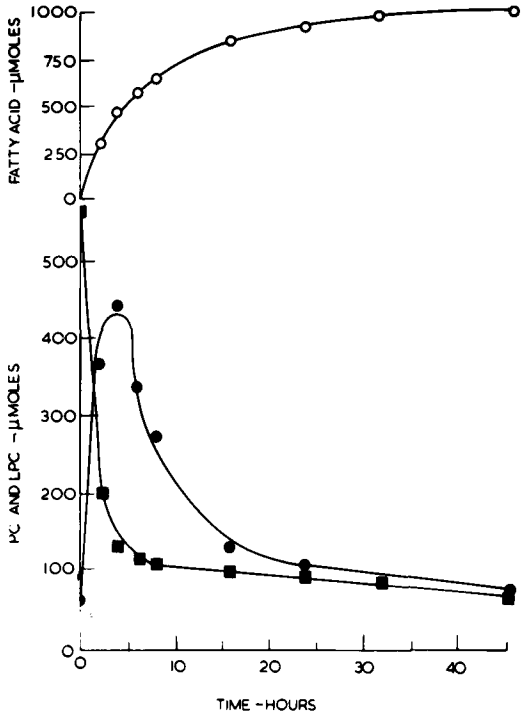

FIG. 5. Change in concentrations with time of phosphatidylcholine (PC, lysophosphatidylcholine (LPC), and liberated fatty acids during hydrolysis of PC by mycelia of *Mucor javanicus*. The reaction mixture consisted of 2.5 ml of 20% (w/v) lecithin in diisopropyl ether, 100 μl water, and 150 mg of mycelia. PC (■), LPC (●), and fatty acid (○).

appears to be present both phospholipase A and lysophospholipase type activities.

It would be expected that the fatty acids liberated in the earlier part of the reaction would be those split off from PC by phospholipase A, while those liberated subsequently would also include those arising from the action of lysophospholipase on LPC. It was shown by GLC analysis that the fatty acid liberated during the first 4 hr of the reaction was 90.4% saturated whereas the total fatty acid liberated over 48 hr was 46.1% saturated. This indicates attack at the predominantly saturated 1-position of PC by phospholipase A_1 activity, followed by lysophospholipase liberation of the residual, mainly unsaturated acids of the 2-position. However, since phosphatidylethanolamine was known to be present in the lecithin used, it was considered that a more accurate estimation of the positional specificity of the phospholipase A component towards PC could be obtained by analysis of the fatty acids residual in the LPC.

Fatty Acid Composition of LPC Formed upon Enzymic Hydrolysis of Lecithin by Mycelia of *R. arrhizus* and *M. javanicus* and by Pancreatin

In some initial experiments, it was confirmed that preparations of pig pancreas using the systems described here showed phospholipase A_2 activity. This was demonstrated by analysis of the fatty acid composition of the lyso-derivative formed, and these results are shown for comparison with those obtained using the mycelial enzyme preparations. In these experiments, 20% (w/v) lecithin solutions were incubated with 75 mg enzyme preparation over a period of 8 hr. The LPC fractions were separated by TLC and analyzed for fatty acid composition. The results shown in Table II indicate that the two types of mycelia have phospholipase A_1 activity in contrast to the phospholipase A_2 activity of pancreatin.

Identification of Other Products Formed during Lecithin Hydrolysis

The data shown in Figures 4 and 5 had indicated the presence of lysophospholipase. The

TABLE II

Comparison of Fatty Acids in Lysophosphatidylcholine Produced from Phosphatidylcholine by the Action of *Rhizopus arrhizus, Mucor javanicus,* and Pancreatin

	Esterified fatty acids (mol %)				
	Egg phosphatidylcholine[a]				
Fatty acid	1-position	2-position	*R. arrhizus*	*M. javanicus*	Pancreatin
14:0	0.5		0.1	0.1	0.3
16:0	61.3	1.6	6.2	10.6	59.2
16:1	1.1	1.2	1.1	1.2	2.2
18:0	27.0	0.1	4.6	5.4	27.7
18:1	8.5	52.1	47.5	46.2	7.8
18:2	1.2	33.2	25.3	21.7	1.3
18:3		0.1	0.6	0.6	0.3
20:0	0.2		0.3	0.3	0.3
20:1	0.1	0.1			
20:3		0.1	0.3	0.3	0.2
20:4		6.5	7.1	6.8	0.6
22:3		0.5			
22:4		1.0		0.4	
22:5		0.1	0.8	0.8	
22:6		3.5	6.5	5.5	
% saturated/ % unsaturated	89.0/11.0		11.2/89.2	16.4/83.6	87.5/12.4

[a]Data of Kuksis and Marai (22).

TABLE III

Products Obtained from Egg Lecithin by Prolonged Hydrolysis with Mycelia of *Rhisopus arrhizus*

	Product (μM)			
	Incubation period (hr)			
Components of hydrolysis mixture	0	2	8	46
Phosphatidylcholine	137	95	33	1
Lysophosphatidylcholine	14	48	91	40
Phosphatidylethanolamine	30	22	0	0
Lysophosphatidylethanolamine	32	18	26	27
Phosphatidic acid	0	5	2	12
Lysophosphatidic acid	0	0	18	16
Glycerylphosphorylcholine and glycerylphosphorylethanolamine	0	-	-	86
Inorganic phosphate (PO_4^{3-})	0	-	-	19
Phosphorylcholine and phosphorylethanolamine	0	-	-	10
Fatty acid	0	86	184	294
Titratable acidity of aqueous extract (μM H^+)	0	-	-	56
Total phosphate (organic $[P]_o$	213	188	170	96
Total phosphate (aqueous) $[P]_a$	0	20	40	116
$[P]_o + [P]_a$	213	208	210	212

possibility that other enzymes activing on phospholipids were present was examined.

An 8% w/v) lecithin solution was incubated with 150 mg of *R. arrhizus* under standard conditions for periods of 2,8, and 46 hr. Both solvent-soluble and water-soluble components were extracted and analyzed.

The results shown in Table III confirm that there is substantial lysophospholipid formation in the early stages of hydrolysis. Upon incubation for longer periods, the formation of phosphatidic acid is evident and degradation of the lyso-derivatives to water to water soluble products occurs. In terms of phosphate esters, the products isolated appear to account for all the substances destroyed. It is evident from these findings that a number of conversions are involved in the overall breakdown of lecithin in the system described.

Enzymic Activities Following Disintegration of the Cell Walls

Sixty mg quantities of both the soluble and insoluble fractions obtained following the disintegration procedure were examined for enzymic activity using a substrate concentration of 10% w/v under standard conditions. A 60 mg quantity of mycelia, unmilled but otherwise

subjected to the same treatment as the disintegrated samples, was used for comparison. LPC and phosphatidic acid were estimated at intervals throughout the reaction period, and the results in Figure 6 show that the phospholipase A and lysophospholipase activities remain bound to the insoluble fraction. The greater specific activities of the insoluble fraction can be attributed to the removal of water-soluble inactive material.

It can also be seen that with the unmilled preparation there was a considerable formation of phosphatidic acid, presumably due to phospholipase D activity. After milling, the particulate fraction has no phospholipase D activity, although this persists in the soluble fraction at a diminished level.

Various other attempts were made to detach the phospholipase A activity from the mycelia: the use of Triton X-100, butanol (23), and sodium dodecyl sulphate (10) were unsuccessful, and so far it has not been found possible to achieve solubilization of the enzyme.

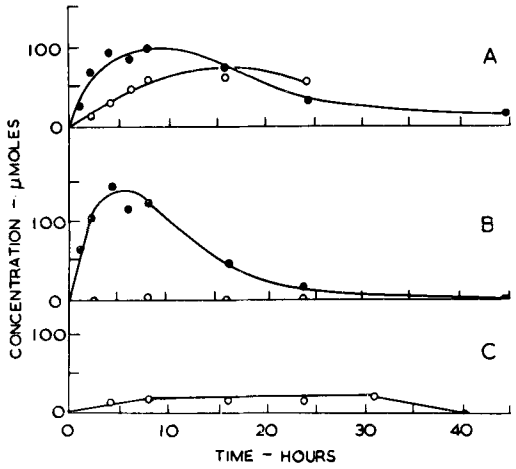

FIG. 6. Effect of disintegration of mycelia of *Mucor javanicus* using a bead mill on phospholipase activities. A = unmilled preparation, B = insoluble fraction after milling, C = water-soluble fraction, lysophosphatidylcholine (•), phosphatidic acid (○).

DISCUSSION

Use of solutions of phospholipids in ether in assays of phospholipase activities arose from the discovery by Hanahan (24) that the phospholipases A_2 of pancreas and snake venoms form ether-soluble aggregates with substrates. It appears that the use of ether for substrate solution has not been previously applied to the less-studied phospholipase A_1 enzymes.

The enzymes studied here are so strongly bound as to be undetachable by methods in common use. In studies on the solid-phase enzymes, solvent systems reduce the physical complexity of the assay and provide high levels of product formation for subsequent analysis. Indeed, such systems may offer the possibility of large scale production of lysophospholipids without need for extensive enzyme purification, and either the 1-acyl or 2-acyl lyso derivative can be produced by appropriate selection of enzyme source.

It is likely that the substrate is presented to the enzyme in the form of much smaller aggregates than would occur in aqueous systems and that these are of an inverted form with the nonpolar fatty acid chains on the outside (25,26). Dawson (27) has pointed out that in the cell phospholipids may exist in a less crowded environment than found in aqueous dispersions. The use of phospholipid substrates in organic solvents may reproduce this aspect of the in vivo situation.

In studying the effect of substrate concentrations on reaction velocities, the expression "apparent Km" has been used. It has been stated by Brockeroff and Jensen (28) that the use of Km as a constant is of little value in phospholipase studies, and they point out that the turnover number cannot have the usual meaning since the substrate may be in varied aggregates on which the enzyme reacts at different rates. Nevertheless, it is of interest that the few published Km values for soluble phospholipase A_1 lie between 3.4×10^{-7} M (from *Echeveria coli*) (10) and 8×10^{-4}M (from rat brain) (7) in aqueous systems. A Km of 8 mM has been obtained when ethereal solutions of lecithin underwent hydrolysis by snake venom phospholipase A_2 (29). Since the apparent Km values for the A_1 enzymes in the work described here were 16 mM and 20 mM, it may be that such high values are characteristic of solvent systems.

Both the phospholipase A_1 and the lysophospholipase activities appear to be tightly bound to the mycelial material, judging by their resistance to solubilization. As indicated in Figure 6, there is, following milling, an increase in both these activities which can be related to the removal of water-soluble material. It might have been anticipated that disintegration would have exposed more enzyme sites and so enhanced the activities, but, at present, there seems to be little basis for speculation on the location of lipolytic enzymes in fungal mycelia.

While well-characterized phospholipase A_1 tightly bound to cell membranes is known to occur in bacteria (10,30), there have been no prior reports of bound phospholipase A in the

filamentous fungi. The only reports relating to the filamentous fungi appear to be on the purified extracellular lipases from *R. arrhizus* (5) and *M. javanicus* (31), which exhibited some phospholipase A_1 activity. In the case of *M. javanicus*, culture filtrates showed a lipase:phospholipase activity ratio of 200-300:1 during latter stages of purification (31). Owing to the difficulty encountered in solubilizing the mycelial enzymes studied here, it is not at present possible to state whether some of the phospholipase A_1 activity may be due to lipase, but work on this is at present being carried out.

The presence of phospholipase D was also indicated. This enzyme has been detected in microorganisms such as *Corynebacterium pseudotuberculosis* (32) and *Haemophilus parainfluenzae* (12), but not in the filamentous fungi.

There appear to have been no previous studies on the phospholipases of fungal mycelia. The methods described in this paper have been found suitable for such studies on bound or insoluble phospholipases. They have shown that phospholipase A_1 activity predominates and that the commoner phospholipase A_2 activity is low or absent in the organisms examined.

Further studies are now being carried out to determine whether the types of phospholipase activity reported here are of general occurrence in the filamentous fungi.

ACKNOWLEDGMENTS

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Lipid Metabolism of the Yellow Clam, *Mesodesma mactroides:* 2-Polyunsaturated Fatty Acid Metabolism

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ABSTRACT

The fate of labeled linoleic, α-linolenic, and higher homologs of α-linolenic acid administered to the yellow clam, *Mesodesma mactroides,* was investigated. It was found that the clam incorporated the acids dissolved in sea water and converted 18:2 (n-6) into 20:2 (n-6) and 18:3 (n-3) into 18:4 (n-3) and 20:3 (n-3). The addition of casein hydrolysate to the sea water increased the desaturation capacity of the clam and allowed the conversion of 18:2 (n-6) into 18:3 (n-6) to be demonstrated. An enhanced desaturation of 18:3 (n-3) into 18:4 (n-3) was also demonstrated. After 12 hr administration of the acid, no radioactivity was found in arachidonic, 20:5 (n-3), or 22:6 (n-3). Feeding the clams a culture of *Phaeodactylum tricornutum* previously incubated with 1-^{14}C-α-linolenic acid demonstrated that all the homologs of the α-linolenic series were found in the clam without any important changes. Six hour administration of labeled linolenic acid resulted in the incorporation of the acid into diglycerides and phospholipids.

INTRODUCTION

In a previous paper (1), the lipid and fatty acid composition of the yellow clam, *Mesodesma mactroides,* collected on the beaches of Mar Azul, Argentina, in different seasons was reported. It was shown that the fatty acid composition of the yellow clam was, in general, a reflection of the fatty acids of the food.

Marine organisms may be classified into different trophic levels in the food chain. It is generally accepted that polyunsaturated fatty acids are mainly synthesized de novo by some species of phytoplankton and are transported to the higher forms of life through the marine food chain (2-5). Therefore, this presupposes the existence of a common dietary origin without excluding possible biosynthesis and transformation at different higher levels.

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Among the phytoplanktophagus organisms, or primary consumers, we find zooplankton, filter-feeder molluscs, and other organisms. *M. mactroides* is a filter feeder, and we have shown that, especially in summer, it is a typical phytoplanktophage (1). Therefore, it is important to investigate whether, in addition to the typical primary feeder ability, the yellow clam has the capacity to convert unsaturated fatty acids into higher homologs. In the present work, we investigated the metabolic fate of labeled linoleic, α-linolenic, and higher homologs of α-linolenic acid in *M. mactroides.*

MATERIAL AND METHODS

Materials

1-^{14}C-linoleic acid (53 mCi/mmol, 99% radiochemically pure, 2% trans isomer) and 1-^{14}C-α-linolenic acid (41.5 mCi/mmol, 99% radiochemically pure, 1% trans isomer) were purchased from Amersham-Searle (Amersham, England).

Organisms

M. mactroides were harvested in March in the sandy beaches of Mar Azul, Argentina, as described in a previous work (1). They were kept for 1 day in a net hung in aerated sea water and filtered through cotton wool before the experiment.

Radioactive Fatty Acid Administration

Three types of media were used. Medium No. 1 was a synthetic sea water solution to which the ammonium salts of the labeled fatty acids were added in a proportion of 0.01 to 0.015 μmol/100 ml of medium. Medium No. 2 was prepared in the same way and supplemented with 1 g/liter Casenolin (casein hydrolysate, Glaxo Arg Co., Argentina).

Medium No. 3 was prepared by suspension of an axenic culture of *Phaeodactylum tricornutum* in synthetic sea water solution. The diatom was provided by the Culture Collection of Algae, Department of Botany, Indiana University (Bloomington, IN). The polyunsaturated fatty acids of the α-linolenic acid family of the diatom were labeled by previous incubation with 1-^{14}C-α-linolenic acid in synthetic sea water solution.

TABLE I

Labeling Distribution in *Mesodesma mactroides* Fatty Acids after Administration of 1-^{14}C-linoleic and 1-^{14}C-α-linolenic Acids Dissolved in Sea Water

Fatty acids	1-^{14}C 18:2n-6			1-^{14}C 18:3n-3		
	3 hr[a]	6 hr	12 hr	3 hr	6 hr	12 hr
16:0	4.6 ± 0.7[b]	2.8 ± 0.8	6.2 ± 0.1	0.6 ± 0.4	1.8 ± 0.7	1.9 ± 0.3
18:0	----	1.2 ± 0.4	3.0 ± 0.7	----	1.1 ± 0.5	1.8 ± 0.1
18:1n-9	----	2.2 ± 0.7	4.0 ± 0.9	----	1.3 ± 0.8	1.7 ± 1.0
18:2n-6	60.8 ± 1.4	63.0 ± 1.2	53.1 ± 1.2	----	----	----
20:1n-9	----	1.5 ± 0.7	2.3 ± 1.0	----	----	----
18:3n-3	----	----	----	90.8 ± 1.2	88.2 ± 1.8	87.3 ± 2.1
18:4n-3	----	----	----	2.3 ± 0.5	3.0 ± 0.5	1.3 ± 0.3
20:2n-6	34.6 ± 2.1	29.3 ± 1.1	31.4 ± 3.0	----	----	----
20:3n-3	----	----	----	6.3 ± 1.2	4.6 ± 0.7	6.0 ± 0.9
Total incorporation (%)	8.0 ± 2.3[c]	15.5 ± 3.6	17.3 ± 1.8	7.5 ± 2.3	16.8 ± 4.0	18.0 ± 2.2

[a]Administration time in Medium No. 1.

[b]Results expressed as percent of total recovered ^{14}C are the mean of the analysis of three groups of two clams each ± SEM. The chromatogram was run till 22:6n-3 peak.

[c]The radioactivity measured in the medium before incubation was considered 100%.

Labeling of Polyunsaturated Fatty Acids of *P. tricornutum*

Aliquots of 100 ml of a suspension of 5 x 10^6 cells/ml that were in the logarithmic phase of growth were incubated with 0.5 μCi of the ammonium salt of 1-^{14}C-α-linolenic acid. After 3 hr incubation at 20 C, the labeled diatoms were separated by centrifugation at 3,000 rpm. After resuspension in 300 ml of synthetic sea water, they were recentrifuged, and this operation was repeated twice. The final precipitate of cells was resuspended in synthetic sea water at a final concentration of 2.5 x 10^6 cells/ml. This suspension was used to feed the clams as Medium No. 3. Aliquots of the diatom suspension were homogenized with a Potter apparatus and extracted with chloroform: methanol (2:1) (6). The lipids were saponified, the unsaponifiables extracted with petroleum ether (bp 30-60 C), and the soaps acidified and esterified 30 min with 3 N HCl acid in methanol. The distribution of the radioactivity in the different fatty acids was determined with a gas liquid radiochromatograph (Pye) equipped with a proportional counter (7).

P. tricornutum incorporated and partially converted 1-^{14}C-α-linolenic acid to labeled 18:4 (n-3), 20:3 (n-3), 20:4 (n-3), 20:5 (n-3), and 22:6 (n-3), indicating that they have desaturating enzymes of the "animal" type.

Procedure

Aliquots of 200 ml of each medium containing the labeled acids were placed in 800 ml bottles with two clams per bottle at room temperature. Filtered air was bubbled into the flasks continuously and then passed through hyamine hydroxide to collect expired labeled CO_2. Radioactivity was measured in a Packard scintillation counter (8).

After determined periods of time, the clams were taken out of the flasks and carefully opened to avoid damage and loss of soft tissues. They were washed with synthetic sea water and the soft tissue homogenized in a VirTis 23 apparatus. The lipids were extracted with $CHCl_3$:CH_3OH (2:1) (1). The extracted lipids were weighed and the radioactivity measured in a Packard scintillation counter. The fatty acid composition was then studied, aliquots of the lipids were saponified, and the fatty acids converted to the methyl esters. The radioactivity distribution of the fatty acids was measured by gas liquid radiochromatography at 180 C in a Pye apparatus equipped with a proportional counter (7). The column was packed with 10% polyethylene-glycol-succinate in Chromosorb W (80-100 mesh). Fatty acids were identified as described previously (1).

For determination of specific radioactivity, fatty acid methyl esters were separated in an F&M chromatograph using the same type of column described previously. The esters were collected at different time intervals in the scintillation solution of diphenyloxazole (PPO) and 5-phenyloxazolylbenzene (POPOP) in toluene. The radioactivity was measured in the Packard scintillation counter. The total activity recovered was 70-72%. To calculate the relative specific activity of each fatty acid methyl ester, the radioactivity of each peak was corrected for 100% and was divided by the area of the peak measured in the mass chromatogram.

Lipid Separation

Aliquots of the total lipids of the clam were

TABLE II

Radioactivity of 1-^{14}C-linoleic and 1-^{14}C-α-linolenic Acids Incorporated and Oxidized by *Mesodesma mactroides*[a]

Fractions	1-^{14}C 18:2n-6		1-^{14}C 18:3n-3	
	Percent[b]	dpm/mg	Percent	dpm/mg
Total lipids	23.3 ± 1.2	2,771 ± 139	21.9 ± 1.6	2,792 ± 213
Fatty acids	20.0 ± 1.7	13,325 ± 1,070	19.3 ± 1.6	12,045 ± 1,139
Water soluble	0.6 ± 0.3	----	0.5 ± 0.3	---
Expired CO_2	5.0 ± 0.9	----	4.4 ± 1.2	---

[a]Labeled acids were administered dissolved in Medium No. 1 during 6 hr. Results are the mean of eight groups of two clams each ± SEM.

[b]The radioactivity of the medium before incubation was considered 100%.

fractionated into "polar" and "nonpolar" lipids by absorption on silicic acid (8). The lipids of each fraction were separated by thin layer chromatography (TLC) by the procedure already described in the first paper of this series (1).

The spots of the "nonpolar" lipids of the TLC were scraped off and extracted with chloroform, determining the mass and radioactivity.

RESULTS AND DISCUSSION

Incorporation of Linoleic and α-Linolenic Acids Dissolved in Sea Water by *M. mactroides*

It has been demonstrated that sea water normally contains a small amount of dissolved fatty acids (10). These fatty acids originate from the decay of the lipids of sea organisms. In a previous communication (11), we demonstrated that *M. mactroides* was able to absorb and incorporate dissolved fatty acids. A similar absorption has also been shown in other filtering organisms (10).

In Table I, it is demonstrated that labeled linoleic and α-linolenic acids dissolved in sea water are assimilated by *M. mactroides* and the incorporation increases with filtering time.

Both labeled linoleic and α-linolenic acids are not only incorporated into the lipids of the clam but also converted to other fatty acids (Table I). The ratio precursor:products decreases with time, demonstrating that there is a continuous conversion of substrate into products.

Palmitic, stearic, and oleic acids were only slightly labeled with ^{14}C. This result may be interpreted as a consequence of β-oxidation of 1-^{14}C linoleic acid and α-linolenic acid with labeled acetyl-CoA production followed by de novo biosynthesis of saturated and monounsaturated acids. The oxidation of the labeled acids by the clam is shown clearly in Table II. It exhibits the distribution of ^{14}C labeling in the different components of the clam after 6 hr incubation with 1-^{14}C linoleic or 1-^{14}C-α-linolenic acids. A measurable amount of the acids is oxidized to CO_2, but the highest labeling is found in the fatty acids incorporated.

Therefore, the clam is able to incorporate unsaturated fatty acids dissolved in the sea water and to use them as a source of energy.

Biosynthesis of Fatty Acids of the Linoleic Acid Family

Research done preferentially with rats has shown that linoleic acid is converted to higher homologs in the animals by a sequence of desaturations and elongations that take place in the microsomes (12-15). The route that leads to the formation of arachidonic acid is apparently initiated by a Δ6-desaturation to γ-linolenic acid followed by an elongation to 20:3 (n-6) and desaturation to arachidonic acid (14-17). In addition, it has been shown that rat microsomes (18) incubated under exclusively elongating conditions are able to evoke the following elongations: 18:2 (n-6) → 20:2 (n-6) → 22:2 (n-6) → 24:2 (n-6). However, the elongation of linoleic and α-linolenic acids is more efficiently carried out by the mitochondria (19). The mitochondrial enzymes are preferentially specific for linoleic and α-linolenic acids, whereas the microsomal enzymes are specifically active with γ-linolenic acid (19).

The administration of 1-^{14}C linoleic acid in the sea water to the clam demonstrates that in this feeding condition the elongation reaction is the only measurable one (Table I), but we cannot envisage which of the two mechanisms evokes the elongation. The clam converts linoleic acid to 20:2 (n-6), but no radioactivity was detected in γ-linolenic and arachidonic acids, indicating no measurable Δ6-desaturation activity. Efficient elongation of linoleic acid to 20:2 (n-6) has been demonstrated in the land mollusc *Cepaea nemoralis* (20) but this mollusc

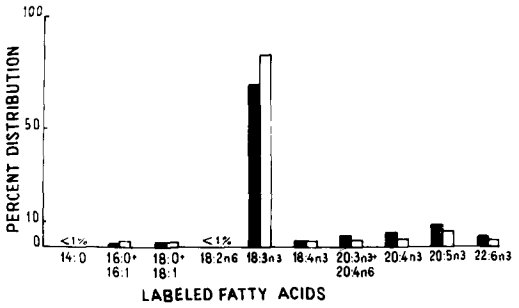

FIG. 1. Labeling distribution of fatty acids of *Mesodesma mactroides* and *Phaeodactylum tricornutum* after feeding the clam with diatoms previously incubated with 1-^{14}C-α-linolenic acid. White and black bars correspond to the clam and diatom fatty acids, respectively. The clams were fed with the diatoms during 12 hr as described in Methods and Materials.

also desaturated the acid, synthesizing 20:3 (n-6) and arachidonic acid.

Biosynthesis of Fatty Acids from the α-Linolenic Family

Experiments with rats (15-21) and fish (22) have shown that the acids 20:5 (n-3) and 22:6 (n-3) are synthesized in the animals from α-linolenic acid by alternate desaturations and elongations similar to linoleic acid.

The results of administration of 1-^{14}C-α-linolenic acid in sea water to the yellow clam (Table I) prove that this mollusc is able to elongate the acid to 20:3 (n-3). Moreover, and differently from what happens with linoleic acid, some radioactivity was also found in 18:4 (n-3), proving the presence of a Δ6-desaturation. The possibility of measuring the Δ6-desaturation of α-linolenic acid and not of linoleic acid may be the consequence of the lower affinity of the enzyme for the second acid that made it undetectable for the sensitivity of the method. In any event, α-linolenic was preferentially incorporated as such into the lipids, and the total conversion to higher homologs was less active for α-linolenic acid than for linoleic acid.

In this experiment, and at least for a period of 12 hr, the yellow clam was unable to synthesize the end product of the series 20:5 (n-3) and 22:6 (n-3). Moreover, feeding the clam diatoms in which the fatty acids of the α-linolenic acid series, including 20:5 (n-3) and 22:6 (n-3), have been labeled shows that the radioactivity of the fatty acids in diet and consumer are similarly distributed (Fig. 1). Since the clams were thoroughly washed after diatoms administration, little contamination with undigested diatoms was expected, except for some amount possibly present in the gut. However,

TABLE III

Labeling Distribution in the Fatty Acids of the Clam after Administration of 1-^{14}C-linoleic and 1-^{14}C-α-linolenic Acids Dissolved in Sea Water in the Presence of Casein Hydrolysate[a]

Fatty acids	1-^{14}C 18:2n-6		1-^{14}C 18:2n-6+protein		1-^{14}C 18:3n-3	1-^{14}C 18:3n-3+protein
	Percent	dpm/peak area	Percent	dpm/peak area	Percent	Percent
14:0	0.8 ± 0.6	64 ± 13	0.8 ± 0.6	21 ± 4	1.4 ± 0.8	0.8 ± 0.4
16:0	1.1 ± 0.1	3 ± 0	---	---	0.5 ± 0.3	0.5 ± 0.3
18:2n-6	63.6 ± 2.2	31,558 ± 437	69.3 ± 2.4	54,359 ± 650	---	---
18:3n-6	---	---	5.6 ± 0.6	3,120 ± 20	---	---
18:3n-3	---	---	---	---	87.4 ± 1.4	86.6 ± 3.4
18:4n-3	---	---	---	---	3.9 ± 0.8	7.1 ± 0.8
20:2n-6	32.5 ± 1.4	12,254	24.3 ± 2.2	12,489 ± 110	---	---
20:3n-3	---	---	---	---	6.8 ± 0.4	4.8 ± 1.4

[a]Results are the mean of two pools of four groups of two clams each fed during 6 hr ± SEM.

TABLE IV

Distribution of Radioactivity in the Lipids of *Mesodesma mactroides* after Administration of 1-^{14}C-linoleic and 1-^{14}C-α-linolenic Acid[a]

Lipid fraction[b]	1-^{14}C 18:2n-6		1-^{14}C 18:3n-3	
	Percent	dpm/mg	Percent	dpm/mg
Total nonpolar lipids	85.0 ± 1.5	3,630 ± 213	79.9 ± 1.3	2,913 ± 122
Origin zone	34.3 ± 0.3	10,856 ± 740	31.2 ± 0.2	8,499 ± 30
Diglycerides	23.9 ± 0.2	6,444 ± 665	21.6 ± 2.5	5.083 ± 743
Free acids	26.8 ± 0.5	10,816 ± 948	27.1 ± 1.1	9,150 ± 100
Total polar lipids	15.0 ± 1.5	1,184 ± 76	20.1 ± 1.3	1,346 ± 97
Origin zone	0.4 ± 0.1	---	0.7 ± 0.4	---
Phosphatidyl choline	1.8 ± 0.1	---	2.5 ± 0.1	---
Phosphatidyl ethanolamine	1.1 ± 0.2	---	1.8 ± 0.1	---
Free acids	11.7 ± 1.6	---	15.1 ± 1.9	---

[a]Results are the mean of two pools of four groups of two clams each fed during 6 hr ± SEM.

[b]Lipids separated in "polar" and "nonpolar" and fractionated by thin layer chromatography as described in Materials and Methods.

after 12 hr feeding, we may assume that most of the lipids of the diatoms have been assimilated. Therefore, results obtained would demonstrate that the administered fatty acids were absorbed and preferentially stored by the yellow clam without very important transformation.

Comparing these results with the changes of the fatty acids composition of the clam evoked by the type of food in the natural habitat (1), this organism, and probably marine fitering molluscs in general, would receive and store highly unsaturated fatty acids of the α-linolenic acid family provided mainly by the phytoplankton.

Effect of Protein on Desaturation of Linoleic and α-Linolenic Acids

Addition of casein hydrolysate to sea water containing labeled linoleic or α-linolenic acid modified the desaturating capacity of the clam. Table III shows that the protein evoked an increase in the Δ6-desaturation of fatty acids, and not only more α-linolenic acid was converted to 18:4 (n-3), but also linoleic acid desaturation to 18:3 (n-6) was measurable. The effect of protein was only evoked in the Δ6-desaturation and not in the elongation that remained practically constant. Therefore, this selectivity would practically rule out the possibility that the increased desaturation could be due to an enhanced assimilation of the labeled acids evoked by the casein hydrolysate. Considerable activation of Δ6-desaturase activity without any effect on the elongation is evoked in rat liver microsomes by administration of hyperproteic diets (23-24). Therefore, the behavior of the clam suggests that the desaturating activity of the microsomes is modified in sea water molluscs by food protein in a manner similar to that in the rat.

Consequently, the yellow clam is able to modify the fatty acid composition of the ingested food by elongation and Δ6-desaturation of linoleic acid and α-linolenic acid. These reactions are generally produced to a very limited extent, but they may be modified by the presence of other dietary components. The acids 20:5 (n-3) and 22:6 (n-3) are possibly provided preferentially by the food.

Esterification of Labeled Fatty Acids in the Lipid Fractions

M. mactroides partially esterified absorbed linoleic and α-linolenic acids and their conversion products (Table IV). The free fatty acid fraction of the extracted lipids was $< 48.5\%$ in all cases. The radioactivity was mainly found in the diglyceride zone of the TLC, little in phosphatidyl choline and phosphatidyl ethanolamine, and nothing in triglycerides, alkoxyglycerides, and sterol esters. Preferential labeling in the clam of diglycerides over phospholipids and triglycerides after 6 hr filtration of the fatty acids dissolved in sea water may be interpreted as inconclusive evidence of biosynthesis of lipids by Kennedy's route (25). At least Bilinsky (26) and Shieh (27) have shown that marine invertebrates can synthesize phospholipids by this route. Since the radioactivity of phospholipids is low and triglycerides are not labeled, a possible interpretation is that diglyceride conversion to the other lipids is slower than diglyceride synthesis.

A relatively high radioactivity was found at the origin during TLC of nonpolar lipids. The nature of this labeling was not investigated, but it may be due to oxidation products of fatty acids and some phospholipids not separated by

silicic acid in the "nonpolar" lipid fractionation.

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Unusually High Levels of C_{24}-C_{30} Fatty Acids in Sponges of the Class Demospongiae

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ABSTRACT

Twenty genera of sponges from the class Demospongiae have been examined for fatty acid composition. All contain unusually high levels (34-79%) of C_{24}-C_{30} fatty acids not generally found in other organisms. These characteristic "demospongic acids" are mostly polyunsaturated.

INTRODUCTION

In 1951 Bergmann and Swift (1) first reported the presence of unexpectedly high mol wt fatty acids in two marine sponges, *Spheciospongia vesparia* and *Suberites compacta.* In addition to surveying fatty acid chain lengths by fractional distillation, they isolated 17,20-hexacosadienoic and 9-hexacosenoic acids from the former organism and octacosatrienoic and octacosenoic acids from the latter. More recently, we have identified 5-*cis*,9-*cis*-hexacosadienoic, 5-*cis*,9-*cis*,19-*cis*-hexacosatrienoic, and other C_{24}-C_{27} homologous acids in the sponge *Microciona prolifera* (2-4).

These findings raise the question of whether the C_{24}-C_{28} fatty acids found in these three sponges are isolated occurrences or whether such ultra long chain acids are characteristic of all sponges. To help answer this question, we have examined the chain length distribution of fatty acids from 20 different genera of the Demospongiae, the most numerous of the three taxonomic classes in the phylum Porifera (sponges).

EXPERIMENTAL PROCEDURES

Living sponges were obtained from the following sources: *Cliona, Halicondria,* and *Haliclona,* near Woods Hole, MA, October 1974 (Northeast Marine Specimens Co., Woods Hole, MA); *Isodictya* and *Mycale*, same source, January, 1975; *Microciona*, near Navesink, NJ, June 1974 (4); *Anthosigmella, Chondrilla, Iotrochota,* and *Tedania,* in the Bahia de Jobos near Guayama, Puerto Rico, December 1974; *Spongilla,* in Brachears Creek near Taylorsville, KY, July 1975; *Axinella, Dysidea, Spongia, Stelletta, Xestospongia,* and *Xytopsene,* near Panacea, FL, April 1975 (Gulf Specimen Co., Panacea, FL); *Lissodendoryx,* same source, March 1972.

Each sponge was cleaned very carefully, and the lipids (ca. 0.5-1.5% of wet wt) were extracted with chloroform:methanol (5). Fatty acid methyl esters were prepared by KOH-catalyzed methanolysis (6) and isolated by thin layer chromatography (TLC). A portion of the methyl esters was hydrogenated using a PtO catalyst in methanol (7) and then purified once more by TLC.

Fatty acid methyl esters were analyzed by gas-liquid chromatography (GLC) on a 1.82 m x 2.4 mm (inside diameter) stainless steel column packed with 10% EGSS-X silicone-polyester at 194 C. Peak areas were measured with an electronic integrator, corrected by appropriate calibration factors, and reported as % by wt.

RESULTS AND DISCUSSION

Previous experiments have shown the fatty acid compositions of marine sponges from the class Demospongiae to be extremely complex. In *Microciona,* for example, we identified 95 different fatty acids in amounts of 0.1% or more (4), and many of these had new and unusual structures. For a comprehensive survey of $>C_{22}$ acids in 20 different Demospongiae genera, therefore, a simplified approach was chosen. Each fatty acid mixture was analyzed by GLC both before and after hydrogenation. Quantitation of the chromatogram from the hydrogenated sample accurately identified the fatty acid chain lengths present, while comparison of unhydrogenated and hydrogenated runs allowed us to estimate whether the major $>C_{22}$ components were saturated, monoenoic, or polyunsaturated acids.

The distribution of fatty acid chain lengths in the 20 sponge genera examined are reported in Table I. All samples contained unusually high levels (34-79%) of C_{24}-C_{30} fatty acids. C_{26} was the most prevalent of these ultra long chain lengths. However, 8-17% C_{28} was present in *Haliclona, Xestospongia, Spheciospongia,* and *Suberites;* and C_{30} chains comprised 38% of *Chondrilla* and 11% of *Cliona.* A major amount (3-8%) of an equivalent chain length (ECL) 23.41-23.47 branched chain acid (presumably *iso*-24:0) was present in five of the

TABLE I

Distribution (% by wt) of Fatty Acid Chain Lengths in Sponges of the Class Demospongiae

SUBCLASS[a] / Order / Genus and species	Carbon atoms in fatty acid chain[b]																	
	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	Σ(24-30)
CERACTINOMORPHA																		
Poecilosclerida																		
Isodictya deichmannae	3	tr[c]	4	1	5	tr	19	1	6	-	1	2	55	-	3	-	-	61
Lissodendoryx sp.	1	tr	3	1	3	4[d]	15	tr	11[e]	tr	16[f]	3	41	tr	1	-	-	61
Iotrochota birotulata	1	tr	7	tr	7	tr	20	tr	1	-	3	5	55	1	-	-	-	64
Microciona prolifera (4)	1	1	7	3	9	1	7	1	22	1	7	2	38	tr	tr	-	-	47
Mycale fibrexilis	-	-	5	1	4	1	22	1	5	tr	8	1	52	-	-	-	-	61
Tedania ignis	1	1	8	tr	7	tr	14	tr	7	tr	11[g]	4	46	1	-	-	-	62
Haplosclerida																		
Haliclona oculata	1	1	4	tr	6	tr	5	tr	17	-	11[h]	tr	43	3	9	-	-	66
Spongilla lacustrus	1	2	10	2	13	tr	15	tr	1	tr	18[i]	6	30	tr	1	-	-	55
Xestospongia halichondroides	3	tr	7	1	10	tr	5	-	13	-	2	1	39	6	13	-	-	61
Xytopsene sigmatum	1	tr	5	2	9	tr	28	tr	8	-	4	1	42	tr	-	-	-	47
Halichondrina																		
Halichondria panicea	4	1	14	2	18	1	15	1	9	tr	5	2	27	tr	1	-	-	35
Dictyoceratida																		
Dysidea camera	tr	2[j]	17[k]	4[l]	17	tr	10	tr	10	tr	8	11	20	tr	-	-	-	39
Spongia sp.	1	6[j]	15	3	13	1	7	tr	15	1	12	25[m]	1	-	-	-	-	38
TETRACTINOMORPHA																		
Astrophorida																		
Chondrilla nucula	tr	5[j]	25	6[n]	10	tr	11	tr	1	-	1	tr	2	-	1	tr	38	42
Stelletta grubii	tr	5[j]	20	1	20	tr	5	-	4	-	3	2	34	5	1	-	-	45
Hadromerida																		
Anthosigmella varians	3	tr	18	tr	27	tr	11	tr	7	tr	5	1	28	-	-	-	-	34
Cliona celata	1	tr	2	1	2	tr	12	1	1	tr	8[o]	4	54	1	1	-	11	79
Spheciospongia vesparia (1)	2	-	12	-	17	-	7	-	4	-	4	-	39	-	15	-	-	58
Suberites compacta (1)	tr	-	10	-	12	-	9	-	18	-	12	-	22	-	17	-	-	51
Axinellida																		
Axinella polycapella	-	-	1	1	2	1	19	-	1	-	7	2	50	15[p]	1	-	-	75

[a]Taxonomic classification follows Levi (9). *Xestospongia* and *Xytopsene* included in the Poecilosclerida (10).
[b]All branched chain acids are included under their estimated carbon numbers: i.e., C_{16} = *n*-16:0 + *iso*-16:0 + *anteiso*-16:0. Any major (>2%) branched chain components are indicated in footnotes.
[c]tr = 0.1-0.5%.
[d]Mainly a mixture of branched chain structures.
[e]ECL 21.45 = 3%, ECL 22.00 = 8% (ECL = equivalent chain length).
[f]ECL 23.41 = 5%, ECL 24.00 = 11%.
[g]ECL 23.44 = 3%, ECL 24.00 = 8%.
[h]ECL 23.42 = 8%, ECL 24.00 = 3%.
[i]ECL 23.47 = 3%, ECL 24.00 = 15%.
[j]All ECL 14.48-14.61.
[k]ECL 15.50 = 3%, ECL 16.00 = 14%.
[l]Mostly ECL 16.64.
[m]ECL 24.48 = 24%, ECL 25.00 = 1%.
[n]Mainly ECL 16.53.
[o]ECL 23.41 = 4%, ECL 24.00 = 4%.
[p]ECL 26.71 = 4%, ECL 27.00 = 11%.

genera. As far as we know, no other group of organisms regularly contains such high levels of C_{24}-C_{30} chains in the total fatty acids.

Comparison of GLC analyses before and after hydrogenation indicated that almost all of the C_{24}-C_{30} peaks moved more than 0.40 ECL units (8) upon hydrogenation. Thus, only small amounts of saturated or monoenoic $\geq C_{22}$ acids could be present; and we conclude that these ultra long chain acids are mostly polyunsaturated. No consistent pattern of C_{24}-C_{30} peaks was observed for the unhydrogenated samples; so exact identification of these polyunsaturates must await detailed structural analyses. In the four genera where such analyses have already been carried out, however, C_{26}-C_{30} polyunsaturates have been definitely identified: i.e., 26:2 and 26:3 in *Microciona* (2,4), 30:4 in *Cliona* (Litchfield and Noto, unpublished data), 26:2 in *Spheciospongia* (1), and 28:3 in *Suberites* (1).

High levels of C_{24}-C_{30} fatty acids are certainly widespread if not ubiquitous throughout all subdivisions of the class Demospongiae of the phylum Porifera. They are common to both the Tetractinomorpha and Ceractinomorpha subclasses, and are present in all seven orders examined. Since these C_{24}-C_{30} fatty acids are so characteristic of the Demospongiae but are not generally found in nonsponge organisms, we propose the term "demospongic acids" as a convenient nomenclature for referring to these compounds as a group. The biochemical significance of demospongic fatty acids in Demospongiae tissue membranes is discussed in detail elsewhere (3).

Do demospongic acids also occur in the other two classes of sponges in the phylum Porifera, i.e., the Calcarea and the Hexactinellida? Our efforts to answer this question have so far been frustrated by the lack of suitable tissue samples. Calcarea specimens received to date have all been so heavily contaminated with algae that reliable data on the sponge fatty acids could not be obtained. The Hexactinellida grow only in deep sea locations (>1000 m), and we have been unable to obtain any living specimens of these animals. We would appreciate hearing from any reader who could help us in obtaining Calcarea or Hexactinellida samples for analysis.

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Effect of Cholesterol and Cholestyramine Feeding and of Fasting on Sterol Synthesis in the Liver, Ileum, and Lung of the Guinea Pig

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ABSTRACT

The effects of feeding diets containing either cholesterol (0.24% w/w) or cholestyramine (2.5% w/w) and of fasting on sterol synthesis in the liver, ileum, and lung of both male and female guinea pigs have been studied by measuring the incorporation by tissue slices of ^{14}C-labeled acetate into total digitonin-precipitable sterols. Cholesterol feeding significantly decreased ($P<0.05$) sterol synthesis in the liver, ileum, and lung of the males and in the ileum of females. Cholestyramine feeding stimulated the rate of hepatic sterol synthesis 13-fold but did not significantly affect sterologenesis in the ileum. Sterol synthesis in the lung was significantly increased ($P<0.05$) but to a much lesser extent than in the liver. Fatty acid synthesis in the liver, ileum, and lung was not significantly affected by either cholesterol or cholestyramine feeding. In guinea pigs fasted for 24 hr, sterol synthesis was inhibited in all three tissues, the most pronounced effect occurring in the liver. Only in the lung was fatty acid synthesis significantly decreased ($P<0.001$) by fasting. Cholesterol feeding resulted in increased concentrations of cholesterol in the plasma and liver. Cholestyramine feeding reduced plasma cholesterol concentration by 81% in females and by 64% in males. However, it did not significantly change the tissue cholesterol concentrations. Fasting resulted in a significant increase ($P<0.05$) in plasma cholesterol concentration but did not affect the concentration of cholesterol in the tissues. It was concluded that in the normal guinea pig, the feedback inhibition produced by both cholesterol and also possibly by bile acids suppresses sterol synthesis in the liver to very low rates compared to those in the small intestine, where sterologenesis is not only less sensitive to the cholesterol negative feedback system than that in the liver, but also is not subject to regulation by the bile acid negative feedback system.

INTRODUCTION

There is now substantial evidence from studies with several mammalian species including man which suggests that hepatic and intestinal sterologenesis and more particularly cholesterogenesis are subject to different mechanisms of control (1). In the rat (2), squirrel monkey (3), and man (4,5), cholesterol feeding markedly inhibits hepatic cholesterogenesis while having relatively little effect on cholesterol synthesis in the intestine. Similarly, fasting has a much greater inhibitory action on hepatic than on intestinal cholesterogenesis in the rat (2) and squirrel monkey (3). In contrast, biliary diversion in these species has a much greater stimulatory effect on intestinal cholesterol synthesis than it has on cholesterol synthesis in the liver (3,6,7). In patients with biliary obstruction, a marked increase in cholesterol synthesis in the intestine has been reported (5), indicating the presence in man of a bile acid negative feedback system similar to that in the rat and squirrel monkey.

The control of sterologenesis in tissues of the guinea pig, particularly the extrahepatic tissues, has not been the subject of detailed study. However, it is known that hepatic sterol synthesis in the guinea pig is markedly depressed by fasting (8) and by feeding with either cholesterol (9,10) or various bile acids (9). There is also evidence that the cholesterol negative feedback system operates in a wide range of guinea pig tissues (10). The effect of the interruption of the enterohepatic circulation of bile acids on sterol synthesis in tissues of the guinea pig apparently has not been previously determined.

Recent studies in this laboratory have demonstrated that in the guinea pig the gastrointestinal tract, particularly the small intestine, is a much more important site of sterol synthesis than is the liver (11). The rate of sterol synthesis in the lung was also found to exceed that in the liver. Thus, the relative rates of sterol synthesis in various tissues of the guinea pig differ markedly from those in the rat (2) and squirrel monkey (3), in which the liver is the principal site of sterol synthesis.

The present study was carried out to examine the factors which control sterol

synthesis in guinea pig tissues, as such a study may help to explain the low rate of hepatic sterologenesis in this species. We have therefore measured the rate of sterol synthesis in vitro in the liver, ileum, and lung of guinea pigs fed diets containing either cholesterol or cholestyramine or which had been fasted for 24 hr. The results of these experiments show that the low rate of hepatic sterol synthesis is the result of strong feedback inhibition by cholesterol and also possibly by bile acids. They also suggest that the guinea pig differs from other species in that intestinal sterologenesis is not subject to feedback inhibition by bile acids.

MATERIALS AND METHODS

Animals and Diets

In the experiments on cholesterol and cholestyramine feeding, male and female guinea pigs of an outbred albino strain bred in the Animal Breeding Establishment of The Australian National University were used. At the commencement of the experiments, the guinea pigs were aged from 12 to 20 weeks. They were housed in groups of from 4 to 6. In the experiments on fasting, outbred albino female guinea pigs from the National Biological Standards Laboratories (Canberra, ACT) were used. These were aged ca. 12 weeks at the time of study and were housed in groups of from three to five. All animals were maintained under controlled lighting with equal periods of light and dark. The details of these conditions have been described earlier (12,13). During an adaptation period of at least 3 weeks, all guinea pigs received a stock diet in the form of pellets identical in composition to the diet used in our earlier studies with rabbits (13). This diet contained 0.02% w/w cholesterol and 0.05% w/w plant steroids. The cholesterol and cholestyramine diets had the same composition as the stock diet except that they contained 0.24% w/w cholesterol and 2.5% w/w cholestyramine, respectively. Commercial grade cholesterol supplied by Townson and Mercer Pty Ltd. (Lane Cove, NSW 2066) was used. Cholestyramine (Cuemid) was obtained from Merck, Sharp and Dohme (West Point, PA). These additives were incorporated into the diet, which was then pelleted (14). In the experiments in which the effect of these diets on sterol synthesis was examined, the stock, cholesterol, and cholestyramine diets were fed to the females for 15, 16, and 14 days, respectively, and to the males for 14, 15, and 12 days, respectively. In the experiment on the effect of fasting on sterol synthesis, the animals were fed the stock diet. All diets were fed ad libitum, except in the experiment on fasting in which one group of animals was fasted for 24 hr. The guinea pigs had access at all times to drinking water containing 0.010% w/v L-ascorbic acid (pro analysi grade E. Merck, 61 Darmstadt, Germany), which allowed an approximate intake of 15 mg/kg body weight/day. Body weights were measured at the commencement of the experiments and on the day of study.

Sampling of Blood and Tissues and Preparation of Tissue Slices

At the commencement of the experiments on the effects of cholesterol and cholestyramine feeding on sterologenesis, the animals were bled from an ear vein into heparinized micro blood collecting tubes (280 μl volume, Sherwood Medical Industries Inc., St. Louis, MO). At the termination of all experiments, the animals were killed between 3.5 and 5 hr after the commencement of the dark period. They were stunned and exsanguinated by severing the jugular veins. The blood was collected into tubes containing EDTA (final concentration 10 mM EDTA) and the plasma separated from the chilled blood by centrifugation. The liver, distal ileum, and lungs were quickly excised and placed in ice-cold saline. The gall bladders were removed from the livers and the weight of the livers and lungs recorded. The ileum was freed of mesentery, opened longitudinally, and washed thoroughly in ice-cold saline. Samples of tissues were taken for the preparation of slices and for the extraction of lipids. Slices of liver, ileum, and lung were prepared freehand with a razor blade to a thickness of ca. 0.8-1.0 mm.

Assay of Sterol and Fatty Acid Synthesis

Tissue slices (200 mg) were incubated in triplicate in Krebs-Ringer phosphate buffer containing sodium 1-^{14}C-acetate (100 μCi/mmol; The Radiochemical Centre, Amersham, Bucks, England). The liver was incubated at pH 6.5 and the lung at pH 6.0, both in buffer containing 10 mM acetate, while the ileum was incubated at pH 7.5 in buffer containing 5 mM acetate. The details of the incubation procedure and the preparation and counting of the digitonin-precipitable sterol fractions have been previously described (11). When fatty acid synthesis was also measured, the saponification mixtures were acidified after extraction of the nonsaponifiable lipids, and then extracted with hexane (2 x 5 ml) which was subsequently evaporated to dryness. The residues from the hexane extracts were dissolved in 1 ml methanol and the radioactivity measured as described for the digitonide

TABLE I

Effect of Cholesterol and Cholestyramine Feeding on Sterol Synthesis in the Liver, Ileum, and Lung of Female Guinea Pigs[a]

Diet	Number of animals	Sterol synthesis[b]		
		Liver	Ileum	Lung
Stock	6	13.1 ± 4.40[c]	86.4 ± 9.79	12.8 ± 1.47
Cholesterol	6	3.6 ± 2.94	54.9 ± 9.83[e]	11.5 ± 2.09
Cholestyramine	6	177.5 ± 33.48[d]	110.9 ± 9.86	23.2 ± 3.99[e]

[a]Guinea pigs were fed a stock diet or a diet containing either 0.24% w/w cholesterol or 2.5% w/w cholestyramine. The animals fed the stock, cholesterol, and cholestyramine diets were killed on days 15, 16, and 14 of the experiment, respectively. Slices of liver, ileum, and lung were incubated in triplicate in Krebs-Ringer phosphate buffer containing 1-^{14}C-acetate as described in Materials and Methods.

[b]Sterol synthesis is expressed as mμmol of 1-^{14}C-acetate incorporated into total digitonin-precipitable sterols/g wet tissue/hr.

[c]Values are the mean ± SE.

[d,e]Comparison by Student's *t*-test with corresponding value for animals receiving stock diet: [d]$P<0.001$, [e]$P<0.05$.

precipitates. Sterol and fatty acid synthesis were expressed as mμmol of 1-^{14}C-acetate incorporated/g wet tissue/hr.

Measurement of Cholesterol Concentration in Diet, Plasma, and Tissues

The concentration of cholesterol was measured in representative samples of diet as described earlier (15). Samples of liver, ileum, and lung were extracted with chloroform: methanol (2:1 v/v, 20 ml/g tissue), filtered, and the extract made up to a known volume. The concentration of cholesterol in samples of these extracts and of plasma were estimated (16) after hydrolysis of the cholesteryl esters (17).

RESULTS

The body weight gains of the guinea pigs fed the cholesterol and cholestyramine diets did not differ significantly from those of guinea pigs fed the stock diet. The feed consumption of the guinea pigs, which did not differ between the animals on the different diets, was ca. 60 g/kg body weight/day.

The effects of feeding the diets containing added cholesterol and cholestyramine on sterol synthesis in the liver, ileum, and lung of female guinea pigs are shown in Table I. Cholesterol feeding significantly decreased the rate of sterologenesis in the ileum ($P<0.05$) but not in the liver and lung. The most pronounced effect of cholestyramine feeding was on the rate of hepatic sterologenesis, which was 13 times higher in the cholestyramine-fed animals than in the animals fed the stock diet ($P<0.001$). In contrast, the rate of sterol synthesis in the ileum was not significantly altered, while in the lung it was increased by 81% ($P<0.05$).

In the above experiment, no attempt was made to determine to what extent the changes which occurred in sterol synthesis in the animals fed cholesterol and cholestyramine may have been due to a direct effect of these treatments on the size of the acetyl CoA pool. Therefore, a similar experiment was carried out in which both sterol and fatty acid synthesis were measured in the same incubations. The results of this experiment using male guinea pigs are given in Table II. Cholesterol feeding caused a marked inhibition of sterol synthesis in the liver ($P<0.01$) and to a lesser extent in the ileum ($P<0.05$). Sterologenesis in the lung was also significantly decreased ($P<0.05$), a finding which differs from that shown with females. No significant differences were found in the rates of fatty acid synthesis in any of the tissues between animals fed cholesterol and those receiving the stock diet.

Cholestyramine feeding in males also resulted in a 13-fold increase in the rate of hepatic sterologenesis ($P<0.01$) but did not significantly affect sterol synthesis in the ileum. In rats, cholestyramine feeding has been reported to markedly stimulate both hepatic (18-20) and intestinal (7) cholesterogenesis. In the cholestyramine-fed male guinea pigs, sterol synthesis in the lung was significantly increased ($P<0.01$) but to a lesser extent than in females. The rate of fatty acid synthesis in the liver, ileum, and lung was not significantly affected by cholestyramine feeding.

The marked inhibition of hepatic sterol synthesis described here is consistent with the results of Swann and Siperstein (10), who demonstrated that in guinea pigs fed a 5% cholesterol diet for 7 days, liver sterol synthesis was inhibited by 93%. They also demonstrated marked inhibition of sterologenesis in several

TABLE II

Effect of Cholesterol and Cholestyramine Feeding on Sterol and Fatty Acid Synthesis in the Liver, Ileum, and Lung of Male Guinea Pigs[a]

Diet	Number of animals	Sterol synthesis[b]			Fatty acid synthesis[b]		
		Liver	Ileum	Lung	Liver	Ileum	Lung
Stock	4	20.7 ± 4.74[c]	118.1 ± 18.29	16.7 ± 1.11	29.3 ± 5.70	174.1 ± 8.64	346.9 ± 20.32
Cholesterol	4	1.1 ± 0.61[d]	63.2 ± 4.12[e]	8.5 ± 2.58[e]	37.7 ± 6.44	152.6 ± 17.63	232.8 ± 36.65
Cholestyramine	4	270.2 ± 55.34[d]	98.4 ± 22.05	22.8 ± 0.77[d]	92.4 ± 28.67	143.8 ± 29.09	304.9 ± 53.00

[a]Guinea pigs were fed a stock diet or a diet containing either 0.24% w/w cholesterol or 2.5% w/w cholestyramine. The animals fed the stock, cholesterol, and cholestyramine diets were killed on days 14, 15, and 12 of the experiment, respectively. Slices of liver, ileum, and lung were incubated in triplicate in Krebs-Ringer phosphate buffer containing 1-^{14}C-acetate as described in Materials and Methods.

[b]The data are expressed as mμmol of 1-^{14}C-acetate incorporated into sterol (as total digitonin-precipitable sterols) and fatty acids/g wet tissue/hr.

[c]Values are the mean ± SE.

[d,e]Comparison by Student's *t*-test with corresponding value for animals receiving stock diet: [d]$P<0.01$, [e]$P<0.05$.

other tissues, including the intestine and lung. Except for the liver and brain, the inhibition of sterol synthesis was proportional to the tissue cholesterol accumulation. In the present study with males, a diet containing only 0.24% cholesterol was found to also strongly inhibit sterologenesis in the ileum and lung, although to a lesser extent than in the liver.

The effects of cholesterol and cholestyramine feeding on the plasma and tissue cholesterol concentrations in both female and male guinea pigs are presented in Table III. Cholesterol feeding increased the plasma cholesterol concentration, but this was significant only in the females ($P<0.05$). The concentration of cholesterol in the liver was higher in the animals fed cholesterol, but only in the females was the increase significant ($P<0.01$). The concentration of cholesterol in the ileum and lung was not significantly affected by cholesterol feeding. Cholestyramine feeding was found to have an extreme hypocholesterolemic action in both females and males. In females, plasma cholesterol concentration was decreased by 81% and in males by 64%. However, the tissue cholesterol concentrations were unaffected.

The effect of fasting on sterol and fatty acid synthesis was studied in two experiments and the data pooled. As shown in Table IV, fasting for 24 hr markedly inhibited sterol synthesis in the liver ($P<0.01$) and, to a lesser extent, in the ileum ($P<0.001$) and lung ($P<0.01$). However, only in the lung did fasting significantly inhibit fatty acid synthesis ($P<0.001$).

The plasma cholesterol concentration in the fasted guinea pigs (72.4 ± 6.61 mg/100 ml) was significantly higher ($P<0.05$) than that in the animals which had been fed ad libitum (44.0 ± 7.37 mg/100 ml). Fasting did not significantly affect the concentration of cholesterol in any of the tissues. The increased plasma cholesterol concentration in the fasted animals could have been produced by a decreased efficiency of clearance of plasma β-lipoproteins from the circulation (21).

DISCUSSION

In the rat (2), squirrel monkey (3), and man (5), sterologenesis in the liver, intestine, and various other tissues is considered a direct measure of cholesterogenesis because the principal sterol produced is cholesterol. In the present study, the incorporation of labeled acetate into total digitonin-precipitable sterols by guinea pig tissues has not been termed cholesterogenesis because, although the principal sterol produced in guinea pig liver is cholesterol, most of that produced by the intestine is

TABLE III

Effect of Cholesterol and Cholestyramine Feeding on Plasma and Tissue Cholesterol Concentrations in Female and Male Guinea Pigs[a]

Sex	Diet	Number of animals	Days on diet	Plasma cholesterol concentration (mg/100 ml)		Tissue cholesterol concentration (mg/g wet tissue)		
				Initial	Final	Liver	Ileum	Lung
Female	Stock	6	15	52.8 ± 7.49[b]	57.2 ± 5.63	1.8 ± 0.08	1.8 ± 0.08	4.0 ± 0.12
	Cholesterol	6	16	62.7 ± 8.89	125.6 ± 22.72[c]	3.1 ± 0.34[e]	1.9 ± 0.11	4.1 ± 0.07
	Cholestyramine	6	14	58.9 ± 7.10	10.9 ± 0.81[d]	1.6 ± 0.04	1.6 ± 0.08	3.9 ± 0.09
Male	Stock	4	14	58.7 ± 2.83	57.7 ± 3.49	1.7 ± 0.09	1.7 ± 0.05	4.0 ± 0.10
	Cholesterol	4	15	56.1 ± 5.36	78.1 ± 14.3	2.5 ± 0.40	1.8 ± 0.11	4.2 ± 0.29
	Cholestyramine	4	12	56.1 ± 5.29	20.0 ± 1.25[d]	1.8 ± 0.05	1.5 ± 0.07	3.9 ± 0.22

[a]Female and male guinea pigs were fed a stock diet or a diet containing either 0.24% w/w cholesterol or 2.5% w/w cholestyramine. The concentrations of cholesterol in plasma and the various tissues were determined at the end of the experiment as described in Materials and Methods.

[b]Values are the mean ± SE.

[c,d,e]Comparison by Student's *t*-test with corresponding value for animals receiving stock diet: [c]$P<0.05$, [d]$P<0.001$, [e]$P<0.01$.

TABLE IV

Effect of Fasting on Sterol and Fatty Acid Synthesis in the Liver, Ileum, and Lung of Female Guinea Pigs[a]

Treatment	Number of animals	*Sterol synthesis*[b]			*Fatty acid synthesis*[b]		
		Liver	Ileum	Lung	Liver	Ileum	Lung
Fed (ad libitum)	8	19.9 ± 4.31[c]	121.7 ± 15.37	20.4 ± 2.68	50.1 ± 21.65	113.2 ± 19.47	416.6 ± 42.52
Fasted 24 hr	9	4.0 ± 0.93[d]	43.8 ± 5.86[e]	9.6 ± 1.12[d]	8.9 ± 1.95	67.7 ± 12.67	184.8 ± 21.38[e]

[a]Guinea pigs which had been fed the stock diet either continued to receive the diet ad libitum or were fasted for 24 hr. They were then killed and slices of liver, ileum, and lung incubated in triplicate in Krebs-Ringer phosphate buffer containing 1-^{14}C-acetate as described in Materials and Methods.

[b]The data are expressed as mμmol 1-^{14}C-acetate incorporated into sterol (as total digitonin-precipitable sterols) and fatty acids/g wet tissue/hr.

[c]Values are the mean ± SE.

[d,e]Comparison by Student's *t*-test with corresponding value for animals fed ad libitum: [d]$P<0.01$, [e]$P<0.001$.

lathosterol and 7-dehydrocholesterol (22). Despite the difference in the type of sterol synthesized by guinea pig liver and intestine, the principal sterol contained in both tissues is cholesterol (22).

Studies recently reported from this laboratory showed that in guinea pigs the average daily rate of sterol synthesis in the ileum was ca. 8 times that in the liver, while in the lung it was about twice that in the liver (11). Depending on the time of day, the rate of sterologenesis in the ileum was from 6 to 14 times that in the liver, while in the lung the rate was up to 3 times that in the liver. Because the average rate of hepatic sterol synthesis was so low, all measurements of sterologenesis in the present study were made between 3.5 and 5 hr after the commencement of the dark period when the rate of hepatic sterol synthesis was maximal. Under these circumstances, the rate of sterologenesis in the ileum was ca. 6 times that in the liver, while the rates of sterol synthesis in the liver and lung did not differ significantly. These findings confirm those reported earlier (11).

The present findings agree with those of Swann and Siperstein (10) in that the extent of feedback inhibition of extrahepatic sterologenesis by cholesterol is much greater in the guinea pig than in other species (2,3,5). However, the degree of inhibition of sterol synthesis in the ileum and lung was less than that in the liver. The failure of cholesterol feeding to inhibit fatty acid synthesis in any of the tissues clearly shows that the low rates of sterol synthesis were produced by a specific inhibitory effect of cholesterol on sterologenesis.

The effect of cholestyramine feeding on sterol synthesis varied markedly between the three tissues. In the liver and lung, sterologenesis was enhanced, the effect being particularly pronounced in the liver, where the rate of sterol synthesis was increased 13-fold. In contrast, sterol synthesis in the ileum was not significantly altered. Under these conditions, the liver and not the intestine would be quantitatively the most important site of sterologenesis.

The lack of an effect of cholestyramine feeding on sterologenesis in the ileum strongly suggests that intestinal sterol synthesis in the guinea pig is not subject to feedback inhibition by bile acids. In rats, cholestyramine feeding enhances cholesterol synthesis in all regions of the small intestine, although the effect is not as striking as that produced by biliary diversion (7). Dietschy (7) has shown that the bile acids in bile are responsible for the inhibition of intestinal cholesterol synthesis and that there is an inverse relationship between the intraluminal bile acid concentration and the rate of cholesterogenesis in the adjacent intestinal wall. More recent studies by Shefer et al. (23), however, indicate that dietary sterols as well as bile acids have a role in the regulation of intestinal β-hydroxy-β-methylglutaryl (HMG) CoA reductase.

The hypocholesterolemic effect of cholestyramine in guinea pigs is much greater than that described in other species. In chickens (19) and humans (24), cholestyramine feeding lowers plasma cholesterol concentration by <25% and has no effect in rats (18,19). However, in guinea pigs as in chickens and rats (19), cholestyramine feeding does not affect liver cholesterol concentration.

In rats which received a dose of cholestyramine (ca. 1 g/kg body weight), the absorption of dietary and endogenous cholesterol was reduced by over 70%, resulting from a decreased concentration of bile acids in the intestinal lumen (25). The fecal excretion of bile acids is markedly enhanced under these conditions (18). As plasma cholesterol concentration does not decrease in rats fed cholestyramine, the rate of hepatic and intestinal cholesterogenesis must be increased sufficiently to compensate for the increased fecal loss of bile acids and neutral steroids. In contrast, the guinea pigs fed cholestyramine (1.5 g/kg body weight/day) in their diet showed an extreme reduction in plasma cholesterol concentration, even though the liver produced sterol (principally cholesterol, 22) at 13 times the normal rate. This suggests that the plasma clearance of cholesterol was increased and that the absorption from the intestine of both exogenous and endogenous cholesterol was decreased. The increased excretion of bile acids produced by cholestyramine feeding also results in their decreased intestinal absorption. Thus, the enhanced rates of sterologenesis in both the liver and lung may have occurred because of a release of the inhibition imposed by either bile acids or cholesterol or both, although the relative importance of each cannot be determined from the present study. After the present study was completed, Swann et al. (26) reported that the injection of Triton WR-1339 into guinea pigs receiving a relatively low cholesterol diet resulted in a great stimulation of hepatic sterologenesis. This suggests that most of the feedback control is probably imposed by endogenous cholesterol, although the possibility that bile acids also directly impose some feedback control on hepatic sterol synthesis in the guinea pig cannot be excluded. It has been previously shown that the feeding of bile acids markedly suppresses liver cholesterol synthesis in this species (9). In addition, the results of studies with rats suggest that bile acids may

directly inhibit the activity of hepatic HMG CoA reductase (27,28).

In guinea pigs fasted for 24 hr, marked inhibition of sterologenesis occurred in the liver, ileum, and lung, although the effect in the liver was greater than in the other tissues. These findings contrast with those reported for the rat (2) and squirrel monkey (3), in which fasting markedly inhibits cholesterol synthesis in the liver but has relatively little effect on cholesterogenesis in the extrahepatic tissues. It is unlikely that the strong inhibitory effect of fasting on sterologenesis in guinea pig tissues represents only an increased dilution of the labeled acetate by the endogenous acetyl CoA pool, because in liver homogenates prepared from guinea pigs fasted for 40 hr, the rate of incorporation of acetate into acetoacetate was not different from that in preparations from fed animals (8). Moreover, studies with rat liver preparations have demonstrated a specific inhibitory effect of fasting on the activity of HMG CoA reductase (29).

The results of these studies can, therefore, explain the low rates of hepatic sterol synthesis relative to the rates of sterologensis in the small intestine of the guinea pig. In the normal animal, the feedback inhibition produced by both cholesterol and possibly also by bile acids suppresses sterol synthesis in the liver to very low rates compared to those in the small intestine, where sterologenesis is not only less sensitive to the cholesterol negative feedback system than that in the liver but also is not subject to regulation by the bile acid negative feedback system. Consequently, the small intestine alone makes a greater contribution to total body sterol synthesis than does the liver.

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SHORT COMMUNICATION

Oleic and Vaccenic Acid Levels in Lipid Classes of Tumors

ABSTRACT

The isomeric octadecenoate composition of triglyceride, phosphatidylcholine, and phosphatidylethanolamine classes from a variety of rat and mouse tumors was examined. Phosphatidylethanolamine from the tumors contained a higher percentage of octadecenoate than reported for many normal tissues. The octadecenoate fractions of the three lipid classes from various tumors consisted of ca. 75% or greater oleate, with vaccenate making up the balance. These data indicate that the loss of lipid class specificity for isomeric octadecenoates reported in hepatomas (*Lipids* 10:746, 1975, and *Lipids* 9:987, 1974) also occurs in other tumors.

INTRODUCTION

Recent data from this laboratory have demonstrated that the major lipid classes of liver contain different proportions of oleic and vaccenic acids (1), whereas lipid classes from host grown hepatoma (1) and cultured hepatoma cells (2) contained the same isomeric octadecenoic acid composition. In order to determine whether the loss of lipid class specificity for isomeric octadecenoates is unique to hepatomas, the isomeric octadecenoate composition of a variety of tumors was examined. The oleic and vaccenic acid compositions of triglycerides (TG), phosphatidylcholines (PC), and phosphatidylethanolamines (PE) obtained from several experimental tumors are reported in this communication.

METHODS AND MATERIALS

Total lipids, isolated from a number of rat and mouse tumors (3), were resolved into neutral lipid and phospholipid fractions by silicic acid chromatography (4), and the TG, PC, and PE classes were isolated from these fractions by thin layer chromatography as described previously (3,5). Methyl esters were prepared, octadecenoate fractions isolated by preparative gas liquid chromatography (GLC) (6), and the positional monoene isomers analyzed quantitatively by GLC of the ozonides (2). The methyl ester fractions were also analyzed by analytical GLC to determine the percentage of the octadecenoate fraction in each lipid class.

All solvents, reagents, standards, etc., were from the same sources and of the same purity as reported previously (2).

TABLE I

Octadecenoic Acid Content of the Three Major Lipid Classes Isolated from a Variety of Tumors

Tumor[a]	Percentage of 18:1 in:[b] PE	PC	TG
Walker carcinosarcoma 256	18.1	17.4	29.5
R3259/96A Sarcoma	30.1	33.7	46.0
Nontransplantable fibroadenoma[c]	11.6	17.1	33.9
Sarcoma 180	15.4	22.3	34.5
Mammary tumor KHZ	16.4	20.9	40.9
Sarcoma T-241	16.6	21.3	36.7
Taper liver tumor	22.2	21.4	25.3
Melanoma B-16	33.8	36.9	43.9
Adenocarcinoma E077	23.4	26.2	33.1
Friend virus leukemia	12.5	10.5	32.9
Ehrlich ascites carcinoma	17.9	19.6	21.6

[a]The first three are rat tumors and the remainder are mouse tumors.

[b]PE = Phosphatidylethanolamine, PC = phosphatidylcholine, TG = triglyceride.

[c]Occurred spontaneously in a few percentage of rats ca. 1 year after 800R total body irradiation.

TABLE II

Isomeric Composition of Octadecenoic Acid Derived from Individual Lipid Classes Obtained from Several Rat and Mouse Tumors

Tumor	Class 18:1 isomeric percentages[a]					
	PE		PC		TG	
	Δ9	Δ11	Δ9	Δ11	Δ9	Δ11
Walker carcinosarcoma 256	80	20	68	32	84	16
R3259/96A sarcoma	81	19	75	25	89	11
Nontransplantable fibroadenoma	80	20	69	31	83	17
Sarcoma 180	77	23	77	23	93	7
Mammary tumor KHZ	72	28	75	25	93	7
Sarcoma T-241	76	24	80	20	93	7
Taper liver tumor	86	14	85	15	95	5
Melanoma B-16	83	17	87	13	92	8
Adenocarcinoma E077	75	25	76	24	92	8
Friend virus leukemia	75	25	69	31	90	10
Ehrlich Ascites Cells	83	17	81	19	83	17
Average ± SD	78.9 ± 4.25	21.1 ± 4.25	76.5 ± 6.35	23.5 ± 6.35	89.7 ± 4.2	10.3 ± 4.2

[a]Percentages represent the mean of duplicate analyses of samples from a pooled lipid sample extracted from tumors obtained from several animals. PC = Phosphatidylcholine, PE = phosphatidylethanolamine, TG = triglycerides.

RESULTS AND DISCUSSION

The percentage of 18:1 in TG, PC, and PE for a variety of rat and mouse tumors is given in Table I. As illustrated in the table, the octadecenoate fraction is a major component of all three classes. One of the most frequently reported abnormalities in the lipids of tumors in general, and hepatomas in particular, is elevation of the "oleic acid" levels, which has been previously discussed (2). The percentage of 18:1 in PC and TG from most of the tumors may be higher than for most normal tissues; however, because the compositions of these two lipid classes from normal tissue are subject to dietary changes (7,8), comparisons have to be made cautiously. However, comparison of the 18:1 percentage in tumor PE with the percentage of 18:1 in PE of normal tissue can be made with more confidence because the differences are more pronounced and the 18:1 composition of PE is affected very little by diet (8). Comparison of 18:1 percentages from PE in Table I with the 18:1 percentages of PE from many normal tissues and fluids (9) shows that most of the percentages of 18:1 in tumors are higher than in normal tissue percentages, except neural tissue. Interestingly, of all the tumors studies, the nontransplantable fibroadenoma had the lowest percentage of 18:1 in PE. It is possible that the elevated percentage of 18:1 in PE is a characteristic of most malignant neoplasms which may be of diagnostic value. Additional work on a variety of human tumors will be needed to confirm this observation.

The percentage of oleate and vaccinate, the major octadecenoate isomers isolated from PE, PC, and TG classes of several tumors, is given in Table II. Oleate was the predominant isomer of each class in all the tumors. Although some variation existed between different tumors, generally, oleate represented 75-80% of PC and PE and ca. 90% of TG 18:1 fractions, with vaccinate making up the balance. These data are in good agreement with the isomeric octadecenoate composition of host grown and cultured hepatoma cells (1,2), which showed that oleate represented 70% or more of the octadecenoate fractions in all classes. The predominance of oleate in these tumors and the hepatomas examined earlier is in sharp contrast to liver, particularly in PC and PE classes, where vaccinate concentrations were the same or greater than that of oleate (1). These data indicate that a variety of other neoplasms exhibit the loss of lipid class specificity for isomeric octadecenoates previously observed in hepatomas. The significance of this apparent metabolic error may not become evident until the biological importance of isomeric octadecenoates becomes known.

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Identification of Plant Sterols in Plasma and Red Blood Cells of Man and Experimental Animals

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ABSTRACT

Direct gas liquid chromatography (GLC) of total plasma lipids showed small peaks (0.5-1.5% of total free sterol area) corresponding to free C_{28} and C_{29} sterols in ca. 50% of some 3,000 normal subjects and patients with hyperlipemia. Comparable proportions of similar peaks were present in the sterol fraction isolated from the red blood cells of many of these subjects. The maximum levels of these components in the plasma and red blood cells of domestic and laboratory animals were up to 10 times higher than those seen in man. Detailed gas chromatography/mass spectrometry analyses of the plasma lipids from a much more limited number of subjects and animals showed that the GLC peaks corresponding to the free C_{28} and C_{29} sterols were largely due to the plant sterols campesterol, stigmasterol, and β-sitosterol. In all instances, variable amounts (0.05-0.2% of the total free sterol area) of 7-dehydrocholesterol, desmosterol, lanosterol, and cholesterol α-oxide were also detected. While the total content and composition of the plasma plant sterols appeared to vary greatly among the subjects, it never exceeded 2% of total sterol in the normal subjects and patients examined. There was no evidence for a significant increase in the plant sterol content of the plasma of patients with hypercholesterolemia or hypertriglyceridemia.

INTRODUCTION

Routine determinations of plasma lipid profiles of normal subjects and patients with hyperlipemia by gas liquid chromatography (GLC) have revealed the presence of small peaks with retention times corresponding to those of plant sterols (1-3). Other reports have claimed the occurrence of plant sterols in plasma and tissues of normal subjects (4) and cancer patients (4,5). In most of these instances, the estimated plant sterol levels have averaged 0.5 mg% or less. Recently, however, Bhattacharyya and Connor (6) have described a new lipid storage disease characterized by β-sitosterolemia and xanthomatosis in which the total plasma plant sterol levels reached 40 mg%. Furthermore, Mellies et al. (7) have shown that the plasma of infants on formula diets rich in plant sterols had plant sterol levels of 6 mg% compared to 1.5 mg% or less for babies on milk or ad lib diets.

Since plant sterols are common components of most normal diets and are known to be absorbed by adults (8,9), we have monitored the GLC peaks for C_{28} and C_{29} sterols in the plasma of over 3,000 subjects participating in the hyperlipemia prevalence study. To distinguish the plant sterol peaks from the peaks of the companion sterols of cholesterol also reported to occur in plasma or serum in variable amounts (10-14), we have performed detailed mass spectrometric analyses on the free and esterified sterol fractions on a significant number of the plasma samples. As a result, we have shown that under normal conditions sterols other than cholesterol do not reach significant levels in the plasma of adult subjects, although molecular ions characteristic of both plant sterols and cholesterol companions can be obtained from the appropriate regions of the chromatogram even when discernible mass peaks are not seen in the total lipid profile.

MATERIALS AND METHODS

Standards

Purified desmosterol, campesterol, stigmasterol, and β-sitosterol were obtained from Applied Science Laboratories, State College, PA. Cholestanol, coprostanol, and cholesterol α-oxide were obtained from Mann Research Laboratories, New York, NY, while the Aldrich Chemical Co., Milwaukee, WI, supplied 7-dehydrocholesterol, lanosterol, and dihydrolanosterol. α-Tocopherol was obtained from Distillation Products Industries, Rochester, NY.

Sources of Plasma and Serum Samples

The bulk of the plasma samples (over 3,000) were obtained from a study of the prevalence of hyperlipemia in a free-living population in the Toronto area conducted by the Toronto-McMaster Lipid Research Clinic. The blood samples were collected in ethylenediamine

tetraacetic acid (EDTA) tubes according to a procedure published by the Lipid Research Clinics Program (15). Plasma of patients with various types of hyperlipemia (2 Type I, 22 Type II, 2 Type III, 9 Type IV, and 4 Type V) were obained through the courtesy of J.A. Little of St. Michael's Hospital and G. Steiner of Toronto General Hospital, Toronto, Canada.

Isolation of Plasma or Serum Lipids

Total lipid extracts of whole plasma or neutral lipid extracts of plasma following digestion with phospholipase C were prepared by extraction with chloroform:methanol, 2:1, as previously described (16). For a detailed examination of the free sterol and steryl ester fractions, the plasma lipid extracts were resolved into the lipid classes by thin layer chromatography (TLC) according to previously published methods (16,17). The steryl ester fraction was saponified with 0.5 N KOH in 70% ethanol and the unsaponifiable matter recovered by extraction with diethyl ether (18). *NOTE*: Care should be taken not to contaminate the plasma lipid samples with plant sterols present on laboratory equipment. Thus, the latex rubber bulbs commonly employed to control Pasteur pipettes upon contact with organic solvents release readily detectable amounts of β-sitosterol and other terpenoid impurities of comparable mol wt (unpublished results).

Trimethylsilylation

The dried lipid extracts were transferred to sealed conical vials of 0.5 ml capacity, and 150-250 μl of TRISIL/BSA or a solution of pyridine:hexamethyldisilazane:trimethylchlorosilane, 12:5:2, were added (19). After 2 hr at room temperature, the silylation was complete and the reaction mixture ready for injection into the gas chromatograph.

Gas Liquid Chromatography

Routine determination of the plasma or serum lipid profiles was made by the manual (16) or automated (20) GLC techniques. High resolution GLC of plasma sterols was performed on 6 ft x 1/8 in. ID glass columns packed with 1% SE-30 or 3% SILAR 5CP on 100-120 mesh Gas Chrom Q (Applied Science Laboratories). The columns were installed in a Varian model 2700 Gas Chromatograph equipped with a flame ionization detector. The carrier gas was nitrogen at 40 ml/min. Isothermal runs were made at 200 C with the injector and detector heaters at 250 C. Temperature programmed runs were made in the range 180-250 C at a heating rate of 4 C/min.

Gas Chromatography/Mass Spectrometry (GC/MS)

Combined GC/MS analyses of the trimethylsilyl (TMS) ethers of sterols and of the fatty acid esters of sterols were made with a Varian Mat CH-5 single focusing mass spectrometer coupled to Varian Data 620/i Computer and a peak matcher (21). The TLC separations of the TMS ethers of sterols were made on a Varian Model 2700 Moduline gas chromatograph equipped with a 6 ft x 1/8 in. ID glass column packed with 1% SE-30 on Gas Chrom Q. The carrier gas was helium at 10 ml/min. The gas chromatograph which did not have a separate detector was operated at 180 C isothermally with an injector heater at 225 C. The transfer line was maintained at 275 C. The mass spectrometer was operated at an ionization voltage of 3,000 V, electron emission energy of 100 μA, and an ion source temperature of 270 C. Scanning was done at 4 sec/decade at a resolution of 800-1,000. Mass acquisitions were made exponentially in a cyclic manner. All total spectra taken over the GLC peaks were corrected for total ion current variation. Also, spectra were taken of the column bleed and were subtracted from the total spectra of any minor components by the computer using Varian Module Sub. GC/MS of total plasma lipid extracts was obtained by means of 3 ft x 1/8 in. ID glass columns packed with 3% OV-1 on Gas Chrom Q (100-120 mesh). These columns were temperature programmed from 175-310 C, using helium as the carrier gas at a flow rate of 10 ml/min. For this purpose, the temperature of the injector heater was raised to 300 C and that of the transfer line to 325 C, while the helium separator and the ion source temperatures were adjusted to 310 C. Under these conditions, the presence of free plant sterols could be monitored by searching for the molecular ions of campesterol, stigmasterol, and β-sitosterol, and that of the plant sterol esters could be monitored by searching for the M-fatty acid ions of these steryl esters. For a more sensitive detection of the plant sterols, the steryl ester fraction was saponified and free sterols isolated by TLC. The sterols were then resolved as the TMS ethrs on the 6 ft GLC columns described above and the appropriate molecular ions monitored by the peak matcher. For this purpose, the low mass channel of the peak matcher was focused on m/e 458 and this channel used to monitor the elution of the TMS ether of cholesterol. After the appearance of cholesterol, the second channel of the peak matcher, preset to focus on m/e 472, was used to monitor the TMS ether of campesterol. Finally, after elution of campesterol, the dial settings of the second channel were switched to

allow the monitoring of m/e 486, which is the molecular ion for the TMS ether of β-sitosterol. Peak height values were corrected for retention time variation and differences in response by using a standard mixture made up by weighing out predetermined amounts of cholesterol, campesterol, and β-sitosterol. The presence of α-tocopherol in the cholesterol peak was detected by monitoring m/e 502, which represents the molecular ion of the TMS ether of α-tocopherol and m/e 237, which is a major fragment. The molecular ions and other appropriate fragments (M-15, M-90, and M-90+15) were used to monitor the presence of Δ^8-methostenol, Δ^8-lanosterol, methostenol, lanosterol, and the α-oxide of cholesterol in the GLC elution pattern.

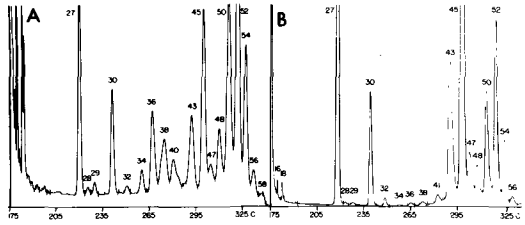

FIG. 1. GLC profiles of plasma lipids of patients with hyperlipemia. A) Total lipids of a patient with Type I hyperlipemia; B) neutral lipids of a patient with Type IIb hyperlipemia. Peaks 16 and 18, TMS esters of C_{16} and C_{18} fatty acids; Peaks 27, 28, and 29, TMS ethers of cholesterol, campesterol, and β-sitosterol; Peak 30, tridecanoylglycerol internal standard; Peak 34, TMS ether of palmitoylsphingosine; Peaks 36-40, TMS ethers of diacylglycerols of 36 to 40 acyl carbons; Peaks 43-47, cholesterol esters of C_{16} to C_{20} fatty acids; Peaks 50-54, triacylglycerols with 50-54 acyl carbons. GLC conditions and samples as given in text.

RESULTS AND DISCUSSION

GLC Identification of Plant Sterols in Total Lipid Extracts of Plasma and Red Blood Cells of Man

An examination of the GLC elution profiles of total plasma lipids from normal subjects and patients with hyperlipemia showed that many samples possessed small peaks in the regions occupied by plant sterols. Figure 1 shows the plasma lipid profiles obtained for a Type I and for a Type IIb hyperlipemia patients. By decreasing the attenuation, it was possible to increase the size and to improve the shape of the peaks, which then allowed the recognition of the characteristic plant sterol profile of various vegetable oils and fats. The minor amounts of plant sterols could be recovered along with free cholesterol by TLC of the plasma lipids in a nautral lipid system. However, TLC occasionally removed some of the nonsterol material emerging in the free sterol region of the total lipid profile. In all instances where plant sterols were found in the free sterol fraction, peaks corresponding to the C_{28} and C_{29} sterols were also recovered in the unsaponifiable matter of the steryl esters isolated from the total lipid

TABLE I

Plant Sterol Levels in Plasma and Red Blood Cells of Normal Subjects and Patients with Hyperlipemia as Estimated by Gas Liquid Chromatography

Subjects[a]	Free sterols		Esterified sterols		Total plant sterols[b]
	Campesterol	β-sitosterol	Campesterol	β-sitosterol	
	(mg/100 ml plasma or cells)				
Normal					
Plasma [35][c]	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	1.2 ± 0.2
Cells [20]	0.3 ± 0.2	0.3 ± 0.1			0.6 ± 0.3
Hypercholesterolemics					
Plasma [22]	0.5 ± 0.1	0.3 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	2.4 ± 0.4
Cells [10]	0.7 ± 0.2	0.5 ± 0.2			1.2 ± 0.2
Hypertriglyceridemics					
Plasma [15]	0.3 ± 0.2	0.3 ± 0.1	0.6 ± 0.2	0.6 ± 0.2	1.8 ± 0.3
Cells [5]	0.4 ± 0.1	0.5 ± 0.1			0.9 ± 0.2

[a]The normal subjects had plasma-free cholesterol level of 45.9 ±11.0 mg % and a total cholesterol level of 171.5 ± 38.5 mg %. Total plasma triglyceride was 100 ± 20 mg %. The hypercholesterolemics had plasma-free cholesterol levels of 100-200 mg % and total cholesterol levels of 300-600 mg %. The total plasma triglyceride ranged from 100-200 mg %. The hypertriglyceridemics had plasma-free cholesterol levels of 70-100 mg % and total cholesterol levels ranging from 200 to 300 mg %. The total plasma triglyceride levels ranged from 400 to 1,000 mg %.

[b]The presence of plant sterols was confirmed by GC/MS in all normolipemic [35] and hyperlipemic [12] plasma samples examined in this manner.

[c]The numbers in square brackets represent the number of individuals in each test group.

TABLE II

Plant Sterol Levels in Plasma or Serum and Red Blood Cells of Domestic and Laboratory Animals as Estimated by Gas Liquid Chromatography

Animal species[a]	Plasma or serum[b]		Red blood cells	
	Campesterol	β-sitosterol	Campesterol	β-sitosterol
	(percent of total sterol in plasma, serum, or cells)			
Rat [10][c]	3.4 ± 1	5.7 ± 1	2.0 ± 1	5.0 ± 1
Mouse [2]	2.3	6.4		
Guinea pig [3]	5.9	3.0	2.0 ± 1	0.5
Rabbit [3]	0.6	3.7	0.6	2.5 ± 1
Dog [2]	1.0	3.0	1.0	2.0
Cow [1]	0.4	5.3	1.0	1.0
Pig [2]	0.5	3.0		
Horse [2]	1.5	2.8		
Sheep [2]	0.9	6.2		
Chicken [5]	1.0 ± 0.5	4.3 ± 1	1.5 ± 0.5	3.5 ± 1
Goose [1]	0.8	5.4		
Duck [1]	2.0	4.0		
Pigeon [2]	3.0	7.0		
Frog [2]	3.0	10.0		
Turkey [1]	0.4	1.8		

[a]The total plasma cholesterol levels ranged from 70 mg % in the rat to 250 mg % in the pigeon, while the corresponding total plasma or serum triglycerides ranged from 25 to 50 mg %. The total red blood cell cholesterol averaged 100 mg % per 100 ml of cells.

[b]The presence of plant sterols in the plasma of the rat, chicken, and rabbit was confirmed by GC/MS. The plasma sterol samples from other animal species were not examined by GC/MS.

[c]Total number of animals in each test group.

extracts of the plasma of normal subjects and patients. Similarly, small amounts of the plant sterols were found in the total lipid extracts of the red blood cells where such examinations were made.

On the basis of the GLC profiles of the total plasma lipids, we have been able to show that > 50% of the 3,000 normal subjects examined exhibited small peaks with retention times corresponding to C_{28} and C_{29} sterols. In a much smaller number of patients with hyperlipemia, the incidence of occurrence of detectable plant sterol peaks appeared to be somewhat higher and the absolute content of these sterols somewhat greater, although the relative proportions of cholesterol and plant sterols remained about the same as those in normal subjects.

Table I summarizes the findings on the plasma and red blood cells of man. The values for total free plant sterol in the plasma of normal subjects averaged ca. 0.4 mg %, of which campesterol and stigmasterol made up ca. 50% and β-sitosterol 50%. It was estimated that the steryl ester fraction contained about twice as much plant sterol, which raised the total plasma plant sterol level in normal subjects to ca. 1.2 mg %. This estimate is in the upper range of the values given by Gray et al. (12) for plasma β-sitosterol. The average values for the hyperlipemic subjects were nearly twice as high as those in the normals and averaged 1.8-2.4 mg %. The latter estimates are of the order reported by Salen et al. (22) for patients ingesting amounts of β-sitosterol normally found in the diet. There was not significant difference in the plant sterol content of plasma of patients with hypercholesterolemia and hypertriglyceridemia, but the differences between normal subjects and patients with the hyperlipemias were statistically significant ($P<0.01$). The present values for plasma plant sterols in adults are somewhat lower than those reported by Mellies et al. (7) for children on cholesterol-poor, plant sterol rich diets, which averaged 2-6 mg %. In no case did the patients with hyperlipemia and xanthomatosis reach the plant sterol levels (up to 40 mg %) reported by Bhattacharyya and Connor (6) for their xanthomatosis subjects. In most instances, the red blood cells showed a plant sterol proportion comparable to that seen for the plasma sterols. In several cases, however, the peak for the C_{28} sterol was somewhat greater than that for the C_{29} sterol. This was probably due to a preferential uptake of campesterol by the red blood cel membrane, as already demonstrated for the membranes of the intestinal mucosa of the dog (23), chicken (24), and pigeon (25).

GLC Identification of Plant Sterols in Total Lipid Extracts of Plasma and Red Blood Cells of Domestic and Laboratory Animals

An examination of the total lipid profiles of

the plasma and serum samples collected from a variety of laboratory and domestic animals also revealed minor peaks for plant sterols. Table II summarizes the results obtained for the total plant sterols of both plasma or serum and red blood cells. Although only a few individuals of each species were assayed, their plant sterol levels appeared to be significantly higher ($P<0.01$) than those seen in adult men, and in several instances exceeded 10 mg %. Since the proportions of plant sterols to cholesterol were about the same in the free and esterified fractions, only total values for plant sterols in the animals have been indicated. The red blood cells contained plant sterols in about the same proportion to cholesterol as the plasma or serum. Plant sterol levels approaching 10-15% of total lymph cholesterol had been previously reported in dogs (23) receiving concentrated soybean sterols in their food. The plasma of chicken has been shown (24) to contain up to 3% of total sterol as plant sterols, depending on the dietary supplementation. Since the plasma cholesterol levels of the birds were of the order of 200 mg %, it can be seen that these plant sterol levels also are in the range of 6 mg % as noted in the present work. The plasma of pigeons receiving plant sterols in the diet has been shown to contain ca. 5 mg % of plant sterol (25). There was somewhat more campesterol than β-sitosterol in the plasma of these dogs and birds despite an excess of β-sitosterol in the diet (23-25). In the present studies, the plant sterol composition of the diet was not known and it is not possible to relate it to that of the plasma sterols.

Since neither man nor experimental animals are capable of biosynthesis of the plant sterols (22,26,27), it may be suggested that the higher levels of these sterols in the animal blood are due either to a lesser selectivity of the intestinal mucosa in the sterol uptake, or to a higher content of plant sterols in the animal diet. It is known that the extent of plant sterol absorption is also dependent upon the nature of the dietary fat (24).

Identification of Plasma Plant Sterols by GC/MS

The identity of the GLC peaks corresponding in retention time to C_{28} and C_{29} sterols with campesterol and β-sitosterol was established by means of mass spectrometry. This was accomplished first by pooling larger amounts of plasma (5 ml), preparing total lipid extracts, and isolating the total free sterol fraction by TLC. GC/MS examination of the TMS ethers of the sterols allowed the recording of well-defined peaks for both campesterol and β-sitosterol, which yielded mass spectra identical to those reported in the literature for purified plant sterols (28). Figure 2 shows the GC/MS spectra recorded for the β-sitosterol and campesterol present in a sample of normal plasma along with the spectra of the corresponding standards. In both instances, all the characteristic peaks of the mass spectra of campesterol and β-sitosterol are seen, including the molecular ions. However, due to the low mass of the plant sterols, some smaller ions are missing as a result of being below the intensity threshold of the system.

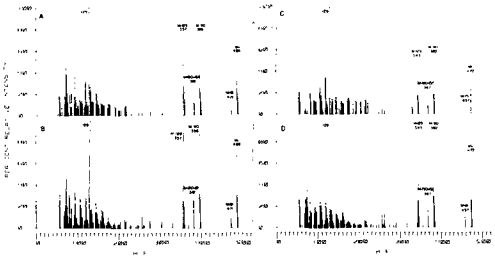

FIG. 2. GC/MS spectra of TMS ethers of plant sterols obtained with SE-30 columns. A) Plasma β-sitosterol; B) standard β-sitosterol; C) plasma campesterol; D) standard campesterol. Operating conditions as given in text. Sample: 1 μl of a silylation mixture containing 1-5 μg of plant sterols.

Since the plant sterol peaks overlapped partially or completely with the GLC peaks of various minor companion sterols of cholesterol, also reported (12-14) to be present in plasma lipid extracts, we made detailed GC/MS examination of the appropriate regions of the gas chromatograms. As a result, we were able to demonstrate that several of the plasma free sterol preparations contained up to 0.1 mg % 7-dehydrocholesterol, 0.1 mg % desmosterol and lanosterol, and 0.1 mg % of α-oxide of cholesterol. Small amounts (0.1-0.3 mg %) of these sterols were also found in the plasma steryl ester fraction. In no instance, however, was the plasma 7-dehydrocholesterol level as high as that (up to 25 mg %) reported by Kohler and Hill (14) on the basis of colorimetric measurements. There was a somewhat higher incidence of the mass fragments due to α-oxide of cholesterol in the plasma samples from patients with hypercholesterolemia in comparison to patients with hypertriglyceridemia, as suggested by Gray et al. (12). In one hypercholesterolemia patient, the peak attributed to campesterol in the GLC profile was found to be largely due to the α-oxide of cholesterol when examined by GC/MS. The amounts of the α-oxide of cholesterol, however, were too variable to attribute any significance to these correlations to total

plasma cholesterol levels.

A GC/MS examination of the cholesterol peak revealed that it overlapped completely with small amounts (0.05%) of cholestanol and coprostanol under our GLC conditions. Small amounts of cholestanol (29) and coprostanol (11,12) have been identified previously in the plasma of normal subjects and patients. There was some evidence that the absolute amounts of the cholesterol companion increase with increasing levels of plasma cholesterol so that the relative proportions of cholesterol and its precursors and oxidation products remain nearly constant. During the GC/MS scanning we also observed the presence of α-tocopherol in the descending limb of the cholesterol peak. The mass spectrum of the TMS ether of α-tocopherol was similar to that of the free compound (30). The molecular ion shifted to m/e 502 was the base peak (28% of the ion summation), and the major fragment shifted to m/e 237 had an intensity of ca. 47%. By means of mass fragmentography, we were able to demonstrate the presence of significant amounts (0.1-1.6 mg %) of α-tocopherol in most samples of plasma of normal subjects and patients. The presence of α-tocopherol in plasma has been reported previously (31), and it ought to have been anticipated to be extracted, derivatized, and eluted along with free cholesterol and other sterols.

Only the rat, chicken, and rabbit plasma and red blood cell lipids were examined for the presence of plant sterols by GC/MS. In these instances, the bulk (75-100%) of the C_{28} and C_{29} sterol peaks were confirmed to be made up of the plant sterols campesterol and β-sitosterol, with smaller amounts of stigmasterol. The rest of the material emerging at this time was due to the companion sterols of cholesterol.

It is concluded that plant sterols and the companion sterols of cholesterol are common components of the plasma lipid profile but that normally they do not exceed 1-2 mg % of the total plasma cholesterol of man and 4-6 mg % of the total plasma cholesterol fo experimental animals.

ACKNOWLEDGMENTS

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Plasma Transport Forms of Ingested Fatty Alcohols in the Rat

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ABSTRACT

Previous studies have shown that ingested fatty alcohols are absorbed as fatty acids and fatty acid esters, particularly triglycerides. The present study was carried out to determine whether fatty alcohols are also transported as 0-alkyl glyceryl ethers, alk-1-enyl glyceryl ethers, and as wax esters. Oxidation of fatty alcohols to other lipids was assessed by using a mixture of [1-^{3}H] hexadecanol and [1-^{14}C] hexadecanol of predetermined ratio. The results indicate that the absorption of fatty alcohol, and of its transport forms, parallels the absorption of labeled fatty acids. Six to 25% of plasma radioactivity was present as 1-0-alkyl diacylglyceryl ethers with a smaller proportion of ether lipids in the phospholipid fraction. In addition, 4-13% of the ingested hexadecanol appeared in the plasma as a material having the chromatographic properties of wax ester. Fatty alcohols were not detected in the plasma as alk-1-enyl lipids.

INTRODUCTION

The purpose of the present investigation is to reexamine the transport forms of fatty alcohols in the blood following their absorption from the gastrointestinal tract. Previous studies have shown that fatty alcohols are absorbed not only unaltered, but also, after transformation in the gut, as fatty acids, triglycerides, and phospholipids (1,2). In addition, the gastrointestinal tract is active in fatty alcohol synthesis (3,4), and it has been suggested that fecal fatty alcohols may be a secretory product of the intestine (3,5). Fatty alcohols also serve as precursors for 0-alkyl and alk-1-enyl lipids synthesized by gastrointestinal tissues (6). Furthermore, fed 0-alkyl lipids are absorbed via the intestinal lymph (7-9), but very extensive cleavage of the alkyl moiety with fatty acid formation occurs in this process (8). Fed fatty alcohols are also converted in part in the intestinal lumen to wax esters (5), but the absorption of wax esters has so far not been observed (2). Although fed fatty alcohols are recovered as 0-alkyl glyceryl ethers in tissues such as the liver (10), it is not clear whether this transformation occurs in peripheral tissues or whether some of the alcohols are transported as 0-alkyl or alk-1-enyl lipids.

The extent to which fed fatty alcohols are transported as 0-alkyl lipids, alk-1-enyl lipids, and wax esters requires clarification and forms the subject of this report. The present investigation has been confined specifically to blood rather than lymph since pertubations of blood lipids following feeding of fatty alcohols might be of greater interest in future human studies.

METHODS AND MATERIALS

Materials

Precoated silica gel plates 0.25, 1.0, and 2 mm thick were obtained from Brinkman (Westbury, NY). [1-^{14}C] hexadecanol (sp. act. 25.2 mCi/mmol) was obtained from Amersham/Searle (Arlington Heights, IL). [1-^{3}H] hexadecanol (sp. act. 50 mCi/mmol) was obtained from Tracerlab (Waltham, MA). These isotopic compounds were repurified by thin layer chromatography (TLC) before use. [1-^{14}C] palmitic acid (sp. act. 25.6 mCi/mmol) was obtained from New England Nuclear Corporation (Boston, MA) and was not repurified. Isopropanol was obtained from Fisher Scientific (Fairlawn, NJ). Heptane, ethyl ether, and acetic acid were obtained from Mallinckrodt (St. Louis, MO). Standard fatty alcohols were obtained from Applied Science Laboratories (State College, PA). Standard cetyl palmitate was prepared by a slight modification of the method of Haahti (11). Palmitoylchloride (Eastman, Rochester, NY) 30 mg and 20 mg of hexadecanol were dissolved in 2 ml of toluene, five drops of pyridine was added, and the solution was heated in a tube with a teflon lined screw cap at 60 C for 30 min. Ten ml of hexane was added, and the pyridine was extracted with 5% aqueous hydrochloric acid. The organic phase was washed several times with water. The wax was then purified by preparative TLC on a 2 mm Silica Gel G plate. The developing solvent was benzene:hexane (1:1 v/v).

Chromatographic and Other Procedures

TLC of lipids was done on prepared silica gel plates (Brinkman). Phospholipids were separated in chloroform:methanol:acetic acid:water (50:25:7:3 v/v). 0-alkyl and alk-1-enyl lipids were separated with hexane:ethyl ether:acetic

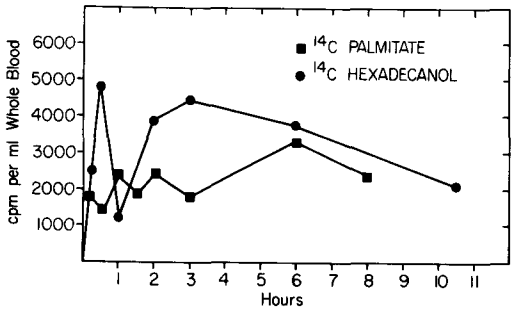

FIG. 1. Time course of appearance of plasma activity following feeding of [1-^{14}C] palmitate and [1-^{14}C] hexadecanol.

acid (20:80:1 v/v). Free fatty alcohols were separated in hexane:ethyl ether:acetic acid (50:50:1 v/v). Other neutral lipids were separated in hexane:ethyl ether:acetic acid (90:10:1 v/v) and wax esters in benzene:hexane (20:80 v/v).

Lipids were extracted by the method of Bligh and Dyer (12) and by the method of Dole (13). Reduction of glyceryl ethers was carried out with "Vitride" (sodium bis [2-methoxyethoxy] aluminum hydride, Eastman) by the method of Snyder (14). Excess "Vitride" was inactivated by the addition of 4% acetic acid. Free fatty acids were isolated by the method of Borgström (15).

Lipoprotein electrophoresis was carried out on cellulose acetate in 0.75 M barbital buffer, at pH 8.6. The strips were stained with oil red 0 (16).

Ultracentrifugation of serum lipoproteins was done by the method of Havel et al. (17). Chylomicrons were first removed by centrifugation at 9,000 x g for 10 min.

Transmethylation was carried out with boron trifluoride methanol:benzene (1:1 v/v). The dried lipids were dissolved in this solution and heated for 2 hr at 100 C in a sealed tube. After the addition of an equal volume of water, the lipids were extracted with petroleum ether.

For the absorption experiments, 10 Sprague-Dawley rats were exsanguinated at 0.75, 1, 2, 3, and 4 hr after administration of [1-^{3}H, 1-^{14}C] hexadecanol by stomach tube. Animals were maintained on standard laboratory diet and fasted 12 hr before the experiments.

Counting Procedures

Radioactive profiles of lipids separated by TLC were carried out as previously described (18) by scraping 4 or 5 mm bands of silica gel into scintillation vials and counting in phosphor:Triton (2:1 v/v) (19).

Radioactive profiles of thin layer chromatograms using mixtures of [1-^{3}H] and [1-^{14}C] hexadecanol were interpreted in this study with certain constraints and in keeping with some known errors. These errors result from the fact that tritium is readily quenched or self-absorbed by the presence of silica gel in the vials. ^{14}C is affected to a lesser extent. The polarity of the lipids also affects the results, since less polar lipids are more easily eluted from the silica by the phosphor solution. The liquid scintillation spectrometer was set so that 0.3% tritium was counted in the ^{14}C channel. However, 30-50% of the ^{14}C counts appear in the ^{3}H channel. Because of the variable quenching and self-absorption, no reliable correction can be made for the ^{14}C spill into the ^{3}H channel. With respect to the present investigation however, these variables pose no problems since the results can easily be interpreted from the fact that conversion of ^{3}H hexadecanol to fatty acids results in *total loss* of ^{3}H. The formation of an aldehyde or an alk-1-enyl glyceryl ether would result in loss of half the tritium and would be suggested by a 30-50% reduction in ^{3}H to ^{14}C. The apparent presence of minor amounts of tritium in some compounds such as the fatty acid and triglyceride peaks represents the ^{14}C spill into the tritium channel. A relative ^{14}C enrichment in the fatty alcohol peak after "Vitride" reduction is the result of in vivo conversion of [1-^{3}H, 1-^{14}C] hexadecanol to palmitate with complete loss of ^{3}H. When lipids are treated with "Vitride," the [^{14}C] palmitate is converted back to [^{14}C] hexadecanol. This reduces the ^{3}H:^{14}C ratio of any unconverted [1-^{3}H, 1-^{14}C] hexadecanol via a ^{14}C enrichment. Thus, in these studies, ^{3}H:^{14}C ratios were used with these constraints: total retention of tritium in a compound was correlated with its chromatographic position to identify 0-alkyl glyceryl ethers, wax esters, and other compounds containing unoxidized hexadecanol. Production of fatty acids was evident by peaks with fewer counts in the ^{3}H than the ^{14}C channel.

RESULTS

Time Course of Appearance of Total Plasma Radioactivity Following Feeding of Hexadecanol

Ten μCi [1-^{14}C] hexadecanol (sp. act. 25.2 mc/mmol) was dissolved in 0.2 ml peanut oil and introduced via a polyethylene catheter into the stomachs of Sprague-Dawley rats weighing ca. 225 g and anesthetized with pentobarbital. One-tenth ml blood samples were collected, at suitable intervals, from the tail into heparinized tubes, over a period of 10-12 hr. The lipids were then extracted. The rate of

appearance of total blood radioactivity from hexadecanol was compared with that following the feeding of [1-^{14}C] palmitate (10 μCi in 0.2 ml peanut oil). The results indicate that the rate of absorption of ^{14}C from [1-^{14}C] hexadecanol, administered orally in peanut oil, requires many hours. This rate is essentially the same as for [1-^{14}C] palmitate (Fig. 1). Random fluctuations were observed in plasma activity after administration of both [1-^{14}C] hexadecanol and [1-^{14}C] palmitate and are illustrated in a typical experiment (Fig. 1). These fluctuations probably reflect fluctuations in intestinal lymph flow (20,21). The absorption curves for palmitate observed in these experiments resemble the classic data for the absorption of fatty acids (22). That fatty alcohols are readily absorbed was determined by homogenization of the gastrointestinal tract 10 hr following oral administration, lipid extraction, and determination of residual activity which was 0.7% of the administered label. These data on the rate of absorption of hexadecanol are in agreement with those of Blömstrand and Rumpf (2).

Distribution of Radioactivity in Plasma Lipids Following Ingestion of Radioactive Hexadecanol

Rats fasted overnight were anesthetized with pentobarbital. A polyethylene tube was passed into the stomach, and 100 μCi of [1-^{3}H, 1-^{14}C] hexadecanol with an absolute activity ratio of 11.3, dissolved in 0.2 ml peanut oil, was instilled. This absolute ratio gave a count ratio between ca. 3:1 and 6:1 in the absence of tritium loss (no oxidation of fatty alcohols to fatty acids). The animals were exsanguinated through the abdominal aorta at 45 min to 4 hr. Lipids were extracted and separated into individual lipid components in several thin layer chromatographic systems. Aliquots of lipid extracts were applied as a band to 0.25 mm thick silica gel plates together with appropriate standards and developed in hexane:ethyl ether:acetic acid (50:50:1 v/v). The position of the standards was recorded, and 5 mm wide silica gel bands were scraped directly into scintillation vials and counted in a 2:1 mixture of phosphor Triton X-100 (19). The results (Fig. 2) show a free alcohol peak with a cpm ratio of ^{3}H:^{14}C of ca. 3. A phospholipid peak at the origin contained considerable amounts of tritium and points to absorption of hexadecanol as ether phospholipids. This was confirmed by further chromatography in methanol:chloroform:acetic acid:water (50:25:7:3). A relatively small amount was absorbed as free fatty acid, but the largest fraction was absorbed as less polar lipids seen at the solvent front.

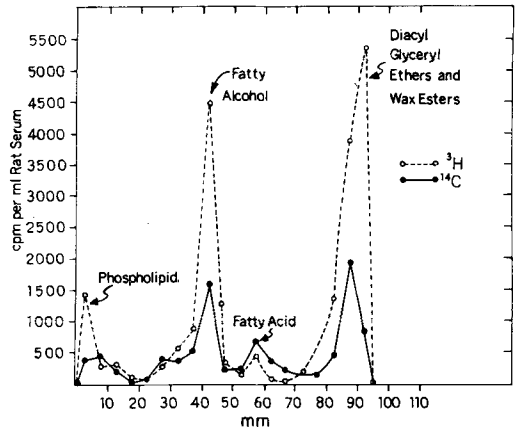

FIG. 2. Radioactive profile of a thin layer chromatogram of plasma lipids following ingestion of [1-^{3}H, 1-^{14}C] hexadecanol. The developing solvent was hexane:ethyl ether:acetic acid (50:50:1 v/v).

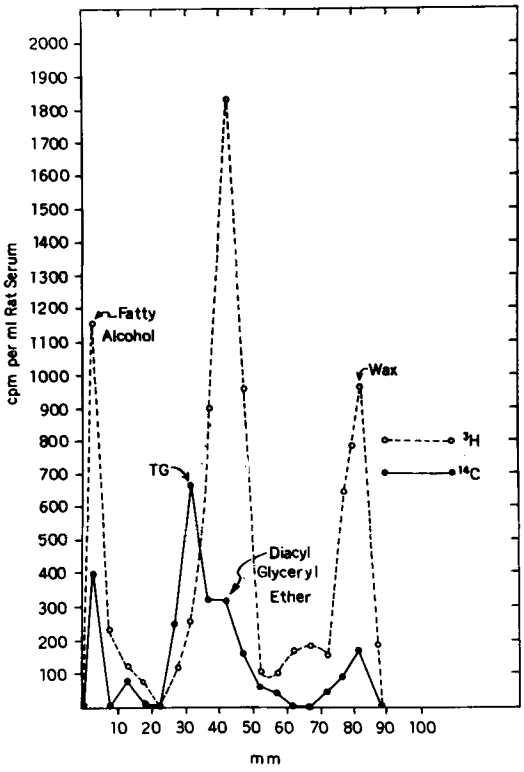

FIG. 3. Radioactive profile of a thin layer chromatogram of plasma lipids following ingestion of [1-^{3}H, 1-^{14}C] hexadecanol. The developing solvent was hexane:ethyl ether:acetic acid (85:15:1 v/v).

The less polar lipids were evaluated first by TLC in hexane:ethyl ether:acetic acid (85:15:1 v/v). The results (Fig. 3) show the presence of

TABLE I

Lipid Class Radioactivity in Plasma Following Oral Administration of Labeled Hexadecanol

Class	Percent total activity
Phospholipid	10 - 14
Fatty alcohol	3 - 23
Free fatty acids	1 - 9
Triglycerides	22 - 43
Diacyl glyceryl ethers	6 - 25
Wax esters	4 - 13

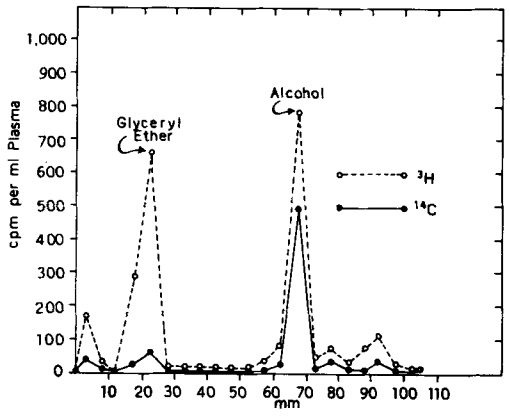

FIG. 4. Radioactive profile of a thin layer chromatogram of plasma lipids treated with "Vitride."

peaks corresponding to triglycerides, diacylglyceryl ethers, and wax esters. The triglyceride radioactivity peak is composed of ^{14}C fatty acids derived from the oxidation of labeled hexadecanol and therefore contains no tritium. Just ahead of the triglyceride peaks is a tritium rich peak corresponding to 1-0-alkyl diacyl glyceryl ethers. Another tritium rich peak can be seen at the solvent front. This peak corresponds to wax esters. The ^3H:^{14}C ratio suggests that the fatty acid moiety of the wax ester fraction is derived almost entirely from nonlabeled sources other than the fed hexadecanol, possibly the peanut oil vehicle. In contrast to this finding is the observation that the triglyceride fraction becomes labeled by the ^{14}C fatty acids derived from the ^{14}C fatty alcohol which is converted to ^{14}C fatty acids and therefore appears in the blood as ^{14}C triglycerides. However, since the fatty acid specific activity is greatly reduced, they do not appreciably decrease the ^3H:^{14}C ratio of the wax esters formed in the process of digestion.

Relative Composition of Lipids Derived from Fatty Alcohols

TLC of lipids obtained from the early absorption phase as compared with samples obtained at 3 or 4 hr failed to demonstrate important differences in lipid composition. Triglycerides ranged from 22 to 43%. Alkylacylglycerolipids ranged from 6 to 25% (Table I).

Absorption as Ether Lipids

To evaluate the relative fraction of lipids absorbed as ether lipids, aliquots were treated with "Vitride." The lipids were separated by TLC on silica gel with hexane:ethyl ether:acetic acid (20:80:1 v/v). It was estimated from these studies (Fig. 4) that ca. 20% of the ingested hexadecanol was absorbed as 0-alkyl lipids. It was also noted that no alk-1-enyl lipids were present. This is in marked contrast to our own observations and those of others indicating that alk-1-enyl lipids are formed in the gastrointestinal tract from hexadecanol (4,8). These alk-1-enyl lipids are, therefore, either not absorbed or are absorbed in trace quantities only. The results obtained following "Vitride" reduction and thin layer chromatography of the intact neutral lipid fraction indicate that the major source of 0-alkyl glyceryl ethers was 1-0-alkyl diacyl glycerol (Fig. 3).

Quantitative Comparison of Lipids in Plasma Following Intravenous Administration of Hexadecanol vs Oral Administration

As indicated above, radioactivity appears in the blood plasma for many hours following oral administration of labeled fatty alcohols or labeled fatty acids. The total activity in the plasma is very low at all time periods as expected from the slow rate of absorption. The percentage of label in 0-alkylglyceryl ethers obtained in these studies varied from 6 to 25% and was present mostly as diacyl glyceryl ethers. Since lymph was not examined directly in these studies, the question arises as to whether or not the diacyl glyceryl ethers were derived directly from intestinal lymph or from other tissues which had taken up fatty alcohols. Therefore, to determine whether plasma activity represents mostly activity of absorbed materials or activity recirculating from tissues, studies were performed with intravenously injected [1-^{14}C] hexadecanol and compared with oral feeding.

In a typical experiment, 10 μCi of [1-^{14}C] hexadecanol was dissolved in 50 μl of ethanol and injected intravenously into the exposed jugular vein of a pentobarbital anesthetized rat weighing 250 g. Blood samples measuring 0.1 ml were obtained at appropriate intervals from a tail vein. Two hr after injection, the rat was exsanguinated through the abdominal aorta. The blood samples were allowed to clot

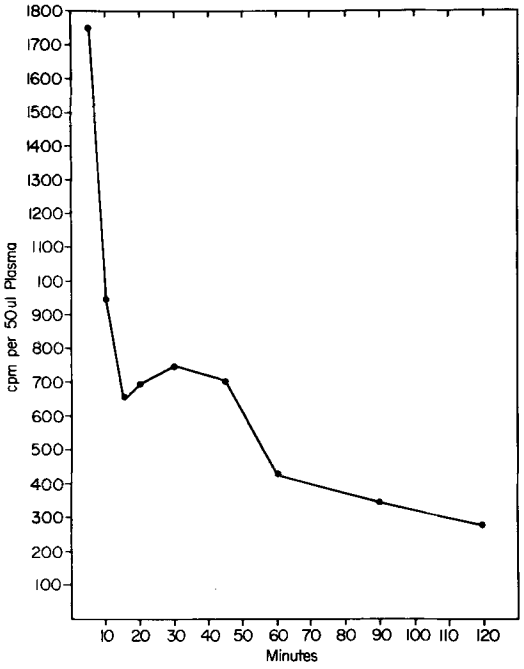

FIG. 5. Plasma activity following intravenous injection of 10 μCi [1-^{14}C] hexadecanol dissolved in 50 μl ethanol.

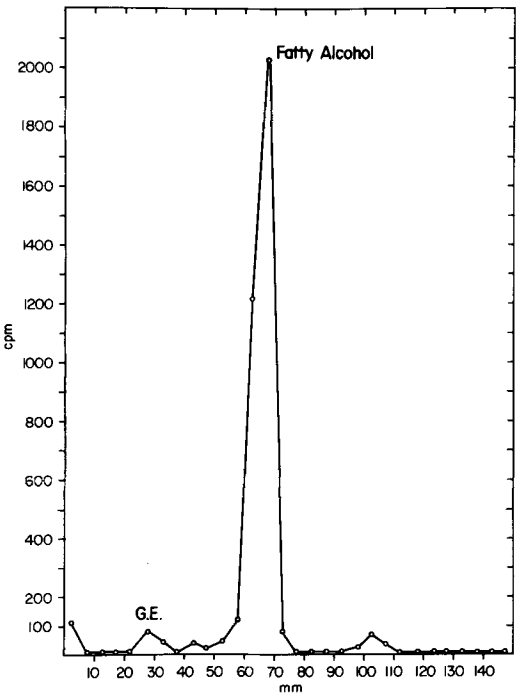

FIG. 6. Radioactive profile of "Vitride" treated plasma lipids obtained 2 hr after the intravenous injection of [1-^{14}C] hexadecanol. Lipids were separated by thin layer chromatography on Silica Gel G. The solvent system was hexane:ethyl ether:acetic acid (20:80:1 v/v). GE = 0-alkyl glycerol.

and serum samples were extracted. Electrophoretic and ultracentrifugal studies of [1-^{14}C] hexadecanol dissolved in 10 μl ethanol and mixed with 1 ml rat serum indicated that binding to α- and β-lipoprotein occurs.

The injected label disappeared extremely rapidly with a half-life estimated at <1 min (Fig. 5). Disappearance was followed by apparent recirculation of label. The highest total plasma activity reached by recirculation of label represented 0.4% of the injected [1-^{14}C] hexadecanol. Two hr after injection of [1-^{14}C] hexadecanol, plasma activity was present mostly as hexadecanol, free fatty acids, triglycerides, with smaller amounts of phospholipids and wax esters. The intravenous administration of [1-^{14}C] hexadecanol was repeated several times in order to examine the tissue distribution of injected [1-^{14}C] hexadecanol. The label was taken up extensively by adipose tissue, muscle, and liver. The amount of label present as 0-alkyl lipids was highest in muscle (20%) and lowest in liver (5%). It is clear, then, that injected hexadecanol is taken up by tissues and converted to 0-alkyl lipids which could be recirculated and could account for the appearance of 0-alkyl lipids in the plasma after feeding hexadecanol. However, 2 hr after the intravenous injection of [1-^{14}C] hexadecanol, plasma lipids after "Vitride" reduction contained only 2-3% of the total lipid activity vs. 6-20% after feeding (Fig. 6). Insufficient activity was present to determine which lipid fractions contained 0-alkyl lipids. Thus, the data show that rapid intravenous administration of 10 μCi of [1-^{14}C] hexadecanol, a relatively large amount of labeled material, was associated with 2 hr with the presence in plasma of very low levels of radioactivity. Of this activity, only a very small fraction was present as 0-alkyl lipids. In contrast, the feeding of 9 μCi of [1-^{14}C] hexadecanol resulted in the appearance in plasma of a much greater fraction of radioactivity as 0-alkyl glyceryl ether. Furthermore, most of the fed hexadecanol was converted to fatty acid or its esters and relatively little was delivered to the plasma as free hexadecanol. Any free hexadecanol might then have been taken up by tissues and recirculated as 0-alkyl lipids. However, the injection experiment shows that a very large amount of 1-^{14}C hexadecanol must be delivered to the plasma to produce even a barely detectable amount of 0-alkyl lipid activity in the plasma after 2 hr. It is concluded that insufficient dietary free fatty alcohol was delivered to tissue to result in the

appearance of measurable recirculated 0-alkyl glyceryl ethers. The early presence of relatively large amounts of labeled 0-alkyl glyceryl ethers in adipose tissue and muscle suggests that the method of injecting labeled hexadecanol is physiologically appropriate to study the recirculating 0-alkyl glyceryl ethers. It is concluded that there are quantitative differences in circulating 0-alkyl lipids following oral vs. parenteral injection of hexadecanol (6-25% vs. 2-3%, respectively).

Absorption of Fatty Alcohols as Wax Esters

A more detailed evaluation for the presence of absorbed wax esters was made by feeding rats a mixture of 56 μCi [1-^{3}H] hexadecanol and 20 μCi [1-^{14}C] palmitic acid plus 5 mg of stable hexadecanol and 5 mg of stable palmitic acid. The mixture was sonicated in 2 ml 7% deoxycholate and fed by stomach tube. Two hr later, the anesthetized animals were exsanguinated through the abdominal aorta, and the lipids were extracted from plasma as described above and separated by TLC on Silica Gel G using benzene:ligroin (1:1 v/v) as the developing solvent. A band corresponding to wax esters was noted just below the cholesteryl ester band. The former was eluted and the chromatographic procedure was repeated after the addition of 2 mg of stable cetyl palmitate. The area corresponding to wax esters was again eluted, dissolved in 2 ml benzene:methanol (1:1) transmethylated with 1 ml boron trifluoride methanol for 2 hr at 100 C in a sealed tube. After the addition of an equal volume of water, the lipids were extracted with petroleum ether and separated by TLC on Silica Gel G. The developing solvent was hexane:ethyl ether:acetic acid (90:10:1). The areas corresponding to free fatty alcohols and fatty acid methyl esters were eluted with ethyl ether and counted. The free fatty alcohols liberated by transmethylation from a material with the same R_f as wax esters contained ca. 10% of the total tritium activity of the serum lipids. The fatty acid moiety of the wax esters contained 0.5% of the serum lipid ^{14}C activity. The ratio of ^{3}H:^{14}C in the wax esters themselves was 6.7, reflecting a relatively small contribution from [1-^{14}C] palmitate. The results suggest that fatty alchols are absorbed as wax esters. However, since the contribution from ingested fatty acid is small, esterification of fatty alcohol may occur mostly with endogenous fatty acids rather than with those which were fed.

DISCUSSION

Fatty alcohols absorbed from the gastrointestinal tract appear in the circulation mostly as fatty acid esters. These observations are in accord with results published earlier by others (1,2). However, higher percentages of fatty acid esters were previously reported. This discrepancy is readily explained by the fact that earlier methods did not detect ether lipids, particularly 0-alkyl diacyl glycerols, which were not separated from triglycerides. The use of [1-^{3}H, 1-^{14}C] hexadecanol and TLC indicates that 6-25% of hexadecanol is absorbed as 0-alkyl diacyl glycerol. Furthermore, earlier studies failed to detect the absorption of wax esters, whereas the present investigation indicates the presence of a compound which contains hexadecanol, which has the chromatographic properties of wax esters and which yields fatty alcohol and fatty acids methyl esters after transmethylation.

The time course of appearance of blood radioactivity following the feeding of labeled hexadecanol is similar to that following the feeding of labeled fatty acid and is generally consonant with the observations of others on the rate of absorption of lymph lipids (22). This and the fact that very low ether lipid activity appears in blood after injection of labeled hexadecanol is compatible with the interpretation that blood activity mirrors the rate of absorption of the hexadecanol and does not reflect recirculation of ether lipids from tissues. Blömstrand and Rumpf found that 10-22.5% of ingested hexadecanol was recovered unchanged from intestinal lymph. These figures are generally comparable with those obtained in the present study in which 3-23% of the label was recovered as unchanged hexadecanol.

If previous studies indicating that fatty alcohols are present in the feces and are secreted by the mucosa into the intestine are correct, it would seem possible that the gastrointestinal tract might provide a continuous source of circulating glyceryl ethers.

Another conclusion from the present investigation is that alk-1-enyl lipids are not a major absorption product of hexadecanol; following reduction of total lipids with "Vitride," 0-alkyl lipids were detected, but no alk-1-enyl lipids were found by TLC. Therefore, although intestinal mucosa is active in alk-1-enyl lipid synthesis, ether lipids appear to be absorbed only in the 0-alkyl form.

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Syntheses of Tetra- and Hexadeuterated Octadecenoates[1]

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ABSTRACT

A study of the metabolism in man of *cis* and *trans* monoenoic acids required the synthesis of tetra- and hexadeutero-9-octadecenoates. Preparation of methyl 9-octadecenoate-8,8,11,11-d_4 (90 mol % deuterium) by the Wittig reaction was accompanied by deuterium scattering with sodium methoxide as the base but not with alkyllithium. Scattering occurred in both the aldehyde and phosphorane moieties only when the aldehyde contained deuterium on the alpha carbon. Methyl 9-octadecenoate-8,8,13,13,14-14-d_6 (98 mol % deuterium) was also prepared by the Wittig reaction. The deuterated octadecenoates were formed principally as *cis* isomers. The *trans* isomers were produced by nitrogen oxide isomerization and separation on a silver ion column.

INTRODUCTION

For a study of the metabolic fate of *trans* isomeric fats in humans, deuterium-labeled oleic and elaidic acids were required. The distribution of the isomeric fats in blood fractions was to be studied by gas chromatography-mass spectroscopy. We have prepared tetra- and hexadeutero-9-octadecenoates since increased accuracy in mass analysis results if the labeled fats contained four or more deuterium atoms per acid radical.

EXPERIMENTAL PROCEDURES

Instruments

Various determinations were made with appropriate equipment: mass spectra on a Nuclide 12-90G spectrometer with 70 eV impact ionization inlet, infrared spectra (IR) on a Perkin-Elmer model 621, gas liquid chromatography (GLC) on an Aerograph 600-B or a Packard Model 7400 equipped with dual flame detectors and temperature programming, and nuclear magnetic resonance (NMR) on a Varian HA 100 spectrometer at 100 MHz.

Preparations

Methyl 8-formyloctanoate-8,8-d_2 and nonanal-2,2-d_2: Methyl 8-formyloctanoate and nonanal were prepared by the procedure of Pryde et al. (1) from methyl esters derived from safflower oil.

Methyl 8-formyloctanoate-8,8-d_2 and nonanal-2,2-d_2 were prepared by multiple exchanges with pyridine-heavy water mixtures. Tucker et al. (2) used a similar procedure but separated the pyridine-heavy water from the deuterated aldehydes by water washing. To economize on the heavy water, we separated the pyridine-heavy water from the deuterated aldehydes by distillation. The pyridine-heavy water mixtures could then be used in first and second exchanges with subsequent batches of aldehydes. For methyl 8-formyloctanoate-2,2-d_2, the pyridine-heavy water mixtures were separated by distillation in vacuo, whereas for nonanal-2,2-d_2, the pyridine-heavy water mixtures were separated by distillation through a 2-ft Vigreux column.

Methyl 8-formyloctanoate-8,8-d_2 (Table I, mass analysis No. 1) bp 136-138 C/20 torr was obtained in 68% yield.

Nonanal-2,2-d_2 of bp 102 C/40 torr or 90 C/20 torr was obtained in a yield of 78% (Table I, mass analysis No. 2).

1-Nonanol-2,2-d_2 and 1-bromononane-2,2-d_2: 1-Nonanol-2,2-d_2 and 1-bromononane-2,2-d_2 were prepared according to Tucker (2) (Table I, mass analyses 3 and 4).

Methyl 9-octadecenoate-8,8,11,11-d_4: Methyl 9-octadecenoate-8,8,11,11-d_4 was prepared according to the Wittig procedure as described by Tucker (2). By combination of distillation and column chromatography on silica gel, the ester was obtained in 50.7% yield (8% *trans* by IR). Mass analysis showed deuterium scattering (Table I, No. 5). Another preparation carried out over molecular sieve in the reaction vessel gave similar results (Table I, No. 6). Reductive ozonolysis followed by GLC analysis showed no double bond migration (3).

9-Octadecenes-8,8-d_2: The source of the scattering problem was investigated by preparing 9-octadecenes from nonanal and its dideutero-derivative and from 1-bromomonane and 1-bromononane-2,2-d_2 using the Wittig procedure (Table II). The reactions were carried out similarly except that the reactions using phosphonium bromide were run with in situ-made bromide because phosphonium bromide could not be crystallized. The reactions were

[1]Presented at the AOCS Fall Meeting in Cincinnati, September 28-October 1, 1975.

TABLE I

Mass Analysis for Deuterium

Number	Substance	Number of deuterium atoms[a]								Average number per molecule
		0	1	2	3	4	5	6	7	
1	Methyl 8-formyloctanoate-8,8-d_2	4.4	9.9	83.6	2.1	--				1.83
2	Nonanal-2,2-d_2	--	4.8	95.2	--					1.95
3	1-Nonanol-2,2-d_2	2.9	3.5	93.6	--					1.91
4	1-Bromononane-2,2-d_2	0.5	5.1	94.5	--					1.94
5	Methyl 9-octadecenoate-8,8,11,11-d_4	0.7	1.4	13.9	26.1	36.5	17.0	3.9	0.4	3.65
6	Methyl 9-octadecenoate-8,8,11,11-d_4	1.1	1.2	15.9	25.0	35.0	17.5	3.8	0.6	3.77
7	9-Octadecene-8,8-d_2	0.8	5.0	92.4	1.6	0.2	--			1.95
8	9-Octadecene[b]	24.5	35.5	36.1	3.6	0.2	0.1	0.1	--	1.20
9	9-Octadecene[b]	3.6	19.0	41.7	0.8	1.6	3.2	--		1.87
10	9-Octadecene[b]	1.0	4.3	92.4	1.6	0.8	--			1.97
11	9-Octadecene[b]	17.1	37.9	32.8	10.5	1.7	--			1.42
12	9-Octadecene[b]	2.6	4.5	89.8	1.2	1.9	--			1.95
13	Methyl 9-octadecenoate-13,13,14,14-d_4	3.5	1.5	2.3	3.6	84.6	2.1	2.4		3.8
14	Methyl 9-octadecenoate-8,8,13,13,14,14-d_6	0.2	0.6	1.5	1.3	1.4	9.2	76.3	6.4	5.88
15	Nonyl-d_2-diphenylphosphineoxide	0.9	11.8	62.3	21.4	3.3	0.2	0.1		2.14

[a]Figures in table under "number of deuterium atoms" are in percent.

[b]Numbers 1-6, respectively, Table II.

TABLE II

Wittig Syntheses of 9-Octadecene[a]

$$CH_3(CH_2)_6CX_2CH_2P^{\oplus}(C_6H_5)_3Z^{\ominus} \xrightarrow{B} CH_3(CH_2)_6CX_2CH{=}P(C_6H_5)_3$$

$$CH_3(CH_2)_6CX_2CH{=}P(C_6H_5)_3 + CH_3(CH_2)_6CY_2CHO \rightarrow CH_3(CH_2)_6CX_2CH{=}CHCY_2(CH_2)_6CH_3$$

Number	X	Z	Y	B	Solvent	Yield (%)	*cis* (%)
1	D	Br	H	$NaOCH_3$	DMF	85	93
2	H	Br	D	$NaOCH_3$	DMF	33	93
3	H	I	D	$NaOCH_3$	DMF	18	94
4	H	I	D	*t*-BuLi	THF	53	71
5	H	I	D	NaH	THF	22	82
6	H	I	D	*n*-BuLi	THF	64	76

[a]Bu = butyl, DMF = dimethylformamide, THF = tetrahydrofuran.

typically as follows (Table II, No. 3):

In a 50 ml three-necked flask equipped with dropping funnel, N_2 inlet, stirrer, and thermometer were placed 4 g (7.75 mmol) 1-nonyltriphenylphosphonium iodide, 0.5 g molecular sieve (type 3A, 1/16 in. pellets, Linde, Tonawanda, NY), and 20 ml dry THF (tetrahydrofuran [dried over molecular sieve]). The contents were cooled to ca. 1 C with an ice-salt bath and 7.8 ml of 1 N *t*-butyllithium in *n*-pentane was added with a syringe. A deep red color formed at once. After 5 min, nonanal-2,2-d_2 was added slowly over 10 min. The ice bath was removed and the mixture stirred 3 hr at 35 C. The color was then orange. The mixture was added to 200 ml pH 2 water and extracted with four 25 ml portions petroleum ether (PE). The extracts were water-washed, dried, and vacuum stripped to yield 1.99 g colorless oil and solid. The solid was separated and washed with PE. The filtrate was vacuum stripped to yield 1.74 g (88%) of nearly pure 9-octadecene by GLC comparison with authentic 9-octadecene. Thin layer chromatography (TLC) showed no impurities. The separated solid had mp 152-4 C (triphenylphosphine oxide mp 152-3). The 9-octadecene was estimated to have 71% *cis* and 29% *trans* isomer (4) (Table I, Mass analysis No. 9).

Methyl 9-trans-octadecenoate-8,8,11,11-d_4: Methyl 9-octadecenoate-*8,8,11,11-d_4* (Table I, No. 5, 12.16 g) was elaidinized (5) to yield 12.13 g of yellow oil, which was purified on 30 g silica gel to remove probable nitrogen-containing impurities. From the hexane eluate there was isolated 10.57 g colorless oil (pure by TLC). This material was separated on 300 x 5 cm silver resin column (6) to yield 7.47 g (80%) of pure *trans*-isomers-d_4, 1.67 g (18%) of pure *cis*-isomers-d_4 and 0.18 g (2%) of a mixture of *cis* and *trans*-octadecenoates. TLC on silver nitrated silica gel confirmed these assignments.

1-Chlorononane-4,4,5,5-d_4: Benzene (1 liter) in a 2 liter flask was thoroughly degassed by evacuating to ca. 125 torr while stirring magnetically 1 min and repressurizing to 1 atm with N_2. This process was repeated 4 times. The flask was attached to a manometric system (7) and evacuated to ca. 100 torr. Deuterium was added to 1 atm and the solution stirred 20-30 min. The evacuation cycle was repeated again as before. Without stirring, 10 g tris(triphenylphosphine)chlororhodium was added and the flask flushed with deuterium. The stirrer was started and the deuterium pressure maintained at 1 atm over the orange solution automatically while the gas uptake was monitored. In ca. 15 min, the gas uptake stopped with 180 ml deuterium absorbed. The catalyst was not completely soluble. 1-Chloro-4-nonyne (75 ml, ca. 0.43 mol) (Farchan Chemical, location, pls; 99+% by GLC) was injected with a syringe. The reaction was at first exothermic and was cooled with an ice bath. After 4 hr, the gas uptake ceased. The reaction mixture was diluted with 750 ml PE, which caused some precipitate to form. The mixture was applied to a silica gel column (200 g, 5.5 x 30 cm) and eluted with 1 liter benzene:PE (1:1). Evaporation of the solvent from the eluate in vacuo gave 65 g of product.

1-Iodononane-4,4,5,5-d_4: 1-Iodononane-4,4,5,5-d_4 was prepared from the corresponding chloride by reaction with sodium iodide in methylethylketone (1). The iodide (81% yield) was contaminated by ca. 1% of the chloride.

1-Nonyl-4,4,5,5-d_4-triphenylphosphonium iodide: 1-Iodononane-4,4,5,5-d_4

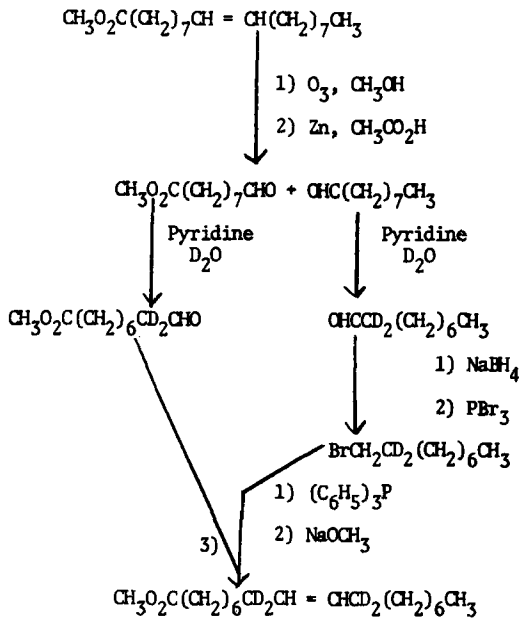

FIG. 1. Synthesis scheme for methyl 9-octadecenoates-8,8,11,11-d_4.

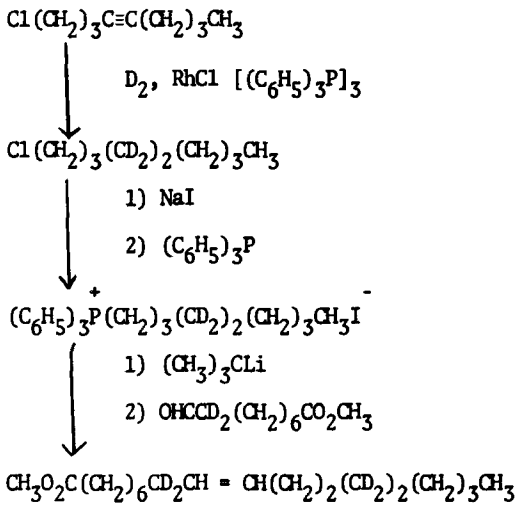

FIG. 2. Synthesis scheme for methyl 9-octadecenoates-8,8,13,13,14,14-d_6.

(120 g, 0.47 mol) triphenylphosphine (135 g, 0.50 mol) and xylene (1.2 liter) were heated at reflux for 7 hr. The lower layer (ca. 300 ml) was separated and stirred with 600 ml ether. The solid which separated was washed with 300 ml ether, filtered, and washed again with ether. The yield was 207 g (85%) mp 81-84 C. TLC indicated no triphenylphosphine but two other apparently minor impurities.

Methyl 9-octadecenoate-8,8,13,13,14,14-d_6: This material was prepared analogously to 9-octadecene-8,8-d_2 using 108 g (0.21 mol) 1-nonyl-4,4,5,5-d_4-triphenylphosphonium iodide, 0.21 mol *t*-butyllithium (1 N solution in pentane), and 39.2 g (0.21 mol) methyl 8-formyloctanoate-8,8-d_2. The yield after purification by distillation and silica gel chromatography was 24% (24% *trans*) (Table I, No. 14, mass analysis).

Methyl-9-octadecenoate-13,13,14,14-d_4: This substance was prepared by the method for the hexadeutero-derivative, described above, by substituting methyl 8-formyloctanoate for its dideutero-derivative. The yield was 27% with 22% *trans* isomer (Table I, No. 13).

DISCUSSION

Tucker et al. (2) reported the synthesis of methyl 9-*cis*-octadecenoate-11,11-d_2 using the Wittig coupling of 1-bromononane-2,2-d_2 and methyl 8-formyloctanoate or coupling of nonanal-2,2-d_2 and methyl 9-iodononanoate. Deuterium was introduced by alpha-deuteration of nonanal by exchange pyridine-heavy water. We attempted to adapt the procedure to the synthesis of tetradeutero-9-octadecenoate by deuterating both nonanal and methyl 8-formyloctanoate (Fig. 1). The alpha-deuterated nonanal was converted to 1-bromononane-2,2-d_2 and coupled with methyl 8-formyloctanoate-2,2-d_2 using the Wittig procedure as described by Tucker. This procedure resulted in extensive scattering of the deuterium label (Table I, Nos. 5, 6). Cleavage of the product with ozone established that the double bond had not migrated.

Also isolated from the Wittig reaction preparation of methyl 9-octadecenoate-8,8,11,11-d_4 was nonyl-d_2-diphenylphosphine oxide, which was identified by mass analysis (Table I, No. 15). Because the average number of deuterium atoms in the phosphine oxide increased, deuterium probably transferred from the alpha positions of the aldehyde.

The Wittig synthesis of deuterated olefins has been plagued by exchange and scrambling. Atkinson et al. (8) found that in reaction in dimethyl sulfoxide solution using methylsulfinylmethane anion as the base, extensive exchange occurred if the carbonyl component contained alpha-deuterium atoms. Little loss of deuterium was found when only the phosphonium halide intermediate contained alpha deuterium atoms. The exchange is probably due to enolization of the carbonyl compound catalyzed by the ylid. However, such a hypothesis is not necessary, as Atkinson's pro-

cedure involved an excess of methylsulfinylmethane anion. Similarly, Malloy et al. (9) encountered scrambling in a Wittig synthesis of methylenecyclopentane using cyclopentanone-2,2,5,5-d_4 and sodium hydride as the base. When butyllithium was the base, no scrambling of the label occurred. Buchanan and Gustafson (10) encountered loss of label in preparing vinylcylcohexane and divinylbenzene from cyclohexanecarbaldehyde and *o*-phthalaldehyde, respectively, in a Wittig synthesis with methyl-d_3-triphenylphosphonium bromide. Marked reduction in loss of label occurred when *t*-butyllithium was used instead of *n*-butyllithium and when the reaction was quenched with deuterium oxide rather than with water. The suggestion was made that exchange occurred by reaction with the intermediate betain.

To determine the cause of the scrambling involved in our work, we prepared 9-octadecenes in which the position of the label or the basic catalyst was varied. When only the phosphonium bromide contained label and sodium methoxide was used, little scrambling occurred (Table I, No. 7, and Table II, No. 1). However, if the label was present in the nonanal, extensive scattering occurred (Table I, No. 8, and Table II, No. 2).

Phosphonium iodide was employed in some experiments because it could be crystallized, whereas the corresponding bromide could not. The iodide allowed exact stoichiometry with the base (Table II, No. 3). The iodide gave results similar to those obtained with the bromide (Table I, No. 9, and Table II, No. 3).

Alkyllithium, when used as the base, essentially eliminated scattering (Table I, Nos. 10 and 12; Table II, Nos. 4 and 6). This finding and the necessity of alpha deuterium in the carbonyl component for scattering are in agreement with Atkinson et al. (8). Sodium hydride was not effective (Table I, No. 11, and Table II, No. 5).

Methyl 9-octadecenoate-8,8,13,13,14,14-d_6 was synthesized by Wittig coupling of 1-iodononane-4,4,5,5-d_4 and methyl 8-formyloctanoate-8,8-d_2 in tetrahydrofuran solution using *t*-butyllithium to avoid scrambling (Fig. 2). Deuteration of 1-chloro-4-nonyne at 1 atm and 25 C with tris(triphenylphosphine)chlororhodium(I) (11) furnished the 1-chlorononane-4,4,5,5-d_4 in 90% yield. The chloride was converted to the iodide by treatment with sodium iodide. The yield of octadecenoate-d_6 (Table I, No. 14, mass analysis) was 24.4%, and the isotopic purity was 76.3%.

By Wittig synthesis between 1-nonyl-4,4,5,5-d_4-triphenylphosphonium iodide and methyl 8-formyloctanoate, methyl 9-octadecenoate-13,13,14,14-d_4 was prepared in 27% yield with 22% *trans* and isotopic purity, 84.6% (Table I, No. 13).

This work has shown successful routes to tetra- and hexadeutero-9-octadecenoates. Also, sodium methoxide is not suitable as a base in the Wittig reaction when the carbonyl moiety contains alpha deuterium because of exchange; alkyllithiums obviate the exchange.

ACKNOWLEDGMENTS

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Mass Spectra of Acetylenic Fatty Acid Methyl Esters and Derivatives[1]

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ABSTRACT

A series of isomeric methyl octadecynoates was analyzed by mass spectrometry; each isomer gave a unique spectrum. The characteristic ions were those resulting from a McLafferty rearrangement of the allenic sites or of the already-rearranged allenic sites. The acetylenic esters were also subjected to oxymercuration whereupon a carbonyl group was formed at either of the original actylenic carbon atoms providing two oxostearates. Further reaction with $NaBH_4$ formed hydroxy esters which, after silylation, gave diagnostic mass spectra indicative of the triple bond location. Applied to esters with both double and triple bonds, this procedure permitted differentiation between the two types of unsaturation. Methoxyl groups marked the original double bond locations and hydroxyls did so for triple bonds.

INTRODUCTION

Mass spectrometric analyses of esters containing triple bonds have been reported (1-4). Although these reports present the pathways for ion formation, none present results from a complete series of esters of one chain length. We analyzed by mass spectrometry an almost complete series of methyl octadecynoates (all but the 3,4 and 16,17 isomers). We found that each mass spectrum was unique and that all but two include the same types of ion fragments. The spectra are complex, and the complexity is greater when olefinic bonds accompany the acetylenic bonds.

By applying oxymercuration procedures (5) to monoacetylenic and ene-yne esters, both double and triple bonds form derivatives which can be used to give definitive mass spectra that locate and differentiate the two types of unsaturation.

MATERIALS AND METHODS

Preparation of the series of octadecynoic acids has been previously reported (6). Each acid was reacted with diazomethane to form the methyl ester. Methyl *trans*-10,16-heptadecadien-8-ynoate and methyl 17-octadecen-9-ynoate were gifts from R.G. Powell, and a quantity of methyl stearolate was from R.O. Butterfield for exploratory studies. Methyl crepenynate (*cis*-9-octadecen-12-ynoate) was isolated from methyl esters of *Crepis alpina* seed oil (7).

Oxymercuration was carried out as previously described (5), except that a large excess of sodium borohydride was used.

IR analyses were performed with a Perkin-Elmer Infracord 137 in 1 mm NaCl cells with $CHCl_3$ as the solvent.

Gas chromatography-mass spectrometry (GC-MS) was conducted as before (8) except that the Packard 7401 gas chromatograph was replaced by a Bendix 2600 instrument for sample introduction into the mass spectrometer during the later stages of this work. Columns were either glass 6 ft x 1/4 in. packed with 3% Silar-5cp on Gas Chrom Q (Applied Science Laboratories, Inc., State College, PA) or stainless steel 4 ft x 1/8 in. packed with 3% Dexsil 300 on Gas Chrom Q.

RESULTS AND DISCUSSION

The mass spectra of the series of methyl octadecynoates analyzed are in general agreement with those published (1-3). The basic mass spectral pattern is one of cleavage with McLafferty rearrangement either of the acetylenic bond or of the isomeric allenes found by rearrangement. Therefore, electron bombardment of each octadecynoate should produce four characteristic ions (Table I). For example, the mass spectrum of methyl 9-octadecynoate (methyl stearolate) showed ions at m/e 196 $[CH_3OCO(CH_2)_7\text{-}CH{=}C{=}CH_2^{\oplus}]$ (A), m/e 210 $[CH_3OCO(CH_2)_7\text{-}CH{=}CH\text{-}CH{=}CH_2^{\oplus}]$ (B), m/e 152 $[H_2C{=}C{=}CH\text{-}(CH_2)_7\text{-}CH_3^{\oplus}]$ (C), and m/e 166 $[H_2C{=}CH\text{-}CH{=}CH\text{-}(CH_2)_7\text{-}CH_3^{\oplus}]$ (D) (Table I). Ions with 32 mass units (CH_3OH) less than ions A and B are also present. These fragments are the same as those reported by Bohlmann et al. (1) and may well be formed in the way he postulated, but we find the relative

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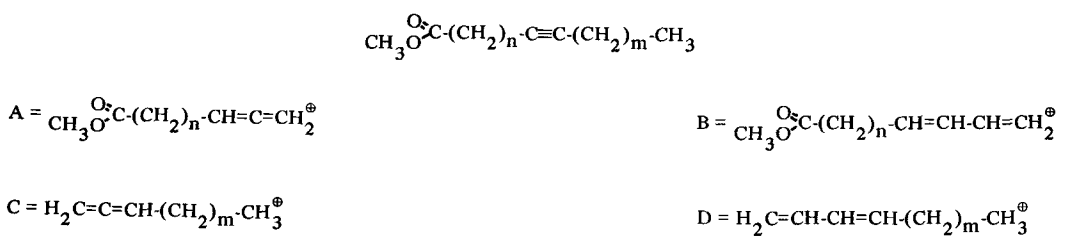
TABLE I

Characteristic Ions (and Relative Intensities) from Methyl Octadecynoates

Triple bond position	n	m	A	B	C	D	A-32	B-32
4	2	12	126(69)	140(13)	222(-)	236(-)	94(23)	108(25)
5	3	11	140(100)	154(1)	208(1)	222(-)	108(13)	122(5)
6	4	10	154(59)	168(2)	194(-)	208(-)	122(26)	136(5)
7	5	9	168(24)	182(4)	180(2)	194(-)	136(33)	150(11)
8	6	8	182(30)	196(18)	166(17)	180(8)	150(81)	164(28)
9	7	7	196(13)	210(26)	152(38)	166(15)	164(24)	178(25)
10	8	6	210(6)	224(14)	138(59)	152(14)	178(10)	192(11)
11	9	5	224(5)	238(13)	124(89)	138(19)	192(4)	206(6)
12	10	4	238(3)	252(5)	110(100)	124(35)	206(3)	220(2)
13	11	3	252(2)	266(-)	96(100)	110(25)	220(1)	234(-)
14	12	2	266(-)	280(-)	82(100)	96(57)	234(-)	248(-)
15	13	1	280(-)	294(1)	68(100)	82(82)	248(-)	262(-)



intensity of these ions compared to the base peak to be lower. However, our spectrum of methyl 9-octadecynoates agrees well with the spectrum reported by Odham and Stenhagen (4).

Ions containing the terminal part of the molecule (Table I, C and D) are the most abundant when the triple bond is close to this part of the molecule. Types A and A-32 are the most intense of the characteristic ions when the acetylenic bond is near the ester function. In fact, when the triple bond is in the 4,5 to 6,7 positions, C and D ions are not detected, and the spectra of the 14,15 and 15,16 isomers do not show ions of type A and B. A small amount of ions $[(M) - (CH_2\text{-}C{\equiv}C\text{-}(CH_2)_x{-}CH_3)]$ is found in esters with 5,6 thru 15,16 triple bonds. For example, methyl 15-octadecynoates produces an ion of m/e 227 $[M - (CH_2\text{-}C{\equiv}C\text{-}CH_2\text{-}CH_3)]$ with relative intensity of 6% and methyl 11-octadecynoates one of m/e 171 $[M - (CH_2\text{-}C{\equiv}C\text{-}(CH_2)_5\text{-}CH_3)]$ 5%. Other ions, which are common to all esters in this series, are M(294), M-31, M-74, 87, and 74.

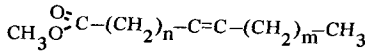
Esters with the two extreme triple bond positions (2,3 and 17,18) do not conform to the general pattern. The mass spectrum of methyl 2-octadecynoates would be expected to contain abundant m/e 98 and 112 ions. Instead, m/e 100, 140, 154, and 168 are more prominent. The last three suggest that the movement of double bonds is more pronounced in this molecule than in esters with acetylenic bonds farther up the chain. The spectrum of the 2,3 isomer does exhibit a small m/e 235 (M-59) ion which, presumably, represents the loss of $CH_3O\overset{O}{\overset{\|}{C}}$-. Methyl 17-octadecynoate also does not show ions indicative of the triple bond position. Its spectrum is understandably much like a saturated methyl ester in containing intense ions of m/e 74 and m/e 87.

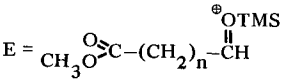
Three esters containing both triple and double bonds were analyzed by mass spectrometry without derivatization. The spectrum of methyl crepenynate (*cis*-9-octadecen-12-ynoate) shows a prominent peak at m/e 236 (29%) which, most likely, represents $CH_3O\overset{O}{\overset{\|}{C}}$-$(CH_2)_7$-HC=CH-$CH_2$-HC=C=$CH_2^{\oplus}$. The analogous fragment (m/e 238) from methyl 12-octadecynoate is found at only 3% relative intensity level (Table I). The most intense ion in the 12-octadecynoate is the hydrocarbon fragment of m/e 110. The comparable C_8 fragment in methyl crepenynate, which would require cleavage between the double and triple bonds, is not found in significant amounts. The position of the double bond is not readily discernible from the mass spectrum of methyl crepenynate. Ions produced from methyl 17-octadecen-9-ynoate are those expected from analogy with methyl 9-octadecynoate. Fragments A and B, m/e 196 and 210, are the same

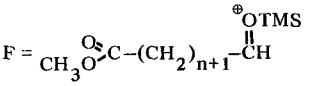
TABLE II

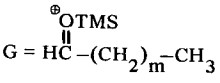
Major Ions of Silylated Hydroxystearates from Methyl Octadecynoates

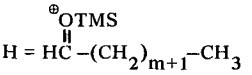
Triple bond position	n	m	E	F	G	H	R	R'
2	0	14	161(1)	175(100)	313(17)	327(-)	132(10)	146(2)
4	2	12	189(-)	203(100)	285(41)	299(-)	160(1)	174(4)
5	3	11	203(40)	217(100)	271(65)	285(18)	174(3)	188(14)
6	4	10	217(73)	231(100)	257(60)	271(45)	188(11)	202(13)
7	5	9	231(78)	245(100)	243(66)	257(61)	202(9)	216(13)
8	6	8	245(64)	259(68)	229(52)	243(44)	216(12)	230(30)
9	7	7	259(59)	273(52)	215(88)	229(80)	230(21)	244(8)
10	8	6	273(87)	287(83)	201(100)	215(85)	244(13)	258(13)
11	9	5	287(25)	301(33)	187(49)	201(56)	258(5)	272(10)
12	10	4	301(51)	315(62)	173(100)	187(75)	272(10)	286(9)
13	11	3	315(43)	329(36)	159(100)	173(73)	286(5)	300(-)
14	12	2	329(11)	343(35)	145(100)	159(42)	300(4)	314(8)
15	13	1	343(8)	357(32)	131(100)	145(35)	314(2)	328(9)
17	15	-	371(3)	-	103(1)	117(100)	342(9)	356(-)

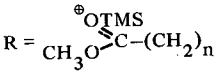


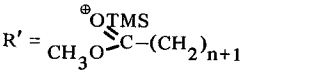












as in 9-octadecynoate and fragments C and D, m/e 164 and 150, are two mass units less. The spectrum of the conjugated ester, methyl *trans*-10,16-heptadecadien-8-ynoate, is not readily interpreted. Almost all its ions are of low mass with no distinct ions indicative of unsaturated sites.

Derivatives of Monoacetylenic Esters

The reaction of mercuric acetate with triple bonds to produce oxo derivatives has long been known (9). The mass spectra of methyl oxostearates formed from methyl octadecynoates, though definitive, are complicated (10). However, reduction of the keto-esters with $NaBH_4$ and silylation produce trimethylsiloxy derivatives which, upon analysis by mass spectrometry, produce spectra that define the position of the oxygen function (11). We used this scheme to locate acetylenic bonds in fatty esters.

Oxymercuration of methyl stearolate produced both 9- and 10-oxostearates. The products were identified by mass spectrometry, nuclear magnetic resonance, and infrared spectra. Addition of excess $NaBH_4$ resulted in a mixture of methyl 9- and 10-hydroxystearates with small amounts of unreacted methyl stearolate and 9- and 10-oxostearates. This reaction sequence was affected in all methyl octadecynoates. The major ions were determined for the silylated hydroxystearates formed from each member of the series (Table II). The fragmentation pattern is one of α-cleavage on each side of the carbon atom bonded to the silylated hydroxyl group (11) and a smaller rearrangement ion (12). Each derivatized octadecynoate produces up to four major characteristic ions and up to two minor characteristic ions (Table II). Ions 32 mass units (CH_3OH) less than fragments E and F (Table II) are also found. Analogous to oxymercuration of olefinic bonds (13), when the triple bond is close to the ester group, the hydroxyl group forms only on the carbon atom farther from this functional group. Therefore, only 3-hydroxystearate is formed from 2-octadecynoate and 5-hydroxystearate from 4-octadecynoate (Table II). The fragments are either hydrocarbons (G and H) or oxygen-containing

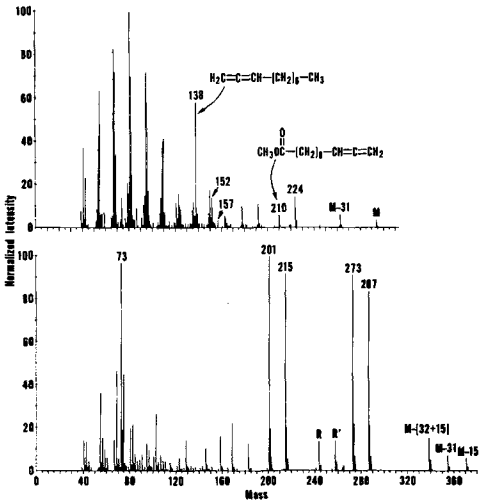


FIG. 1. Top: Mass spectrum of underivatized methyl 10-octadecynoate. Bottom: Spectrum of silylated methyl 10- and 11-hydroxystearates derived from methyl 10-octadecynoate. See Table II for R and R'.

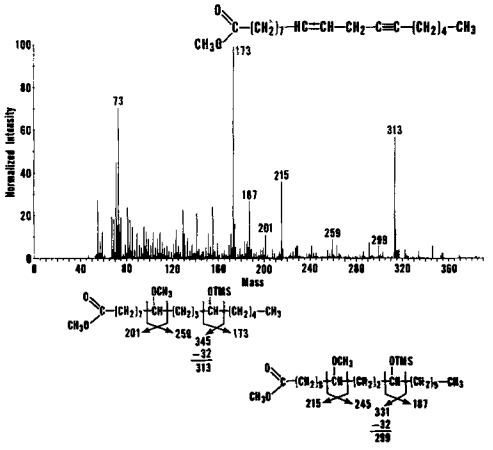


FIG. 2. Mass spectrum of silylated oxymercuration products of methyl crepenynate.

(E, R, F and R'). The former predominates when the OTMS group is close to the ω-methyl group, and the Δ-containing ions are dominant when the functional group is nearer to the ester function. Mass spectra of underivatized and derivatized methyl 10-octadecynoate are reproduced in Figure 1.

Ester with Ene-yne Structures

Oxymercuration in methanol and reduction with $NaBH_4$ result in two isomeric methoxy esters for each double bond and two isomeric hydroxy esters for each triple bond. In other words, from an ester with one olefinic and one

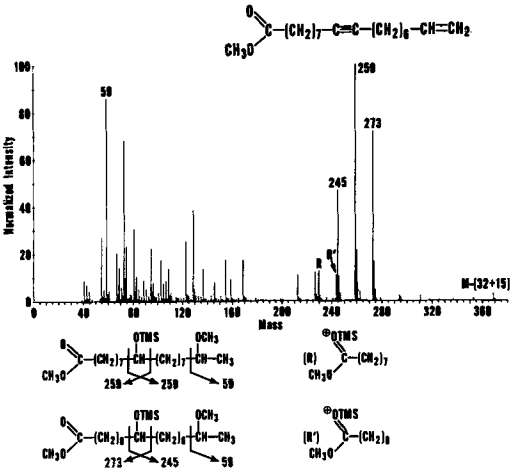


FIG. 3. Mass spectrum of silylated oxymercuration products of methyl 17-octadecen-9-ynoate.

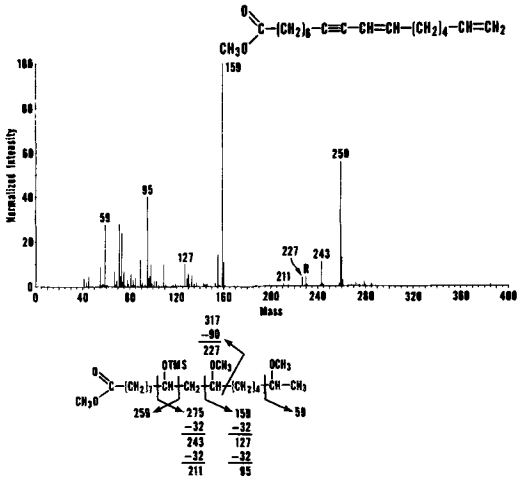


FIG. 4. Mass spectrum of oxymercuration products of methyl *trans*-10,16-heptadecadien-8-ynoate.

acetylenic bond, four different isomers are formed from this reaction. However, after silylation, these reaction products are so similar that GC does not completely separate them and they elute as one broad peak. While spectra taken at different points on the peak show changes in the ratio of the ions, the characteristic ions of all isomers are still present and they can be used to distinguish between double and triple bonds and to establish their location. For example, the mass spectrum of derivatized methyl crepenynate (Fig. 2) clearly locates the original triple bond (m/e 173 and 187) and the double bond (m/e 215 and 201).

In the same manner, Figure 3 shows the fragmentation pattern of the oxymercuration

products of methyl 17-octadecen-9-ynoate. The ions resulting from α-cleavage at the carbon attached to the trimethylsiloxy group are m/e 259 and 273 (fragments containing the ester function) and 245 and 259 (fragments with the terminal end of the molecule). The fragment with m/e 245 could be construed as the ion $CH_3O\overset{O}{\overset{\|}{C}}-(CH_2)_6-HCOTMS^{\oplus}$, which would locate the triple bond at the 8,9 position; however, this position is ruled out since no rearrangement ion occurs at m/e 216, whereas rearrangement ions are found for a silyloxy group at both the 9 and 10 positions (m/e 230 and 244). In addition, significant peaks found at m/e 227 (259-32) and m/e 213 (245-32) are formed by loss of methanol from the original ion. The derivatized olefinic group in this molecule is located by the large m/e 59 found in the spectrum.

The fragmentation pattern from derivatized methyl *trans*-10,16-heptadecadien-8-ynoate (Fig. 4) clearly shows the presence of a silylated hydroxyl group at the ninth carbon atom (m/e 259) and a methoxyl at positions 11 and 16 (m/e 159 and 59). However, these ions define the location of only one of the carbons at each of the unsaturated sites. Obviously, the conjugated nature of the original ester directs the derivatization to only one of the two possible carbon positions. If the relative position of the formed hydroxyl and methoxyl groups prove to be the same for all conjugated ene-yne structures, then the location of the unsaturation could be established. At this time, we have not investigated other conjugated esters.

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Interactions of Phospholipase D with 1,2 Diacyl-sn-glycerol-3-phosphorylcholine, dodecylsulfate, and Ca^{2+}

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ABSTRACT

Some properties of the pure, soluble phospholipase D (phosphatidycholine phosphatido hydrolase, EC 3.1.4.4) interactions with phosphatidyl choline (1,2 diacyl-*sn*-glycerol-3-phosphoryl choline) in a system also containing dodecylsulfate and Ca^{2+} ions were studied. Concentrations of Ca^{2+} greater than 50 mM were necessary both for activity and adsorption of the enzyme to the "supersubstrate." Ethylenediamine tetraacetic acid caused inhibition of activity, greater than one would expect from its chelating capacity. A nonlinear increase in activity with the increase of enzyme protein was observed, suggesting a subunit aggregation into a higher mol wt protein, catalytically more active. Upon centrifugation of the supersubstrate-enzyme complex at 4.5 x 10^5 g·min at 30 C, most of the substrate molecules sedimented regardless of the pH. The reverse was true when centrifugation was done at 1 C. Phospholipase D hydrolyzed phosphatidylcholine molecules present in the supersubstrate at temperatures around 0 C at a rate 1/5 that of a maximal value measured at 30 C. The Arrhenius plot was linear in the range from 0 to 30 C, and at that temperature the curve broke with a smaller slope. Activation energy of 9.1 Kcal/mol, below 30 C, was calculated. Adsorption of the enzyme to the sedimentable supersubstrate occurred at pH 8.0, regardless of temperature. At pH 5.6, a considerable portion of phosphatidylcholine was degraded at 30 C, thus minimizing the capacity of the supersubstrate to adsorb the enzyme. Although Mg^{2+} could replace Ca^{2+} in the formation of sedimentable supersubstrate, it neither assists in adsorption of the enzyme nor in activation of the phosphatidylcholine hydrolysis.

INTRODUCTION

We have recently been able to purify a soluble phospholipase D (phosphatidylcholine phosphatido hydrolase, EC 3.1.4.4) from dry peanut seeds. The mol wt can vary up to 200,000 ± 10,000 in multiples of 22,000 ± 3,000 (1). Catalytically active molecules, however, were observed only with enzyme having the high mol wt (1,2).

We have studied primarily the hydrolysis of egg-lecithin (phosphatidylcholine, or 1,2-diacyl-*sn*-glycerol-3-phosphorylcholine), which consists of a mixture of molecules having both saturated and unsaturated fatty acid esters. Other phospholipids may be hydrolyzed, but no extensive studies have been reported (2-4).

Although the reaction may be stimulated by various agents, detergents and especially dodecyl sulfate have been shown to be the best activators (2). The reaction also requires Ca^{2+}. Optimal hydrolysis rates were obtained with a phosphatidylcholine:dodecylsulfate molar ratio of 2:1 (2,5,6). Under these circumstances, a heterogeneous system is formed containing at least five components, i.e., enzyme, substrates (phospholipid and water), and activators (detergent and metal cation).

With the cabbage phospholipase D, Dawson and Hemington (7) have carried out studies involving similar components in an attempt to elaborate the concept of surface charge density requirements for the action of phospholipases.

Phospholipids, either alone or in association with other amphipaths, may acquire different macromolecular forms and may be treated for enzymological considerations as "supersubstrates" (4,8). This colorful term, which was coined by Brockerhoff (8), describes a "matrix in which a substrate molecule is embedded and refers to a triglyceride droplet, a phospholipid micelle or liposome, or an aggregate of many substrate molecules with or without the inclusion of nonsubstrate amphipaths" (4).

We have chosen to study the reaction catalyzed by the highly purified phospholipase D acting on such a supersubstrate. Its composition may be determined and controlled, but a knowledge of its structure is unfortunately rather limited.

However, this supersubstrate, a macromolecular complex made of phosphatidylcholine (PC), dodecylsulfate, and Ca^{2+} ions, apparently binds the enzyme at the right orientation to bring its active sites to the vicinity of the reactive bonds in the molecules

of the supersubstrate. Our data seem to indicate an adsorption of the enzyme molecules onto the surface of the supersubstrate, followed by some sort of size transformation into a catalytically more active enzyme. We describe some of the conditions for the formation of such a supersubstrate-enzyme complex. A preliminary account of these studies has been previously published (9).

MATERIALS AND METHODS

The preparation of (^{3}H) choline-labeled PC, the purification of phospholipase D, and the assay of its activity have been previously published (1,2).

Interactions of phospholipase D with the PC-dodecylsulfate-Ca^{2+} complex were carried out at pH 5.6 or 8.0 as follows:

1. Reaction mixtures were prepared containing 50 mM acetate, pH 5.6, or Tris-HCl, pH 8.0; 50 mM $CaCl_2$; 5 mM (^{3}H) PC (the concentration of the lipid is based on phosphorous analysis); and 2.5 mM sodium dodecylsulfate in a final volume of 2 ml.
2. The tubes were incubated with continuous shaking in a thermostatic water bath at 1 C or 30 C for 10 min after the addition of the enzyme.
3. After the incubation, total radioactivity was determined on a small aliquot of the reaction mixture.
4. Partitioning of both PC and phospholipase D between sedimentable and soluble fractions was done as follows:
   (a) An aliquot was centrifuged at 30,000 x g for 15 min (4.5×10^5 g•min) at either 1 C or 30 C. The supernatant was carefully and completely removed, and the precipitate was resuspended in fresh buffer solution containing $CaCl_2$.
   (b) Determination of radioactivity was done on aliquots of the supernatant and precipitate.
   (c) Determination of PC in the supernatant and precipitate was performed. Aliquots of each were extracted with chloroform and methanol according to Folch et al. (10), and after phase separation, the radioactivity was determined in the upper (aqueous-methanolic) phase and in the lower (chloroform rich) phase. The former contains only (^{3}H) choline, which is water soluble, whereas the latter contains the unhydrolyzed (^{3}H) PC and the nonlabeled phosphatic acid.
   (d) Assay of phospholipase D activity in the supernatant and precipitate was done with aliquots of each fraction added as enzyme to a complete reaction mixture (2).

RESULTS AND DISCUSSION

We considered the possibility that the enzyme is acting primarily on a "supersubstrate" composed of ovolecithin, dodecylsulfate, and Ca^{2+} ions.

Effect of Divalent Cations

The soluble phospholipase D requires Ca^{2+} for activity, and maximal rates were obtained with 50 mM or higher $CaCl_2$ (2,5,11). Although with 50 or 60 mM Ca^{2+} the activity of the enzyme was 4.95 and 5.4 units/mg protein, respectively, the addition of only 10 mM Na_2EDTA at pH 5.6 reduced the activity to 1.8 and 2 units, respectively. This is far below an anticipated decrease in activity based on the calculated ability of EDTA (ethylenediamine tetraacetic acid) to chelate only equimolar amounts of Ca^{2+}, e.g., 10 mM (12). Dodecylsulfate is expected to bind also equivalent amounts of Ca^{2+}. Therefore, under the experimental conditions, ca. 38.75 mM Ca^{2+} should be free and available.

During incubation at pH 5.6, very turbid suspensions were always formed. Such turbidities could be observed even if Ca^{2+} was replaced by Mg^{2+}.

Qualitative analysis shows that Ca^{2+} or La^{3+} ions form insoluble salts with dodecylsulfate over a wide range of pH values. On the other hand, Mg^{2+} or Mn^{2+} ions form soluble salts (M. Heller, unpublished observations).

The occurrence of such turbid suspensions suggested a formation of aggregates of supersubstrate molecules which might serve as an adequate surface for enzyme binding. Such aggregates might be large enough and sediment at relatively low centrifugal forces. Table I shows that only when Ca^{2+} ions are present does phospholipase D bind to the supersubstrate molecules and sediment at $4.5 \cdot 10^5$ g•min (Exp. 1). It is also obvious that although omission of Ca^{2+} or replacing it with Mg^{2+} does not support enzymatic hydrolysis (Column B), it also prevents binding of appreciable amounts of enzyme to the sedimentable supersubstrate (compare columns C & D). The diminished recovered activities are due to the sensitivity of the enzyme to dodecylsulfate (1). Ca^{2+} ions seem to protect the enzyme from the detergent (2). We have included Exps. 5 and 6 to indicate absence of nonspecific adsorption of enzyme to

TABLE I

Effects of Ca^{2+} or Mg^{2+} on the Sedimentation of Phospholipase D[a]

Exp. no.	Composition (A)	Phospholipase D activity (mUnits) Before centrifugation (B)	After centrifugation PCT (C)	After centrifugation SUP (D)
1	Complete	112	80	1.3
2	Omit Ca^{2+}	1.7	20	14
3	Omit Ca^{2+} and enzyme	1.5	0	6.3
4	Omit Ca^{2+} add Mg^{2+}	1.5	0	46
5	Ditto, (Exp. 4) add 2 x enzyme	3.2	19	62
6	Ditto, (Exp. 4) add 4 x enzyme	1.5	24	215

[a]Phospholipase D (18.5 μg protein) was incubated at 1 C in a final volume of 2 ml containing 50 mM acetate pH 5.6, 50 mM $CaCl_2$ or $MgCl_2$, 5 mM ^{3}H-phosphatidylcholine, 2.5 mM sodium dodecylsulfate (except Exps. 5 and 6, which contained 37 μg and 74 μg enzymatic protein, respectively). After 10 min of incubation, aliquots were removed for determination of phospholipase D activity, and the rest was centrifuged at 30,000 x g, 15 min at 4 C. The precipitates (PCT) were resuspended in acetate pH 5.6, and aliquots of the PCT and supernatants (SUP) were immediately withdrawn and assayed for activity (see Materials and Methods).

TABLE II

Effect of Ca^{2+} or Mg^{2+} on the Sedimentation of Phosphatidylcholine[a]

Exp. no.	Composition (A)	(^{3}H) Phosphatidylcholine (μmol) PCT (B)	(^{3}H) Phosphatidylcholine (μmol) SUP (C)
1	Complete	7.51	0.52
2	Omit Ca^{2+}	3.30	6.54
3	Omit Ca^{2+} omit enzyme	3.95	6.29
4	Omit Ca^{2+}, add Mg^{2+}	7.69	1.91
5	Ditto, (Exp. 4) add 2 x enzyme	7.51	2.04
6	Ditto, (Exp. 4) add 4 x enzyme	7.46	2.15

[a]The conditions were identical to those described in Table I. At the end of the incubation, the tubes were centrifuged at 30,000 x g, 15 min at 4 C. The amounts of ^{3}H-phosphatidylcholine in the precipitate (PCT) and supernatant (SUP) were determined in the chloroform rich, lower phase after extraction according to Folch et al. (10).

an inadequate surface of the supersubstrate.

Since the enzyme does not adsorb to the sediment in the absence of Ca^{2+}, we then examined the composition of the sedimenting supersubstrate under the same conditions described in Table I. The only condition for a sedimentable supersubstrate is the presence of divalent cation (Table II, Column B).

We may point to the possible roles of divalent cations in the catalysis of phospholipase D:

1. Ca^{2+} may be needed for the actual catalytic hydrolysis similar to the process accomplished by phospholipase A_1 (13).
2. The excess inhibitory action by EDTA might result from the removal of an essential metalic cation from the enzyme. This has been shown with phospholipase C (14).
3. It is insufficient to form a sedimentable supersubstrate (e.g., by Mg^{2+}) as long as either the right orientation of the molecules in the supersubstrate is not attained or the denaturing negative charges of the dodecyl sulfate anion are not blocked (e.g., in the case of soluble Mg^{2+} salts).

Effect of Protein Concentrations

We have shown previously that the enzyme is probably composed of subunits having a minimal mol wt of 20,000-25,000 (1). However, only those molecules having a mol wt at 200,000 have been found to be catalytically active. Furthermore, a time-dependent dissociation of the active enzyme having a mol wt of 200,000 into subunits of smaller mol wt was observed (1).

We reasoned that the surface of the sedi-

mentable supersubstrate composed of ovolecithin, dodecylsulfate, and Ca^{2+} ions might assist in the transformation of the noncatalytically active subunits into active, high mol wt species. Using the most purified enzyme (Step 5, Ref. 1), we have found a nonlinear increase in activity exhibiting an upward bend with the increase of protein concentrations (Fig. 1). This behavior was observed on different occasions with several batches of the enzyme. This was not always observed with the cruder enzyme (Step 4, Ref. 1) and the protein concentrations employed. Occasionally, the enzyme at an earlier stage of purification exhibited this kind of behavior.

Dawson and Hemington (7), using a highly purified, soluble phospholipase D from cabbage leaves, showed a time-dependent nonlinear increase in activity with large lecithin particles in the presence of Ca^{2+} ions (cf. Ref. 7, Fig. 2). They have ascribed this "autocatalysis" to the "stimulating formation of the product-phosphatidic acid," but the data could be alternatively interpreted as a time-dependent conversion of the enzyme into a more active form. In addition, in the same report, Dawson and Hemington have shown a nonlinear increase in the rate of hydrolysis with the increase in the concentrations of the purified enzyme (cf. Ref. 7, Fig. 11 [The straight lines drawn in this figure are apparently misleading, because the experimental points do not necessarily comprise a straight line]).

It therefore seems reasonable to assume an initial adsorption of enzyme molecules onto the surface of the supersubstrate, followed by an organizational step of conversion into higher mol wt species (ca. 200,000) which are catalytically more active. At this stage, it will be difficult to describe the exact mechanism for the role of the divalent cations in either the supersubstrate organization, enzyme adsorbance, orientation, or catalysis.

Effects of Temperature and pH

The organization of amphipathic phospholipids in an aqueous environment depends on their composition, concentration, and on temperature (15). The rate of enzyme catalyzed reactions is also affected by temperature (16,17).

Hydrolysis of PC "supersubstrate" in a complex with phospholipase D occurs even at temperatures around 0 C (Fig. 2). The rates increased linearly up to 30 C and then leveled off. Temperatures higher than 50 C cause irreversible denaturation of the enzyme (2,5,11). By plotting the logarithm of the initial velocities vs. 1/T, two straight lines are obtained intersecting at 30 C, with calculated Arrhenius energies of activation of 9.1 Kcal/mol below 30 C. Most hydrolytic enzymes exhibit activation energies of ca. 10 Kcal/mol (16,17).

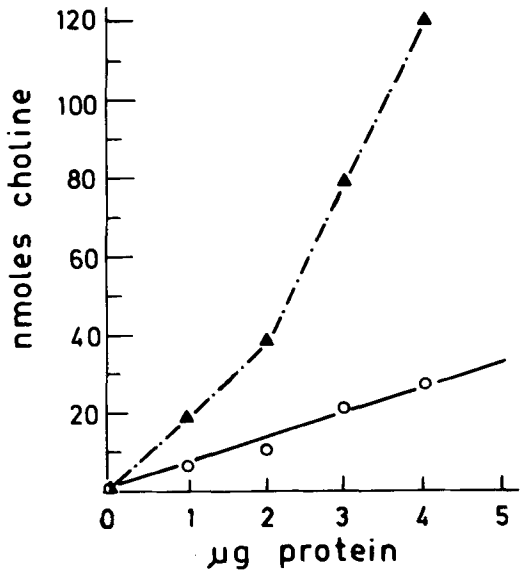

FIG. 1. Effect of protein concentrations on phospholipase D activity The activity was assayed with enzyme preparations at step 4 (○—○) and step 5 (▲—▲) of purification (1,2) as a function of protein concentrations. Aliquots were taken from either enzyme stock solutions containing both 10 µg protein/ml into a final volume of 1 ml. Incubations lasted for 10 min at 37 C. (The experiment is representative of four others.)

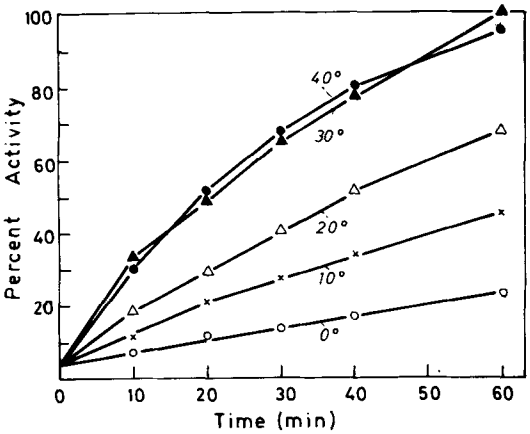

FIG. 2. Effect of temperature on phospholipase D activity. The activity was assayed in the presence of dodecylsulfate and Ca^{2+} ions with constant shaking, as outlined in Materials and Methods, at the temperatures indicated in the figure. 100% activity was taken as the value determined at 30 C after 60 min of incubation.

TABLE III

Phospholipase D Activity of the Sediment and Supernatant after Centrifugation[a]

Shaking at:		Phospholipase D activity (mUnits) after centrifugation at the following temperatures			
		1 C		30 C	
pH	Temperature (C) (A)	PCT (B)	SUP (C)	PCT (D)	SUP (E)
5.6	1	305	0	0	398
	30	288	48	0	530
8.0	1	32	10	191	0
	30	60	13	97	0

[a]Ten μmol of phosphatidylcholine, 5 μmol sodium dodecylsulfate, 100 μmol $CaCl_2$, and 100 μmol acetate pH 5.6 or tris-HCl pH 8.0 in a final vllume of 2 ml were shaken for 10 min in the presence of 18.5 μg phospholipase D at 1 C or 30 C. After incubation, aliquots were withdrawn and centrifuged at 30,000 x g, 15 min at 1 C or 30 C. The supernatant (SUP) was completely removed and the precipitate (PCT) was resuspended with acetate and $CaCl_2$. Aliquots of each fraction were assayed for phospholipase D activity (see Materials and Methods) at a final pH of 5.6.

From phase diagrams and Tm values of PC containing saturated and unsaturated acids, it seems unlikely that the breaking point at 30 C reflects a phase transition of egg-lecithin (15,18-22). Actually, the high degree of unsaturation of egg-lecithin brings the Tm value below 0 C (23). Inclusion of dodecylsulfate, however, might affect the melting characteristics of this PC, yet lack of data on the effects of both dodecylsulfate and Ca^{2+} on the melting profile of egg-lecithin prevents the drawing of a final conclusion.

We have shown the ability of the calcium-containing supersubstrate to adsorb the enzyme, and also the effects of temperature on the enzyme's activity. We consequently examined the effects of temperature and pH on the sedimentation characteristics of the enzyme with the supersubstrate and of the supersubstrate itself. The components were mixed at two temperatures (1 C or 30 C) and at two pH values (5.6 and 8.0). Table III shows the measured activities of phospholipase D which either adsorbed to the sedimenting "supersubstrate" or remained in solution. Since the centrifugation period equals that of the incubation period, the centrifugation was also carried out at these two temperatures.

The incomplete recoveries of the enzymatic activities are, in part, due to instability displayed by the enzyme under these conditions.

The enzyme adsorbed to the sedimenting supersubstrate almost regardless of temperature and pH. An exception was observed when the incubation was conducted at 1 C or 30 C at pH 5.6, then centrifuged at 30 C; the enzyme was found in solution and did not sediment (Table III, Column E). Since these conditions are optimal for efficient hydrolysis of PC in the supersubstrate, a large portion of it was degraded, causing a change in the molar proportion of PC to dodecylsulfate D despite the reduction of PC in the supersubstrate, its sedimentation pattern was not affected (data not shown). Regardless of the pH, PC sedimented as a supersubstrate complex primarily at 30 C. Since at low temperature (ca. 0 C), or at pH 8.0, the degree of PC hydrolysis was considerably smaller compared to pH 5.6 and 30 C, it was not surprising to obtain these results.

We may thus summarize our conclusions as follows: An appropriate surface furnished by the supersubstrate composed of PC, dodecylsulfate, and a divalent cation (Ca^{2+}) adsorbs the enzyme molecules. Subsequently, a conversion into a macromolecular form occurs on the surface either by increasing protein concentrations or with time. With the right spatial organization of supersubstrate and the accumulation of the appropriate macromolecular enzyme species, pH 5.6 and 30 C, maxiaml rates of hydrolysis are obtained.

Our data do not exclude the possibility that any form of the enzyme might also hydrolyze another spatial organization of PC in a different supersubstrate arrangement (D. Lichtenberg and P. Greenzaid, unpublished observations).

The model system presented in these studies may serve for subsequent studies of a more physiological supersubstrate complex in which the dodecylsulfate anion is replaced by other negatively charged amphipatic phospho- or glycolipids in large aggregates furnishing adequate surfaces. We have previously clearly shown that phospholipase D degraded phospholipids in biological membranes, e.g., rat liver microsomes or human erythrocyte membrane vesicles, without the need of any nonsubstrate

amphipathic activator (2,24). Furthermore, in previous studies we described a "natural supersubstrate" in which dodecylsulfate was replaced by rat liver microsomes. Radioactive PC, microsomes, and Ca^{2+} created a "natural" supersubstate with the right orientation for both binding of the enzyme and catalysis (24). On the other hand, forming a supersubstrate with another type of lipoprotein, e.g., plasma β-lipoprotein, caused an inhibitory effect, probably due to a wrong orientation, improper binding, or lack of size transformations (24).

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Kinetics of Absorption, Equilibration (or Distribution), and Excretion of Orally and Intraperitoneally Administered Cholesterol in the Rat

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ABSTRACT

Resorption and metabolism of cholesterol administered intraperitoneally and per os have been described in the rat utilizing a technique of double isotopic labeling. Depending upon the route of administration, these parameters vary to a large degree. Alimentary cholesterol is progressively resorbed over a period of 10 hr, while the maximal blood level of cholesterol is attained after the 6th hr following intraperitoneal injection. On the other hand, cholesterol administered per os is more rapidly utilized in biliary acid synthesis than cholesterol administered intraperitoneally. In the range of concentrations utilized in this work (10-300 μg and 0.2-300 μg, respectively, administered to the rat, orally and intraperitoneally), the rate of cholesterol resorption remained constant.

INTRODUCTION

For many years, we have known that the totality of cholesterol does not form a single pool in mammalian tissues. Chevallier (1) has demonstrated that the rate of exchange between tissular and plasmatic cholesterol varies considerably from tissue to tissue. In addition, the rate of plasmatic disappearance of radioactive cholesterol administered intravenously indicates the existence of at least two different pools (2). Apart from these, quantitatively reduced pools of cholesterol with different rates of metabolization also exist; these conclusions have been derived from in vivo (3,4) as well as in vitro (5,6) experiments. Thus, only a fraction of the cholesterol present in the microsomal membranes is readily available as a substrate for the cholesterol-7α-hydroxylase in the liver (5,6). In fact, the concentration of cholesterol in the liver cell might be an important regulatory factor of this enzymatic activity which catalyzes the rate limiting step in the biliary acid metabolic pathway (7).

Through the use of techniques of double labeling and a sample oxidizer allowing the combustion of organic samples and the separation of the radioactivity linked to ^{3}H (as molecules of water) and ^{14}C (as molecules of CO_2), we have further characterized the resorption and distribution kinetics of orally and intraperitoneally administered cholesterol. The absence of isotopic effects in the distribution and the metabolism of [1α-^{3}H] and [4-^{14}C]cholesterol was also demonstrated, regardless of the route of administration.

MATERIALS AND METHODS

Materials

All the experiments were carried out using 200 g Sprague-Dawley rats (Iffa Credo, Les Oncins, Lyon, France) which were maintained on a standard commercial diet (UAR A04, Villemoisson, France).

[1α-^{3}H]cholesterol (48 Ci/mmol) and [4-^{14}C]cholesterol (55.5 mCi/mmol) were purchased from IRE (Fleurus, Belgium) and purified before use by thin layer chromatography (TLC) on silica gel with chloroform as a solvent. Unlabeled cholesterol (Merck, Darmstadt, Germany) was purified by two successive crystallizations in an ethanol:water (15:5, v/v) system. Tween 80 was bought from Sigma (St. Louis, MO). The various products needed for the measurement of radioactivity by liquid scintillation were obtained from Packard (Downers Grove, IL). All other chemicals and solvents were of analytical grade and were bought from Merck (Darmstadt, Germany).

Measurements of ^{3}H and ^{14}C linked radioactivity were performed by liquid scintillation with a Packard Tri-Carb Model 3375 spectrometer. The radioactivity was measured either directly on tissular organic extracts or after combustion of the samples into H_2O and CO_2 with the aid of a Packard Tri-Carb 306 sample oxidizer (8). This last technique was used each time a mixture of ^{3}H and ^{14}C was present in the analyzed samples or when the radioactivity was measured directly on tissues without any extraction. The advantage of using the sample oxidizer for the determination of radioactivity present in biological samples has been particularly useful when a mixture of ^{14}C and ^{3}H

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TABLE I

^3H:^{14}C Ratio Determined in Organs after One Intraperitoneal Injection of [1α-^3H, 4-^{14}C] cholesterol[a]

Number	Delay after injection (hr)	^3H:^{14}C ratio				
		Blood	Liver	Bowels	Skin	Kidney
1	½	6.09	5.97	5.65	5.87	5.95
2	1	5.90	6.17	6.27	5.86	6.27
3	2	6.22	5.89	5.65	6.13	6.12
4	3	5.94	5.88	6.12	6.18	6.24
5	5	5.82	5.96	6.15	6.18	5.73
6	8	6.02	6.14	6.13	6.15	5.88
7	12	5.97	6.30	6.29	6.10	6.20
8	24	6.15	6.11	6.35	6.35	6.21

[a]The quantities of administered radioactivity were 73,800,000 dpm and 12,200,000 dpm for ^3H and ^{14}C, respectively, with a ^3H:^{14}C ratio of 6.05. One rat was killed at different delays after the injection, and one part of the different organs was directly burned in the sample oxidizer; ^3H and ^{14}C were then counted by liquid scintillation.

occurs in the analyzed sample (8).

Methods

Administration of [1α-^3H]cholesterol and/or [4-^{14}C]cholesterol: The labeled samples of cholesterol were administered to the rats by one of the following two routes: (a) intraperitoneal injection of a Tween 80 (1 mg/ml) micellar solution of cholesterol in saline (9); (b) gastric intubation of a suspension of cholesterol in medicinal oil (*oleum ad injectionem*, Pharmacopée belge V) under mild ether anesthesia.

Removal of tissues: The rats were sacrificed by cervical dislocation and bled from the carotid, whereupon the blood was collected in a heparinized tube. The various tissues (liver, intestine, muscle, skin, adipose tissue, and kidney) were quickly removed and placed in ice-cold isotonic KCl. The bowels were immediately emptied of their contents by washing with isotonic KCl and the intestinal mucosa removed by scraping.

To study the evolution of plasmatic cholesterol radioactivity in individual animals, samples (100 μl) of blood were repeatedly collected by retro-orbital puncture.

Treatment of the samples and measurement of radioactivity: The tissue samples (liver, skin, muscle, and intestinal mucosa) were either directly burned in the oxidizer for determination of the total radioactivity or homogenized in 3 volumes of isotonic KCl. The tissular homogenates were then extracted with 20 volumes of a chloroform:methanol (2:1, v/v) mixture. After filtration over anhydrous Na_2SO_4, the organic extracts were evaporated under reduced pressure and reextracted for 30 min by a mixture of diethylether:heptane:ethanol:water (1:1:1:1, v/v). The two resulting phases were then collected separately and evaporated to dryness under reduced pressure. The organic phase residue, solubilized in a few drops of chloroform, was used after combustion for the measurement of the labeled cholesterol. The aqueous phase residue dissolved in a few drops of warm ethanol was directly burned in the oxidizer for the measurement of labeled biliary acids or submitted to TLC to separate the conjugated from the free biliary acids (10,11). In this method, a mixture of butanol:acetic acid:water (10:1:1, v/v) was used to develop the chromatoplates. The silica gel corresponding to the biliary acid spots localized by comparison with references migrating parallely on the same plate (visually perceived by spraying with a mixture of anisaldehyde:sulfuric acid:acetic acid (1:2:100, v/v) was ultimately scraped off the plate and burned in the sample oxidizer.

In the blood, the only parameter studied was the total radioactivity measured after combustion of the sample.

RESULTS

Absence of Isotopic Effect

Before applying a double isotopic method to the study of cholesterol metabolism, the absence of isotopic effect in the rate of distribution and in the metabolism of [1α-^3H] vs. [4-^{14}C] labeled cholesterol was first verified.

A mixture of these two labeled isotopes was injected intraperitoneally to the rats. The ^3H:^{14}C ratio was analyzed in the blood, liver, bowels, skin, and kidney of these animals (Table I). In addition, specific measurement of the ^3H:^{14}C ratio in terms of purified cholesterol and total biliary acids and salts was also performed in the liver and in the bowel (Table II).

The statistical analysis of the results based

TABLE II

$^{3}H:^{14}C$ Ratio of Hepatic or Intestinal Cholesterol and Biliary Acids after an Intraperitoneal Injection of [1α-^{3}H] and [4-^{14}C]cholesterol mixture[a]

Number	Delay after injection (hr)	$^{3}H:^{14}C$ ratio			
		Cholesterol		Biliary acids	
		Liver	Bowels	Liver	Bowels
1	½	5.94	6.23	5.73	6.03
2	1	5.94	6.30	5.95	6.10
3	2	5.81	6.03	6.55	6.26
4	3	-	6.28	6.11	6.24
5	5	5.89	6.48	6.17	6.38
6	8	5.76	6.13	5.84	6.34
7	12	6.29	6.17	6.01	6.19
8	24	6.27	6.22	5.88	6.11

[a]After killing the rats at different delays, one part of the liver and of the intestinal mucosa was homogenized, and cholesterol and biliary acids were extracted and burned in the sample oxidizer. ^{3}H and ^{14}C were then counted by liquid scintillation. For a description of the mixture injected, see Table I.

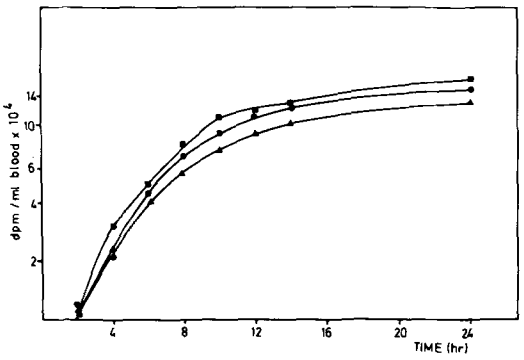

FIG. 1. Evolution of blood radioactivity after gastric intubation of 20 μCi of [1α-^{3}H]cholesterol dissolved in medicinal oil. The amount of administered cholesterol was either 0.2 mg (—■—■—), 2 mg (—▲—▲—), or 20 mg (—●—●—). Each result represents the mean of values obtained from two rats.

on time after injection as well as the analyzed tissue led to the conclusion ($P<0.05$) that the mechanism assuring transport and metabolism of exogenously administered cholesterol was not influenced by the nature of isotopic labeling.

Influence of the Concentration of the Injected Cholesterol on its Rate of Resorption

Before comparing the results of experiments where the rate of a compound administered by two different routes is studied, it is necessary to verify the absence of a rate limiting step at the level of the resorption mechanism itself. To show that the rate of cholesterol resorption by either way was independent from the amount of cholesterol administered, a fixed amount of [1α-^{3}H]cholesterol diluted with increasing concentrations of unlabeled cholesterol was administered to the rat either by gastric intubation or by intraperitoneal injection. As the kinetics of cholesterol distribution are superposable in the blood and in the other tissues (12, Fig. 4), the rate of tritium distribution was followed in the blood only; this procedure allowed us to follow the kinetics in each individual animal (Fig. 1). After oral administration, the exogenous cholesterol appeared 2 hr later in the blood and progressively accumulated to reach a plateau 24 hr later. Figure 1 illustrates that the resorption rate of orally administered cholesterol is independent from the amount of cholesterol absorbed by the animal. At any given time, the concentration of exogenous cholesterol present in the bloo; is directly proportional to the amount initially administered to the rat. Identical results were observed whether the cholesterol was administered as a suspension in medicinal oil or as an aqueous micellar solution with Tween 80. On the other hand, in the case of an intraperitoneal injection, oil suspensions of cholesterol are very poorly resorbed; it is thus necessary to use an aqueous micellar solution of cholesterol in order to obtain a significant resorption. Under these conditions (Fig. 2), the results were comparable to those obtained after an oral administration. The rate of cholesterol appearance in the blood was independent from the amount injected, and the concentration of exogenous cholesterol in the blood was directly proportional to the amount administered.

Nevertheless, the overall shape of the radioactivity curve in the blood was different. In fact, the resorption was more rapid after an intraperitoneal injection. A plateau was already reached 6 hr following the administration, and

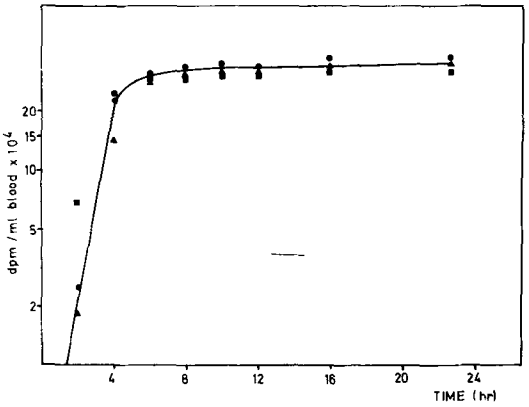

FIG. 2. Evolution of blood radioactivity after an intraperitoneal injection of 20 μCi of [1α-^{3}H]cholesterol dissolved in 1 ml of water with the help of Tween 80 (1 mg/ml). The amount of administered cholesterol was either 10 μg (—■—■—), 30 μg (—▲—▲—), or 300 μg (—●—●—). Each result represents the mean of values obtained from two rats.

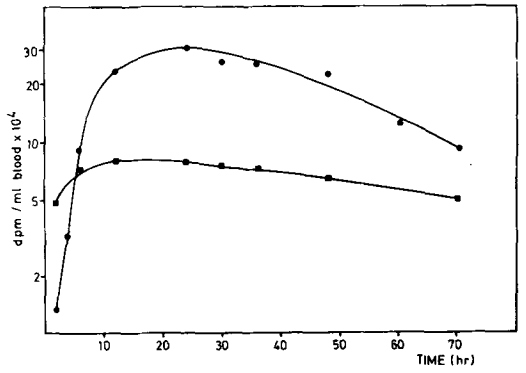

FIG. 3. Evolution of ^{3}H (—●—●—) and ^{14}C (—■—■—) radioactivity in the blood after a simultaneous administration of 30 μCi of [1α-^{3}H]cholesterol (20 μCi/μmol) by gastric intubation and 10 μCi of [4-^{14}C]cholesterol (20 μCi/μmol) by intraperitoneal injection.

it is noteworthy that from the 6th up until the 30th hour following treatment, the blood radioactivity was remarkably stable.

Comparative Metabolism of Orally and Intraperitoneally Administered Cholesterol

[1α-^{3}H]cholesterol and [4-^{14}C]cholesterol were respectively administered per os and intraperitoneally to the animals. The rats were then sacrificed at various times, and the total radioactivity was determined after combustion of blood, liver, intestinal mucosa, muscle, skin, adipose tissue, and kidney tissue samples. In addition, the radioactivity specifically linked to cholesterol and biliary acids was also measured in the liver and intestinal mucosa.

Figure 3 shows the variations of total plasmatic radioactivity during the 3 days following the simultaneous administration of [1α-^{3}H] and [4-^{14}H]cholesterol by two different routes. As already seen in Figure 2, the intraperitoneally injected cholesterol was rapidly absorbed, and its plasmatic concentration did not change very much in the following 3 days. The orally administered cholesterol was resorbed less quickly and its plasmatic concentration decreased more rapidly than in the case of the intraperitoneally injected compound.

To compare the differences in the rates of absorption of the cholesterol administered by these two different routes, ^{3}H:^{14}C ratios were calculated in different tissues (blood, liver, kidney, muscle, skin, and adipose tissue). The general pattern of radioactivity is the same in all tissues, and it was verified that a similar

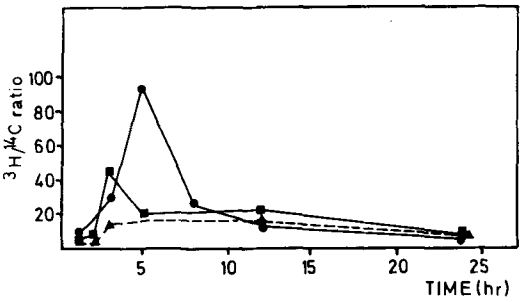

FIG. 4. Evolution of ^{3}H:^{14}C ratio of biliary acids from liver (—■—■—) and from bowels (—●—●—) and of cholesterol (—▲—▲—) from kidney, blood, liver, skin, or adipose tissue. A series of rats are orally administered 50 μCi of [1α-^{3}H]cholesterol and injected intraperitoneally with 10 μCi of [4-^{14}C]cholesterol. Each time one rat is killed, the organs are removed and cholesterol or bile acids are extracted and separated before being burned for radioactivity counting. In each case, ^{14}C radioactivity of biliary acids was above 500 dpm for biliary acids and above 2,000 dpm for cholesterol; ^{3}H radioactivity was always higher.

result was obtained if the radioactivity was measured on the extracted cholesterol.

Figure 4 shows that the ^{3}H:^{14}C ratio, which never exceeds 20 for cholesterol, went up to 45 for biliary acids in the liver and up to 90 for biliary acids in the intestine. Nevertheless, the dissociation between the curves corresponding to cholesterol and to biliary acids is only transient and does not last after the first day. The ^{3}H:^{14}C ratio peak associated with biliary acids (seen between the 2nd and the 10th hr after injection) results from an increase of ^{3}H radioactivity and not from a decrease in ^{14}C radioactivity. This indicates that the orally absorbed [^{3}H]cholesterol is preferentially used for the synthesis of the biliary acids.

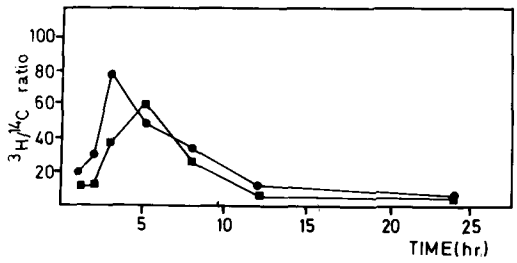

FIG. 5. Evolution of ^3H:^{14}C ratio of free (—■—■—) and conjugated (—●—●—) biliary acids of the intestinal mucosa. The experimental procedure is similar to that described in Figure 4. The pool of bile acids are separated by thin layer chromatography, and the areas corresponding to conjugated and free acids are scraped off, burned in the oxidizer, and ^3H or ^{14}C are counted by liquid scintillation. The radioactivity associated with the free biliary acids varied from 80 to 300 dpm, and that associated with the conjugated ones varied from 250 to 1,000 dpm; ^3H radioactivity was much higher.

To verify that this ^3H:^{14}C ratio peak results from a physiological phenomenon and not from contamination of the bile acid extracts from cholesterol (cholesterol ^3H:^{14}C ratio is very high in the intestine, as part of the exogenous [^3H]cholesterol is not yet resorbed), we have further purified the biliary acid extracts and separated the conjugated from the free biliary acids by TLC.

Figure 5 shows that the results obtained for both conjugated and free biliary acids were similar to those recorded for the crude extract. Slight differences between the two curves may be seen, but due to the low radioactivity associated with conjugated biliary acids, the measurements of ^3H:^{14}C ratio of these compounds are much less accurate.

On the contrary, the radioactivity values of free biliary acids are much higher, and consequently their ^3H:^{14}C ratio is more reliable. It differs radically from the ^3H:^{14}C ratio evolution found in cholesterol.

DISCUSSION

The in vivo study of cholesterol metabolism has always been a difficult problem, particularly due to the fact that different discrete pools of cholesterol exist and turn over at various rates. Sophisticated methods (3,4,13-15) have been described to measure the kinetics of equilibration and metabolism of exogenously absorbed and endogenously synthesized cholesterol under different experimental conditions. To evaluate the relative participation of these "cholesterol" in the formation of pools and in the subsequent synthesis of biliary acids under the best conditions, it is very convenient to utilize a method incorporating both ^3H and ^{14}C as isotopic markers. Consequently, techniques allowing the exact measurement of these radioisotopes mixed in a wide range of proportions are very helpful in solving these problems. In this respect, the new sample oxidizer used in our study was particularly reliable and very well adapted to this kind of work (8). When the appropriate method is used, the rate of resorption is independent from the concentration of cholesterol administered. In oil solution, it is well resorbed when orally administered, while it is not after being intraperitoneally injected. In the latter case, cholesterol must be administered in the form of an aqueous micellar solution with the aid of a detergent. In both cases, the resorption rate is not modified by increasing the concentration of administered cholesterol. The requirement of an emulsifier for the intraperitoneal resorption of cholesterol can be extrapolated to the intestinal one. In effect, in the bowels, the biliary salts (16,17) play the same role as Tween 80 on the resorption of exogenously administered cholesterol. Under our experimental conditions, the resorption rate of orally administered cholesterol is always slower than intraperitoneally injected cholesterol.

Six hours after the treatment of an animal, the blood concentration of intraperitoneally given cholesterol reached an optimal value and then slowly declined to reach a value of ca. 65% of the optimum level 72 hr later. The plasmatic concentration of the orally administered cholesterol increased to reach a peak value 24 hr after administration and then declined more rapidly, reaching, 72 hr thereafter, a value corresponding to ca. 35% of the optimum concentration. The overall shape of the distribution curves of cholesterol in the various tissues being similar to the one in the blood, one might conclude that the rate of exogenous cholesterol metabolism is different depending upon the route of administration.

Comparisng the ^3H:^{14}C ratios, the totality of hepatic cholesterol is not readily available as a substrate for the enzymes involved in biliary salts biosynthesis. This conclusion is further supported by other works (3,4) demonstrating that exogenous cholesterol and newly endogenously synthesized cholesterol are not metabolized at the same rate. In vitro experiments have also shown that endogenous and exogenous cholesterol are not utilized to the same extent by cholesterol-7α-hydroxylase, which most likely catalyzed the rate limiting step of biliary acid biosynthesis (5,6,18). The present study emphasizes that, depending upon the rate of its

administration, the resorption, the distribution, and the metabolism of exogenously administered cholesterol are different.

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Studies on Drug-induced Lipidosis: VII. Effects of Bis-β-diethylaminoethylether of Hexestrol, Chloroquine, Homochlorocyclizine, Prenylamine, and Diazacholesterol on the Lipid Composition of Rat Liver and Kidney

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ABSTRACT

4,4'-Bis (β-diethylaminoethoxy)-α,β-diethyldiphenylethane (DH), which had been shown to induce a type of lipidosis resembling Niemann-Pick disease, was given to rats at a dose of 20, 50, 100, and 150 mg/kg body weight per day for 1 or 2 weeks. An enlargement of the liver with marked increases in free cholesterol, total phospholipids, and phosphatidylinositol took place by administration of a larger dose. The increase in bis (monoacylglyceryl) phosphate (BMGP), which is peculiar to this kind of drug-induced lipidosis, was dependent upon the dose of the drug as well as the length of time. Similar changes were also observed in kidney. Among several other drugs tested, chloroquine and diazacholesterol brought on as much increase in BMGP as treatement with DH.

INTRODUCTION

Administration of 4,4'-bis (β-diethylaminoethoxy)-α,β-diethyldiphenylethane (3,4-bis [p(β-diethylaminoethoxy)phenyl] hexane, hexestrol bis (β-diethylaminoethyl ether)) (DH) caused a specific type of lipidosis characterized by a foam cell syndrome resembling Niemann-Pick disease (1-3). The lipidosis can be induced by this drug in humans as well as in experimental animals (4,5), but there is some difference in biochemical and pathological features between humans and rats which has been shown to be related to the difference in drug-metabolizing ability between animal species (4-6). It is the purpose of the present investigation to show the effect of increasing doses of DH on the lipid composition of rat liver and kidney and to see if there is a dose response relationship between the accumulation of the drug and the increase in acidic phospholipids which is peculiar to drug-induced lipidosis. The effect of chloroquine, homochlorocyclizine, prenylamine, and diazacholesterol was also tested.

MATERIALS AND METHODS

Male albino rats of the Sprague-Dawley strain weighting 200-250 g that had been raised on a commercial stock diet were divided into two groups. A group of rats was divided into eight subgroups, and DH (dihydrochloride) was given as an aqueous solution through a stomach tube at a level of 20, 50, 100, or 150 mg/kg body weight per day for 1 or 2 weeks. Another group of rats was divided into four subgroups and given 1) chloroquine 100 mg/kg body weight per day for 1 week, 2) homochlorocyclizine 100 mg/kg for 1 week, 3) prenylamine 50 mg/kg for 2 weeks, and 4) diazacholesterol 10 mg/kg for 2 weeks. The rats were sacrificed by exsanguination after the end of the experimental period. Five rats were used for each subgroup.

The liver and kidney were removed and homogenized with chloroform:methanol, 2:1, and the crude lipid extract washed by the method of Folch et al. (7). The estimation of total phospholipids, free cholesterol, cholesteryl esters, and triglycerides and the analysis of phospholipids were carried out by thin layer chromatography-colorimetric assay procedures as described by Rouser et al. (8,9) and in our preceding papers (1,5,10).

RESULTS

Effect of Increasing Doses of DH on Rat Liver and Kidney

Changes in weight of the liver and kidney (Tables I and III: There was a significant increase ($P<0.001$) in weight of the liver by increasing the dose of DH. The increase showed a correlation with both the dose administered per day as well as the length of the period of oral administration. A significant increase ($P<0.01$) in weight of the kidney was also observed after 2 weeks of DH at the highest level (150 mg/kg).

Lipid composition of the liver (Table I): There was a significant increase ($P<0.01$) in phospholipid content of the liver after 2 weeks of the administration of the drug at a lower dose level (20 or 50 mg/kg). The increase be-

TABLE I

Effects of Increasing Doses of DH[a] on the Lipid Composition of Rat Liver

Dose and period	Weight of liver (g)	Phospholipid	Free sterol	Esterified sterol	Triglyceride
Control	7.9 ± 0.5	3.13 ± 0.21 (%)[b]	0.18 ± 0.06 (%)	0.06 ± 0.02 (%)	0.37 ± 0.17 (%)
1 Week					
20 mg	7.2 ± 1.1	3.14 ± 0.08	0.19 ± 0.02	0.12 ± 0.02	0.46 ± 0.12
50 mg	8.1 ± 1.1	3.69 ± 0.40	0.19 ± 0.04	0.18 ± 0.07	0.41 ± 0.09
100 mg	8.2 ± 0.5	6.30 ± 0.94	0.35 ± 0.07	0.53 ± 0.19	0.81 ± 0.50
150 mg	11.4 ± 0.7	6.66 ± 0.53	0.47 ± 0.05	0.54 ± 0.04	1.42 ± 0.75
2 Weeks					
20 mg	8.2 ± 0.4	4.68 ± 0.52	0.27 ± 0.02	0.08 ± 0.02	0.43 ± 0.18
50 mg	10.1 ± 0.9	5.32 ± 1.31	0.29 ± 0.08	0.24 ± 0.03	0.79 ± 0.44
100 mg	10.6 ± 0.7	5.89 ± 0.88	0.36 ± 0.04	0.49 ± 0.21	0.47 ± 0.19
150 mg	12.5 ± 0.7	7.07 ± 0.61	0.55 ± 0.09	0.76 ± 0.20	1.10 ± 0.22

[a]DH = 4,4'-bis(β-diethylaminoethoxy)-α,β-diethyldiphenylethane.
[b]Percent of fresh weight of tissues.

came more prominent by increasing the dose, and the maximal increase in total phospholipids was attained already after 1 week by the administration of DH at a level of 100 or 150 mg/kg. Free cholesterol and cholesteryl esters showed the same tendency as total phospholipids.

Phospholipid composition of the liver (Table II): Increases in bis(monoacylglyceryl)-phosphate (BMGP) and phosphatidylinositol (PI) presented a peculiar change in distribution pattern of liver phospholipids. (BMGP is the name of the acidic glycerophospholipid which has been called lysobisphosphatidic acid in our previous papers [1-6, see also Ref. 20].) The increases in these acidic phospholipids were dose dependent. A marked increase in PI was noted at a larger dose level of the drug. Although there were not significant differences in concentration of PI in total phospholipids between 1 and 2 weeks at each dose level of DH, there was a marked increase in BMGP between 1 and 2 weeks of administration. Phosphatidylserine (PS) was also slightly increased after 2 weeks by giving the drug at a larger dose (100 or 150 mg/kg). Cardiolipin (CL) and phosphatidylethanolamine (PE) were slightly decreased, but there was no change in concentration of phosphatidylcholine (PC), lysophosphatidylcholine (LPC), sphingomyelin (Sph), and phosphatidic acid (PA).

Lipid composition of the kidney (Table III): An increase in total phospholipids was also observed in rat kidney by the administration of the drug at a level higher than 100 mg/kg body weight per day, although the rate of increase was not so marked as in liver. Free cholesterol was also slightly increased; however, there was no change in concentration of cholesteryl esters and triglycerides.

Phospholipid composition of the kidney (Table IV): Increases in BMGP and PI were also observed in rat kidney, although the rate of increase was markedly lower than in liver. The increase in PI was dependent upon the dose of DH as well as the length of the period of drug administration. In contrast to the compositional change in liver phospholipids, the increase in BMGP did not show a marked difference between 1 and 2 weeks of administration.

Decreases in CL, PS, and PE were observed after 2 weeks at the highest dose level, while there was no significant change in concentration of the other phospholipid classes.

Effect of Chloroquine, Homochlorocyclizine, Prenylamine, and Diazacholesterol on the Phospholipid Composition of Rat Liver

Increases in concentration of total phospholipids were observed after administration of chloroquine at a level of 100 mg/kg for 1 week. The phospholipid composition of the liver in this subgroup showed marked increases in BMGP and PI almost equal to the change in DH-treated animals. Slight increases in acidic phospholipids were also obtained in rats treated with homochlorocyclizine and prenylamine, although the rate of increase was much less than in the animals treated with DH or chloroquine. An increase in BMGP almost as much as the increase obtained by DH at the dose of 50 mg/kg was observed by the administration of diazacholesterol at a lower dose level, although there was no net increase in total phospholipids.

The change in lipid composition as well as in histological findings on electron microscope was minimal in the subgroup of rats treated with prenylamine, but it is not possible to compare the results exactly in a quantitative manner because the dose of this drug was half as

TABLE II

Effects of Increasing Doses of DH on the Phospholipid Composition of Rat Liver[a]

Dose and period	BPA	BMGP	CL	PE	PC	Sph	PI	PS	LPC	PA
Control	–	0.0	5.9 ± 0.3	26.8 ± 0.6	50.3 ± 1.3	4.9 ± 0.2	7.3 ± 0.6	3.7 ± 0.5	0.6 ± 0.2	0.1 ± 0.1
					(Percent of total phospholipids)					
1 Week										
20 mg	0.1 ± 0.1	1.3 ± 0.4	4.9 ± 0.4	25.1 ± 0.7	51.1 ± 0.9	4.5 ± 0.7	7.8 ± 0.4	3.8 ± 0.3	1.0 ± 0.4	0.1 ± 0.1
50 mg	0.1 ± 0.1	1.6 ± 0.3	4.7 ± 0.6	23.1 ± 1.7	51.4 ± 2.9	4.4 ± 0.6	9.1 ± 1.0	3.4 ± 0.2	1.1 ± 0.4	0.3 ± 0.3
100 mg	0.1 ± 0.1	2.2 ± 0.7	4.0 ± 0.2	16.6 ± 1.4	53.9 ± 3.0	4.1 ± 0.8	13.3 ± 1.1	4.0 ± 0.3	0.9 ± 0.4	0.3 ± 0.3
150 mg	0.2 ± 0.1	2.4 ± 1.0	3.7 ± 0.4	15.5 ± 1.8	55.3 ± 1.6	4.7 ± 0.9	11.3 ± 0.8	3.9 ± 0.3	1.6 ± 0.1	0 ± 0
2 Weeks										
20 mg	–	1.4 ± 0.4	4.8 ± 0.4	25.1 ± 0.7	50.4 ± 0.7	4.6 ± 0.3	7.9 ± 0.4	3.9 ± 0.3	1.3 ± 0.2	0.1 ± 0.1
50 mg	0.4 ± 0.2	2.8 ± 0.3	3.9 ± 0.5	22.2 ± 1.9	50.0 ± 1.4	4.5 ± 0.3	10.4 ± 1.1	3.8 ± 0.4	0.6 ± 0.2	0.1 ± 0.1
100 mg	0.1 ± 0.1	2.9 ± 0.6	3.2 ± 0.2	17.4 ± 1.2	52.4 ± 0.8	3.9 ± 0.4	13.4 ± 0.8	4.5 ± 0.3	0.8 ± 0.4	0.1 ± 0.1
150 mg	0.4 ± 0.1	4.3 ± 0.3	3.3 ± 0.1	16.3 ± 0.4	50.4 ± 3.3	5.5 ± 0.8	12.4 ± 0.3	5.1 ± 0.3	1.1 ± 0.2	0.2 ± 0.2

[a]DH = 4,4'-bis(β-diethylaminoethoxy)-α,β-diethyldiphenylethane, BPA = bisphosphatidic acid (tentatively identified), BMGP = bis(monoacylglyceryl)phosphate, CL = cardiolipin, PE = phosphatidylethanolamine, PC = phosphatidylcholine, Sph = sphingomyelin, PI = phosphatidylinositol, PS = phosphatidylserine, LPC = lysophosphatidylcholine, PA = phosphatidic acid.

TABLE III

Effects of Increasing Doses of DH[a] on the Lipid Composition of Rat Kidney

Dose and period	Weight of both kidneys (g)	Phospholipid	Free sterol	Esterified sterol	Triglyceride
Control	1.9 ± 0.1	2.48 ± 0.05 (%)[b]	0.40 ± 0.03 (%)	0.02 ± 0.02 (%)	0.55 ± 0.12 (%)
1 Week					
50 mg	1.9 ± 0.1	2.24 ± 0.15	0.37 ± 0.12	0.02 ± 0.01	0.37 ± 0.12
100 mg	2.2 ± 0.2	3.03 ± 0.13	0.40 ± 0.02	0.05 ± 0.03	0.97 ± 0.12
150 mg	2.2 ± 0.3	3.67 ± 0.71	0.51 ± 0.11	0.02 ± 0.02	0.55 ± 0.13
2 Weeks					
50 mg	1.9 ± 0.1	2.76± 0.35	0.53 ± 0.07	0.02 ± 0.02	0.74 ± 0.07
100 mg	2.1 ± 0.2	3.07± 0.18	0.51 ± 0.03	0.05 ± 0.02	0.37 ± 0.07
150 mg	2.6 ± 0.3	3.46± 0.18	0.54 ± 0.04	0.03 ± 0.02	0.41 ± 0.07

[a]DH = 4,4'-bis(β-diethylaminoethoxy)-α,β-diethyldiphenylethane.

[b]Percent of fresh weight of tissues.

TABLE IV

Effects of Increasing Doses of DH on the Phospholipid Composition of Rat Kidney[a]

Dose and period	BPA	BMGP	CL	PE	PC	Sph	PI	PS	LPC	PA
Control	–	0.1 ± 0.1	5.8 ± 0.1	28.8 ± 0.3	36.0 ± 0.4	14.1 ± 0.5	4.9 ± 0.4	8.2 ± 0.4	1.1 ± 0.2	0.4 ± 0.3
					(Percent of total phospholipids)					
1 Week	0.0									
50 mg	0.3 ± 0.1	0.9 ± 0.2	6.9 ± 0.3	29.4 ± 0.8	31.1 ± 1.5	14.8 ± 0.8	5.4 ± 0.7	7.1 ± 0.5	1.6 ± 0.2	1.3 ± 0.2
100 mg	–	0.9 ± 0.4	6.6 ± 0.3	27.7 ± 0.7	35.8 ± 0.8	13.9 ± 1.1	6.0 ± 0.7	7.1 ± 0.5	0.7 ± 0.2	0.6 ± 0.5
150 mg	0.2 ± 0.1	1.5 ± 0.5	5.6 ± 0.2	28.8 ± 0.7	35.0 ± 1.0	13.3 ± 0.9	6.7 ± 0.4	6.0 ± 0.7	1.1 ± 0.2	0.4 ± 0.3
2 Weeks										
50 mg	0.1 ± 0.1	1.1 ± 0.6	6.0 ± 0.4	28.5 ± 0.4	36.2 ± 3.0	12.7 ± 1.2	6.1 ± 0.3	7.6 ± 0.3	0.6 ± 0.3	0.4 ± 0.3
100 mg	0.2 ± 0.2	0.7 ± 0.1	5.7 ± 0.4	26.4 ± 0.8	36.3 ± 0.3	12.6 ± 0.6	7.0 ± 0.5	7.4 ± 0.3	0.9 ± 0.2	0.7 ± 0.3
150 mg	–	1.2 ± 0.5	4.8 ± 0.2	24.9 ± 0.9	39.8 ± 0.9	12.0 ± 0.3	7.9 ± 0.4	7.0 ± 0.4	1.0 ± 0.1	0.1 ± 0.1

[a]DH = 4,4'-bis(β-diethylaminoethoxy)-α,β-diethyldiphenylethane, BPA = bisphosphatidic acid (tentatively identified), BMGP = bis(monoacylglyceryl)phosphate, CL = cardiolipin, PE = phosphatidylethanolamine, PC = phosphatidylcholine, Sph = sphingomyelin, PI = phosphatidylinositol, PS = phosphatidylserine, LPC = lysophosphatidylcholine, PA = phosphatidic acid.

TABLE V

Effects of Chloroquine, Homochlorocyclizine, Prenylamine, and Diazacholesterol on the Phospholipid Composition of Rat Liver

	Control	Chloroquine	Homochloro-cyclizine	Prenylamine	Diaza-cholesterol
Total phospholipids[a]	3.1 ± 0.2	4.8 ± 1.2	3.5 ± 0.3	2.6 ± 0.5	3.2 ± 0.1
BMGP	0.0	3.2 ± 0.9	1.1 ± 0.4	0.9 ± 0.2	2.7 ± 0.6
CL	5.9 ± 0.3	3.6 ± 0.3	3.5 ± 0.4	4.7 ± 0.2	3.8 ± 0.6
PE	26.8 ± 0.6	22.8 ± 1.0	20.4 ± 0.4	28.7 ± 1.0	22.6 ± 0.3
PC	50.3 ± 1.3	47.0 ± 1.2	56.6 ± 0.9	48.7 ± 1.5	55.2 ± 1.4
Sph	4.9 ± 0.2	4.5 ± 0.5	3.6 ± 0.5	5.9 ± 1.2	3.0 ± 0.1
PI	7.3 ± 0.6	10.8 ± 1.3	9.3 ± 0.5	6.5 ± 1.3	8.3 ± 1.2
PS	3.7 ± 0.5	4.8 ± 0.1	3.0 ± 0.5	3.2 ± 0.2	3.5 ± 0.3
LPC	0.6 ± 0.2	1.4 ± 0.4	1.6 ± 1.0	0.8 ± 0.2	0.7 ± 0.2
PA	0.1 ± 0.1	0.4 ± 0.2	0.6 ± 0.2	0.4 ± 0.2	0.3 ± 0.1

[a]BMGP = bis(monoacylglyceryl)phosphate, CL = cardiolipin, PE = phosphatidylethanolamine, PC = phosphatidylcholine, Sph = sphingomyelin, PI = phosphatidylinositol, PS = phosphatidylserine, LPC = lysophosphatidylcholine, PA = phosphatidic acid.

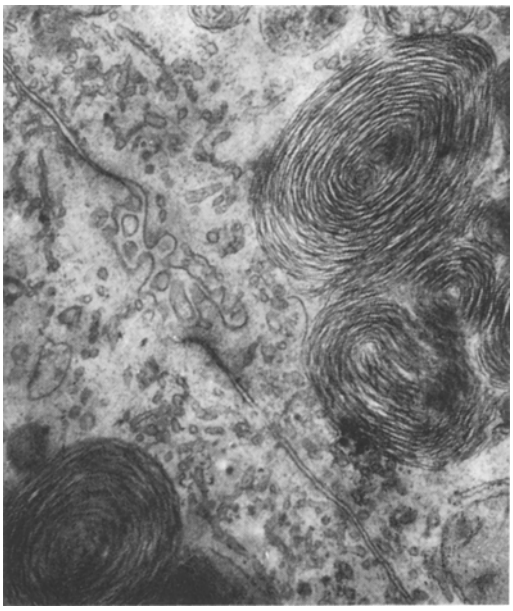


FIG. 1. Electron micrograph of liver parenchymal cells of a chloroquine-treated rat.

much as the dose of homochlorocyclizine and chloroquine in the present experiment.

DISCUSSION

Our previous experiments showed that the administration of DH at a level of 20 mg/kg body weight per day resulted in a significant increase ($P<0.001$) in a peculiar acidic glycerophospholipid, BMGP, in rat liver and spleen (4,5). There was a good correlation between the length of the period of the drug administration and the concentration of BMGP in liver and spleen. However, in contrast to the finding in human cases (1), hepatosplenomegaly did not take place in rats by using this dose level of DH, which was ca. 7 times as much as the dose used for human cases. The increase in PI was never observed in rat liver in previous experiments (5). The increase in this phospholipid was observed in human liver at an early stage of the drug-induced lipidosis (1).

By the oral administration of increasingly large doses of DH in the present experiment, an enlargement of the liver with a marked increase in free cholesterol as well as significant increases ($P<0.001$) in both acidic phospholipids, BMGP and PI, took place in rats and showed a feature very similar to the one observed in human cases. The increases in these lipid components were clearly dependent upon the dose level as well as the length of the period of drug administration, but still there is a problem that could not be solved in this experiment. Even by giving DH at a very high level (150 mg/kg) that resulted in a marked hepatomegaly and a marked increase in total phospholipids, the increase in BMGP was less prominant than in the previous experiment (5) in which DH was given at a smaller dose for a longer period. Among the two acidic phospholipids, the response of PI was much faster than BMGP when the drug was given at a larger dose. Rapid response of PI is also a pattern of change in phospholipid composition in human cases and in monkeys (Matsuzawa et al., in preparation). It seems probably that the increase in BMGP takes the place of PI when the concentration of the drug in some peculiar cell organella is increased to a very high level. This hypothesis will explain the fact that BMGP increased slowly without any change in PI when the drug was given at a lower dose level.

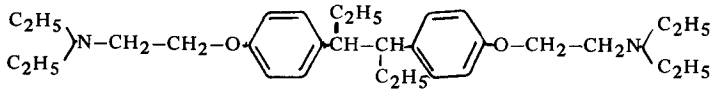
It must also be noted in the present experiment that cholesteryl esters were markedly increased in rat liver; this has never been experienced in our previous experiments and in human cases (1,5). It is likely that the synthesis of cholesterol was enhanced in rat liver to a much greater extent than needed for the formation of membranous structure, or the increase in cholesteryl ester could be due to the fact that rats can esterify cholesterol much more rapidly than humans.

In contrast to liver, there was not such a significant increase in cholesterol in rat kidney. Normal kidneys of mammalians are rich in cholesterol, almost all of which is in the free state (11,12). It was also reported that the turnover rate of cholesterol in kidney is very slow compared with cholesterol in liver (13). This must be one of the reasons why there was no significant increase in cholesterol in kidney at an early stage of the DH treatment. Another experiment in which DH was given at a dose of 20 mg/kg for a longer period of time (8 weeks) showed an increase in free cholesterol in rat kidney (14). The increase in total phospholipids and BMGP was also less conspicuous than in liver. The difference between two tissues seems to be related both to the amount of the drug stored in tissue cells and the rate of synthesis of phospholipids.

Hruban et al. (15) reported the appearence of myelin-like inclusions in liver of rats which were given chloroquine, homochlorocyclizine, and some other drugs. Lüllmann-Rauch et al. (16,17) reported the appearance of abnormal lamellated cytoplasmic inclusions after oral administration of an anorectic drug (chlorphentermine) and various tricyclic antidepressants. Lüllmann-Rauch showed that the introduction

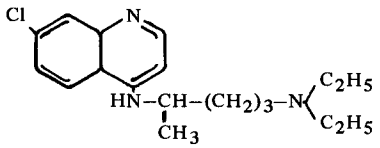
TABLE VI

Molecular Structures of Compounds Investigated



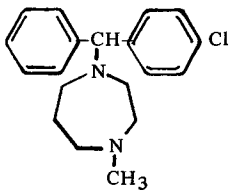
DH

4,4′-bis(β-diethylaminoethoxy)-α,β-diethyldiphenylethane



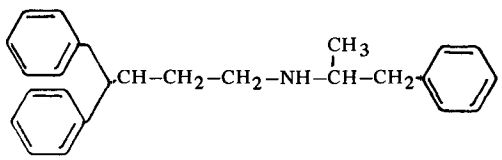
Chloroquine

7-chloro-4-(4-diethylamino-1-methylbutylamino)-quinoline



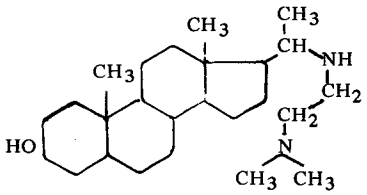
Homochlorocyclizine

1-(p-chloro-α-phenylbenzyl)hexahydro-4-methyl-1H-1,4-diazepine



Prenylamine

N-(3,3-diphenylpropyl)-α-methylphenethylamine



Diazacholesterol

of a chlorine atom in 1 (para)-position enhances the apolarity of the aromatic ring system, producing a compound of higher amphilphilia, which is more potent in inducing a lipidosis. Our experimental results showed that the increase in acidic phospholipids (BMGP and PI) occurred at the same time as the appearence of lamellated cytoplasmic inclusions. It was shown that chloroquine was just as effective as DH in increasing total phospholipids and BMGP plus

PI in rat liver as well as in the formation of cytoplasmic inclusions (Fig. 1). Diazacholesterol seems to be as effective as these two compounds. These compounds which caused a marked increase in acidic phospholipids contain nitrogen base and migrate on silica gel thin layer chromatography close to each other and not very far from sphingomyelin by using chloroform:acetone:methanol:acetic acid:water, 100:40:30:20:12, as a developing solvent. However, there is no structural similarity in molecular organization of these chemical compounds (Table VI). Similarity in physicochemical characteristics which presents a proper amphiphilic nature leading to the induction of the myelin-like inclusions should be investigated in the future.

BMGP is a positional isomer of phosphatidylglycerol in which each of the two glycerol moieties is esterified with only one fatty acid (18-20). The phospholipid was identified by Gray and Body in pig lung (18) and also shown in the tissue of Niemann-Pick disease by Rouser et al. (19) and Seng et al. (20). The phospholipid is a minor component in usual tissues, but it was found by us that this phospholipid shows a slight increase up to 4% in liver of hyperlipemia and lipodystrophy (10). The present experimental results showed that the increase in BMGP takes place when drugs of amphiphilic nature are given in large amount and the lamellated cytoplasmic inclusions are formed in tissue cells. Wherret and Hutterer (21) isolated lysosome fraction from rat liver homogenate after treatment with Triton and detected BMGP in relatively high concentration. The results obtained in this laboratory showed that there is a curvilinear correlation between the concentration of the drug accumulating in liver and the concentration of acidic phospholipids (Matsuzawa et al., in preparation). Acidic phospholipids, BMGP and PI, are probably needed for binding the alkaline substance with amphilphilic nature to the membranous structure in cytoplasm. Works are now in progress to follow the difference in the role of these two phospholipids.

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Age-related Changes in the Lipid Metabolism of Fisher 344 Rats

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ABSTRACT

Lipid metabolism of male Fisher 344 rats aged 2-24 months was studied. Serum and liver cholesterol levels did not display the age-related gradual increase seen in other rat strains. An increase in the serum plus liver cholesterol pool from 2 to 6 months was followed by a plateau through 18 months and then another increase at 24 months of age. The triglyceride pool increased from 2 to 6 months and then remained unchanged through 24 months of age. Cholesterol synthesis from acetate decreased 50% between 2 and 9 months and fell only slightly through 24 months of age. Assay of 3-hydroxy-3-methyl glutaryl Coenzyme A (HMG-CoA) reductase showed a similar pattern but did not decrease further after 9 months of age. Cholesterol 7α hydroxylase activity was not significantly altered by age. These age- and strain-related differences present an opportunity for a comparative study of the aging process using the parameters of lipid metabolism as indicators.

INTRODUCTION

Age-related increases in the levels of several classes of lipids have been reported. Carlson et al. (1) reported increases in plasma cholesterol, triglycerides, and phospholipids in Sprague-Dawley rats aged from 1 to 18 months. In a comparison of age-related lipid changes in various strains of rats (2), a randomly bred strain (Wistar) showed increased serum cholesterol with advancing age while inbred strains (Lewis, BN, and DA) did not. Dupont et al. (3) have shown an increase in serum cholesterol in both male and female rats of the CFE strain. Serum cholesterol levels increased gradually up to 12 months and then increased ca. 100% between 12 and 18 months of age.

It has been suggested that changes in lipid levels arise from an age-related decrease in all phases of lipid metabolism. Yamamoto and Yamamura (4) have reported decreases in rats in hepatic cholesterogenesis in vitro, in acetate incorporation into serum and hepatic cholesterol in vivo, in bilary and fecal excretion of cholesterol, and in the absorption of dietary cholesterol. Hruza and Wachtlova (5) reported increased time for clearance of a single intravenous injection of tritiated cholesterol.

The present study examines the lipid metabolism of the Fisher 344 strain of rat, a strain used extensively in aging studies.

METHODS AND MATERIALS

Male Fisher 344 strain rats were obtained from Charles River Breeding Laboratories, Inc., North Wilmington, MA. All animals were cesarean-derived and barrier-reared by Charles River and fed diets of guaranteed composition and source. The 50% mortality age is ca. 28-29 months for the Fisher 344 rats, with the maximum lifespan being 45+ months. Sprague-Dawley rats, raised under identical conditions, have 31-32 month 50% mortality and 48+ month maximum age. In Experiment 1, groups of 6 rats at ages 2, 6, 12, 18, and 24 months were used. Experiment 2 comprised groups of 6 rats of 2, 9, and 12 months and one group of 5 rats of 24 months of age. The animals were fasted 12 hr before being killed by decapitation and blood and liver samples taken for analysis. Animals in experiment 1 were killed at ca. 11 p.m. in an attempt to maximize the cholesterol 7α hydroxylase and 3-hydroxy-3-methyl glutaryl Coenzyme A (HMG-CoA) reductase levels. Experiment 2 was conducted at 10 a.m. Serum cholesterol and triglycerides were determined by the methods of Pearson et al. (6) and Levy and Keyloun (7), respectively. Aliquots of liver were homogenized with chloroform:methanol (2:1) for extraction and subsequent determination of cholesterol (6) and triglycerides (7).

For lipid biosynthesis experiments, 0.5 g slices of liver were incubated under oxygen at 37 C for 3 hr in 5 ml phosphate buffer (pH 7) containing 0.6 mM $MgCl_2$, 30 mM nicotinamide and 0.5 μCi [1-^{14}C]-acetate. The reaction was stopped by addition of 15% alcoholic KOH. Cholesterol was extracted from the saponified sample, isolated as the digitonide, and the mass and radioactivity of cholesterol were determined (8,9). The aqueous residue was acidified to pH 1, and fatty acids were extracted into ether and counted directly.

Cholesterol 7α hydroxylase and HMG-CoA reductase activities in liver microsomal suspensions were measured as previously described (10-12). Liver microsomal preparations were

made using a solution of 0.3 M sucrose, 0.075 M nicotinamide, 0.002 M ethylenediaminetetraacetic acid, and 0.02 M mercaptoethanol. For assay of cholesterol 7α hydroxylase activity, 0.2 ml of the microsomal suspension was incubated with [1, 2-^{3}H]-cholesterol (0.15 μmol, 1 μCi/μmol) and solubilized in phosphate buffer (pH 7.4) with 2 mg Tween 20 and a reduced nicotinamide adenine dinucleotide phosphate (NADPH) generating system. After incubation at 37 C for 30 min, the reaction was stopped by the addition of 7.5 ml methylene chloride:ethanol (5:1). Steroids were separated by thin layer chromatography (TLC) on Silica Gel G with ethyl acetate: hexane (8:2). The bands were visualized with iodine vapor, scraped from the plates, and assayed for radioactivity by liquid scintillation spectrometry. Assay of HMG-CoA reductase was carried out using 0.2 ml of microsomal suspension with [3-^{14}C] HMG-CoA (0.2 mM, 3.75 μCi/mM) in phosphate buffer (pH 7.4) with a NADPH generating system. After incubation at 37 C for 30 min, the reaction was stopped by addition of 0.4 ml in H_2SO_4 and incubated at 37 C for 1 hr to lactonize all the mevalonic acid which had been formed. Carrier [2-^{3}H]-mevalonolactone (3 mg; 3.2 x 10^5 dpm) was added and the mevalonolactone then extracted with ether and isolated by TLC on Silica Gel G developed in acetone:benzene (1:1). The radioactivity in the nevalonolactone was determined and the tritium radioactivity used to determine the efficiency of extraction procedures, which was used to correct the ^{14}C radioactivity and to calculate the activity of the enzyme, since the specific activity of the substrate was known.

The [1, 2-^{3}H]-cholesterol, [1-^{14}C]-acetate, and [3-^{14}C]-HMG-CoA were purchased from New England Nuclear Corporation, Boston, MA. The [2-^{3}H]-mevalonolactone was purchased from Amersham/Searle Corporation, Arlington Heights, IL. The 1, 2-^{3}H]-cholesterol and [3-^{14}C]-HMG-CoA were checked for purity by TLC and paper chromatography, respectively.

RESULTS

Tables I and II contain the body and liver weight and serum and liver lipid data from two experiments.

Body weights did not increase significantly after 12 months of age in either experiment. Liver weight increased slowly through 12 months of age and continued to get larger from 12 months through 24 months while body weight remained constant, resulting in a relative increase in liver weight per unit body weight.

Serum cholesterol levels were fairly constant through 18 months and then increased by 61%. Differences in serum cholesterol levels observed between Experiments 1 and 2 can largely be accounted for by variation in time of year (January vs. June), time of day (11 p.m. vs 10 a.m.), and the laboratory variation in serum cholesterol determinations. We have no ready explanation for this discrepancy. However, comparison of the cholesterol levels among age groups in each experiment shows that the ratios relative to the 2-month group were the same in both experiments. Thus, in Experiment 1, if the 2-month group cholesterol level is considered to be 1.00, then the comparable levels for the 12- and 24-month groups are 1.02 and 1.66, respectively. In the second experiment, the ratios for the 12- and 24-month groups relative to the 2-month groups are 0.98 and 1.87, respectively. Little or no change was observed in liver cholesterol levels. However, when serum plus liver cholesterol pool levels were calculated using estimated of serum volume and liver weights, a much different pattern emerged. A two-fold increase through 9 months is followed by a plateau through 18 months and then another increase to 272% of the 2-month level. (Cholesterol and triglyceride pools were calculated using 3% of body weight as the estimate for serum volume. This proportion of serum volume may change with age. However, this calculation is only an estimate of a nonhomogeneous, rapidly exchanging pool which helps account for differences in liver and body weight.)

Serum triglycerides did not change until 18 months, when they increased 40% and remained at that level. Liver triglyceride concentrations increased significantly (160%) ($P<0.001$) through 9 months and then dropped steadily between 9 and 24 months, at which point they were 60% higher than the 2-month levels. When body size differences were accounted for in the triglyceride pool levels, an increase in total triglycerides was observed through 9 months. Levels reached a plateau at 18 months at about 3 times the 2-month level.

Cholesterol and fatty acid synthesis in vitro by liver slices are shown in Table III. Cholesterol synthesis from acetate decreased significantly ($P<0.05$) from 2 to 9 months and then did not change significantly through 24 months. Fatty acid synthesis from acetate was not affected by age.

Levels of liver microsomal HMG-CoA also decreased from 2 to 9 months and then stabilized at that level through 24 months of age.

Liver microsomal cholesterol 7α hydroxylase

TABLE I

Serum and Liver Lipids of Fisher Rats of Different Ages (Exp. 1)[f]
(Mean ± SEM)

	2 Months	6 Months	12 Months	18 Months	24 Months
Body weight (G)	230 ± 3	376 ± 7[a]	430 ± 13[ab]	430 ± 10[ab]	430 ± 10[ab]
Liver weight (g)	7.7 ± 0.1	11.1 ± 0.4[a]	11.6 ± 0.7[a]	12.7 ± 0.6[ab]	14.6 ± 0.9[abc]
Liver (% body weight)	3.36 ± 0.03	2.94 ± 0.11[a]	2.70 ± 0.10[a]	2.95 ± 0.08[a]	3.38 ± 0.19[c]
Cholesterol					
Serum (mg/dl)	46.2 ± 6.7	37.0 ± 4.7	47.3 ± 3.6	53.0 ± 8.1	76.8 ± 13.0[b]
Liver (mg/100 g)	345 ± 19	365 ± 21	375 ± 20	383 ± 21	375 ± 16
Pool (mg)[e]	29.7 ± 1.9	44.9 ± 3.7[a]	49.6 ± 3.5[a]	55.4 ± 3.3[a]	64.4 ± 4.7[abc]
Triglycerides					
Serum (mg/dl)	133 ± 36	123 ± 18	126 ± 23	186 ± 22[b]	251 ± 42[bc]
Liver (mg/100 g)	142 ± 3	211 ± 7[a]	254 ± 24[a]	267 ± 20[ab]	174 ± 7[abcd]
Pool (mg)[e]	18.3 ± 1.3	37.2 ± 2.9[a]	45.9 ± 5.6[a]	54.4 ± 3.8[ab]	57.3 ± 5.0[ab]

[a]Significantly different (at least $P<0.05$) from 2-month animals.
[b]Significantly different (at least $P<0.05$) from 6-month animals.
[c]Significantly different (at least $P<0.05$) from 12-month animals.
[d]Significantly different (at least $P<0.05$) from 18-month animals.
[e]Serum plus liver pool estimated for total liver weight and serum volume.
[f]Six animals per group.

TABLE II

Serum and Liver Lipids of Fisher Rats of Different Ages (Exp. 2)[c]
(mean ± SEM)

	2 Months	9 Months	12 Months	24 Months
Body weight (g)	208 ± 7	386 ± 8[a]	405 ± 10[a]	426 ± 12[ab]
Liver weight (g)	7.3 ± 0.4	10.8 ± 0.2[a]	11.7 ± 0.2[ab]	14.4 ± 1.7[ab]
Liver (% body weight)	3.50 ± 0.10	2.81 ± 0.07[a]	2.89 ± 0.07[a]	3.35 ± 0.22[b]
Cholesterol				
Serum (mg/dl)	105 ± 8	106 ± 5	103 ± 8	196 ± 48
Liver (mg/100 g)	240 ± 6	318 ± 10[a]	346 ± 18[a]	372 ± 15[a]
Pool (mg)	24.1 ± 1.7	46.7 ± 1.4[a]	53.2 ± 3.5[a]	78.7 ± 11.4[ab]
Triglycerides				
Serum (mg/dl)	287 ± 46	264 ± 39	279 ± 55	296 ± 48
Liver (mg/100 g)	166 ± 10	432 ± 44[a]	400 ± 50[a]	321 ± 46[a]
Pool (mg)	30.0 ± 3.1	77.2 ± 6.5[a]	80.7 ± 8.7[a]	84.1 ± 14.4[a]

[a]Significantly different (at least $P<0.05$) from 2-month animals.

[b]Significantly different (at least $P<0.05$) from 9-month animals.

[c]Six rats in age groups 2, 9, and 12 months, and 5 rats aged 24 months.

TABLE III

Lipid Synthesis in Vitro by Liver Slices from Fisher Rats at Different Ages[b]
(mean ± SEM)

	2 Months	9 Months	12 Months	24 Months
Cholesterol synthesis from acetate (dpm/mg cholesterol (x 10^3)	14.20 ± 2.04	7.68 ± 1.52[a]	4.34 ± 0.61[a]	5.19 ± 1.30[a]
HMG-CoA reductase[c] (pmol/mg protein/min)	85.7 ± 11.1	43.1 ± 24.6	38.5 ± 8.4[a]	42.6 ± 6.7[a]
Fatty acid synthesis from acetate (total dpm) (x 10^4)	1.83 ± 0.16	2.73 ± 0.49	2.04 ± 0.26	2.85 ± 0.61

[a]Significantly different (at least $P<0.05$) from 2-month animals.

[b]Data from animals in Exp. 2.

[c]HMG-CoA = 3-hydroxy-3-methyl glutaryl Coenzyme A.

TABLE IV

Cholesterol 7α Hydroxylase Activity of Aging Fisher Rats[a]
(mean ± SEM)

	2 Months	6 Months	12 Months	18 Months	24 Months
Cholesterol 7α hydroxylase (pmol/mg protein/min)	52.5 ± 10.2[a]	51.6 ± 7.1	42.6 ± 3.7	44.9 ± 4.2	47.7 ± 6.2

[a]Data from animals in Exp. 1.

activity results are shown in Table IV. Although there was a slight decrease in such activity with age, the differences were not significant.

DISCUSSION

Changes in weight and lipid levels of the Fisher 344 rat are much different than those reported for other strains of rats. Both Wistar and Sprague-Dawley rats gain weight throughout their lifespan and reach maxima usually greater than the 430 g observed in these experiments (3-5,13-15). Accumulated data on lipid levels has been restricted to observations in animals 12 months of age and younger. Serum and liver lipid concentrations have been observed to increase slightly but not always significantly with age (1-4). However, when the increases in serum volume and liver weight are accounted for, these increases become much more significant. The most noticeable difference observed with the Fisher 344 rat was the difference in the pattern of weight gain and increases in lipids. Earlier reports in Sprague-Dawley and

Wistar rats have always indicated a gradual increase in body weight and lipids throughout the lifespan. The fisher rats, however, seem to gain weight rapidly up to 12 months and maintain that weight through 24 months. Cholesterol levels in serum and liver do not change appreciably until 24 months of age. When effects of serum volume and liver weight are included, cholesterol levels increase from 2 to 9 months, level off through 18 months, and then increase at 24 months. Serum and liver triglyceride levels also respond independently, with liver reaching a maximum early and serum late in the lifespan. Triglyceride pool levels seem to closely parallel changes in body weight. Examination of these variables in rats older than 24 months of age to determine if cholesterol levels reach another plateau and if triglycerides maintain the observed plateau would be most interesting.

Zucker and Zucker (16,17) have suggested that liver lipid levels are better correlated with body weight than with age. Correlation coefficients calculated from our data in Fisher 344 rats indicated a significant correlation between cholesterol pool and age ($r = 0.79$) or body weight ($r = 0.77$). Triglyceride pools were also well correlated with age ($r = 0.79$) and body weight ($r = 0.83$). All of these correlation coefficients were significantly different from zero ($P << 0.001$), but no better correlation was observed with body weight than with age.

The decreases in cholesterol synthesis from acetate confirm earlier reports (4,18,19). The strain difference was again observed in that all cholesterol synthesis and HMG-CoA reductase measurements decreased from 2 to 9 months and then remained relatively constant through 24 months. Earlier reports have indicated a more gradual decrease with advancing age. Measurement of HMG-CoA reductase had not been carried out in previous experiments with aging rats. These strain differences need further examination since all previous work mentioned involved animals of no more than 12 months of age.

Hruza and Zbuzkova (15) observed decreased excretion of an injected dose of tritiated cholesterol in the feces of old rats (12 months). This would suggest a decrease in bile acid synthesis which would in turn suggest a decrease in cholesterol 7α hydrolase activity, the rate limiting step in bile acid synthesis. In a study with Wistar rats (20), cholesterol 7α hydroxylase in 18 month rat liver preparations was 68% of that observed in preparations from 2-month-old rats. Strain differences could explain the lack of such an alteration with age in the Fisher 344 rats. However, the increase in excretion could also be explained by differences at another level, e.g., lower substrate availability as a result of lowered cholesterol synthesis or inhibition of bile acid reabsorption from the intestine. Again, an elucidation of strain differences would help explain this lack of agreement.

Lipid metabolism in the Fisher 344 rat differs quite markedly from that in other strains of rats. Alterations in cholsterol synthesis and degradation cannot fully explain the observed changes in serum and liver lipid levels. Further study of all facets of lipid metabolism in Fisher 344, as well as other rat strains, is needed to fully understand the mechanism of these age- and strain-related differences. Such information is basic to a determination of the underlying causes for the strain differences in lipid metabolism in rats.

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A Study of the Heparin-Manganese Chloride Method for Determination of Plasma α-Lipoprotein Cholesterol Concentration

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ABSTRACT

To assess the limits of the heparin-$MnCl_2$ precipitation method for quantitation of α-lipoprotein cholesterol (C-HDL), effects of varying final $MnCl_2$ and heparin concentrations were studied, and the precipitation method was compared to preparative ultracentrifugation. In 65 parallel plasma aliquots, C-HDL ($\bar{X} \pm$ SE) determined by ultracentrifugation (54.3 ± 1.8 mg/dl) correlated significantly ($r = 0.98$, $P < 0.001$) with the precipitation method (56.0 ± 1.9 mg/dl). C-HDL by ultracentrifugal and precipitation methods were also similar in 16 subjects with triglycerides ranging from 150 to 312 mg/dl (41.4 ± 2.6, 43.4 ± 2.8, $r = 0.97$, $P < .001$). A constant amount of cholesterol in the supernatant was measured over a final $MnCl_2$ range of 0.046-0.23 *M*, and cholesterol values in the supernatant at final $MnCl_2$ concentrations of 0.046, 0.05, and 0.055 *M* did not differ from each other, $P > 0.1$. However, cholesterol levels in the supernatant at final $MnCl_2$ concentration of 0.042 *M* differed from those at concentrations of 0.046, 0.05, and 0.055 *M*, $P < 0.05$, and the amount of supernatant cholesterol increased as the final $MnCl_2$ concentration was reduced from 0.042 to 0.02 *M*. A constant amount of cholesterol in the supernatant was measured over a heparin concentration range of 92-734 USP units/ml. The final $MnCl_2$ and heparin concentrations of 0.046 *M* and 184 USP units/ml, which are incorporated in widely used procedures, gave C-HDL values for the precipitation method which were in close agreement with the ultracentrifugal method. There is no evidence for a heparin-Mn++ precipitation of HDL and systematic underestimation of HDL by the precipitation method. However, the final $MnCl_2$ concentration is very near the minimum required for accurate measurement of C-HDL. To preclude incomplete precipitation of low and very low density lipoproteins by insufficient manganese concentration, an increase of the manganese concentration should be considered.

INTRODUCTION

A number of recent reports reinforce the importance of accurate measurement of C-HDL. In several early studies of lipoproteins (1-4), it was realized that low levels of α-lipoprotein cholesterol (C-HDL) and elevated levels of β-lipoprotein cholesterol (C-LDL) were associated with premature atherosclerosis. Miller and Miller (5) and Castelli et al. (6) proposed that low plasma HDL concentration, independent of C-LDL or total cholesterol concentration, may be associated with an increased incidence rate of coronary heart disease. In familial hyperalpha-lipoproteinemia, probands and affected relatives had distinctive elevations of C-HDL with no elevations of C-LDL; kindred members demonstrated elongated life expectancy and apparent rarity of premature cardiac events (7,8).

C-HDL has generally been measured using either the heparin-manganese precipitation method of Burstein and Samaille (9) or as the lipoprotein cholesterol after ultracentrifugation of plasma at density 1.063 g/ml (10). The heparin-manganese method measures the cholesterol content of the supernatant following precipitation of low and very low density lipoproteins, and this quantity is often referred to as α-lipoprotein cholesterol (an electrophoretic term) or as high density lipoprotein cholesterol (an ultracentrifugal term). Measurement of the cholesterol content of the supernatant is the most commonly used method of quantifying (this supernatant), although the apolipoprotein concentration could also be quantitated as another approach. The ultracentrifugal fraction ($d > 1.063$ g/ml) includes not only high density lipoprotein but also very high density lipoproteins (V-HDL). The cholesterol content of both HDL and V-HDL is measured by the ultracentrifugal methods (10) used comparatively in this study.

[1]A portion of this work was supported by General Clinical Research Center Grant No. RR00068-14, and a portion of it was done during Dr. Glueck's tenure as an Established Investigator of the American Heart Association, 1971-1976.

The accurate measurement of C-HDL is essential for the indirect determination of C-LDL by several ultracentrifugation techniques (10-12) and by the Friedewald formula (C-LDL = total cholesterol - C-HDL - triglyceride/5). Recently Srinivasan et al. (13) have suggested that heparin and high density lipoproteins interact in the presence of Mn^{++}, with an overall effect that would lead to the precipitation of some C-HDL and a resultant underestimation of C-HDL after heparin-$MnCl_2$ precipitation. An additional problem with the heparin-$MnCl_2$ precipitation method also appears when the plasma triglycerides are elevated (usually >300 mg/dl), and a "floating precipitate" appears in the supernate after addition of heparin and manganese, providing an overestimation of C-HDL by the precipitation method as compared to d>1.063 g/ml ultracentrifugation.

In the development of a micro-gas liquid chromatography method to measure C-HDL (14), it was noted that minute reductions of the final $MnCl_2$ concentrations below 0.046 *M* led to incomplete precipitation of LDL and VLDL. The current study was designed to assess effects of varying heparin and manganese chloride concentrations on C-HDL quantitation and to provide a comparison of C-HDL measured by ultracentrifugation and precipitation (9-11).

MATERIALS AND METHODS

Subjects

Fasting plasma samples from 65 subjects studied in the outpatient section of the General Clinical Research Center were obtained using vacutainers containing crystalline disodium ethylenediaminetetraacetic acid (EDTA), and plasma was separated following the Lipid Research Clinic Protocols (11). Plasma cholesterol and triglyceride levels ranged, respectively, from 137 to 325 and from 43 to 312 mg/dl.

To assess the effects of varying the final concentration of $MnCl_2$ and/or heparin on the completeness of the precipitation of LDL and VLDL, seven normal plasma pools with triglycerides <200 mg/dl and five lipemic plasma pools with triglyceride >200 mg/dl were used (Fig. 1-4).

Comparison of C-HDL Quantitation by Heparin-$MnCl_2$ Precipitation and Ultracentrifugation

To assess the accuracy of the heparin-$MnCl_2$ precipitation method (9-11), duplicate aliquots of 65 plasma samples were processed in parallel, with quantitation of C-HDL by precipitation and ultracentrifugation (11) (Table I). The ultracentrifugal method was arbitrarily desig-

TABLE I

Comparison of C-HDL Measurement[a] between Manganese-Heparin Precipitation and Ultracdntrifugation Fraction >1.063 g/ml

	N	Mean	SD	SE	Pearson-r	P	Regression equation[b]	
							a (slope)	b (intercept)
Plasma samples								
Fraction >1.063 g/ml	65	54.3	14.3	1.8	0.98	<0.001	1.062	-1.65
Mn:Heparin		56.0	15.5	1.9				
Elevated C-HDL (>70 mg/dl)								
Fraction >1.063 g/ml	14	74.6	9.3	2.5	0.94	<0.001	1.031	+0.46
Mn:Heparin		77.4	10.3	2.7				
Plasma triglyceride (150-312 mg/dl)								
Fraction >1.063 g/ml	16	41.4	10.1	2.6	0.97	<0.001	1.041	-0.21
Mn:Heparin		43.4	10.8	2.8				

[a]Cholesterol measured by Technicon AA-II method (11).
[b]General regression equation: Y = aX + b.

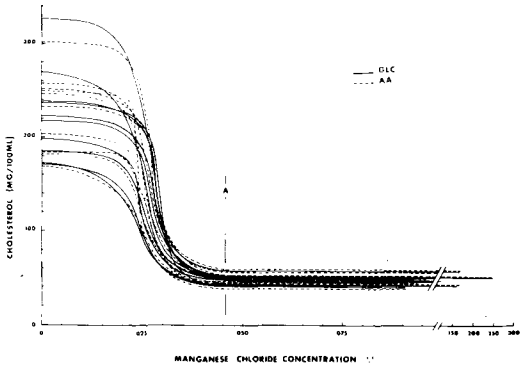

FIG. 1. Concentrations of cholesterol in the supernatant after precipitation of LDL and VLDL by heparin:manganese. Final heparin concentration constant at 184 USP units/ml, final $MnCl_2$ reagent concentration varied from 0 to 0.23 *M*; (A) represents the $MnCl_2$ concentration commonly reported, 0.046 *M*.

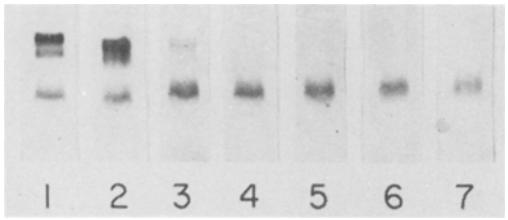

FIG. 2. Agarose gel electrophoresis of the total plasma (1) and the supernatant after the precipitation of LDL and VLDL using a final concentration of heparin, 184 USP units/ml, with the following molar concentrations of $MnCl_2$: (2) 0.018, (3) 0.037, (4) 0.042, (5) 0.046, (6) 0.055, and (7) 0.23.

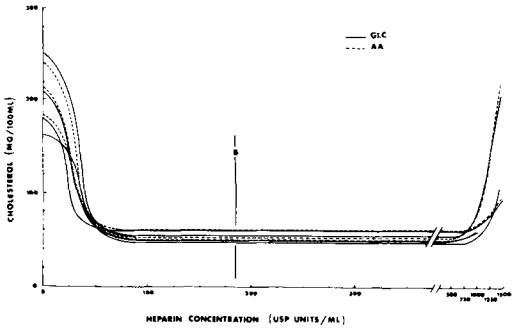

FIG. 3. Concentrations of cholesterol in the supernatant after precipitation of LDL and VLDL by heparin:manganese. Final $MnCl_2$ concentration (B) constant at 0.046 *M*, final heparin reagent concentration varied from 0 to 1,468 USP units/ml; (B) represents the heparin concentration commonly reported.

nated as the reference method in this comparison.

Preparation of Ultracentrifugal Fractions of Plasma

A 5 ml sample of plasma was adjusted to background density 1.063 g/ml with solid KBr according to the following calculation (15):

$$X = \frac{V_i (d_f - d_i)}{1 - \bar{V} d_f}$$

in which X is the g solid KBr to be added, V_i is the initial volume of the plasma, d_f is the final density after adjustment, d_i is the initial density, and $\bar{V}$ is the partial specific volume of KBr. In a cellulose nitrate tube, the adjusted sample is overlaid with KBr solution, d = 1.063 g/ml, to fill the tube and then centrifuged at 105,000 x g for 18 hr at 10 C. Lipoprotein fractions of d>1.063 g/ml were recovered using a tube slicer to cut the cellulose nitrate tube (11). The fraction >1.063 g/ml was then assayed for cholesterol levels, using both the Technicon AA-II (11) and gas liquid chromatographic (GLC) methods (16).

Precipitation of LDL and VLDL

The following conditions were used to assess the effects of changes in final $MnCl_2$ and heparin concentrations on the precipitation of LDL and VLDL and the subsequent measurements of C-HDL:

1) Mn^{++} concentration was varied while heparin concentration was held constant.
2) Heparin concentration was varied while Mn^{++} concentration was held constant.

To determine the effects of varying $MnCl_2$ concentrations, the final heparin concentration was maintained at the commonly used 184 USP units/ml (9,17,18), while the final Mn^{++} concentration was varied from 0 to 0.23 *M* using $MnCl_2$ tetrahydrate (Mallinckrodt No. 6126, St. Louis, MO) (Figs. 1 and 2). Concentrations of Mn^{++} in various $MnCl_2$ solutions were verified by titration with EDTA (19).

To determine the effects of heparin concentration variation on the precipitation of LDL and VLDL, analysis of the cholesterol in the resulting supernatant was performed at a constant final $MnCl_2$ concentration of 0.046 *M*, a final concentration which has been shown to be necessary for complete precipitation of VLDL and LDL (9,10). The heparin (Liquaemin Sodium "400," Organon, Inc., West Orange, NJ) final concentration was varied from 0 to 1,468 USP units/ml (Figs. 3 and 4). The potency of the sodium heparin preparation was 149 USP units/mg. The weight of heparin added to plasma aliquots for C-HDL (precipitation method) was 1.34 mg/ml plasma for the experiment comparing quantitation by precipitation and ultracentrifugation. Heparin from different sources does not always have a constant equivalency factor which can be used

to translate from USP units/ml to mg/ml. There may be considerable mol wt variation of commercial sodium heparins (20). In commercial heparin preparations, there can be a 6% variation in USP units/mg between different lots and sources. Each individual heparin lot was checked for its ability to completely precipitate VLDL and LDL by agarose gel electrophoresis (11). These minor variations in heparin potency are probably not of great practical significance, however, since the commonly used concentration of heparin (184 USP units/ml) is quite far removed from the low or the high concentration limits.

In all precipitation reactions, volumes were held constant as follows: a) 1 ml plasma, b) 0.04 ml heparin, and c) 0.05 ml $MnCl_2$ solution.

Measurement of C-HDL

All samples were kept on ice throughout the procedure. Heparin was added to the plasma and the sample vortexed. This was followed by the addition of $MnCl_2$, repeat mixing, and return to the ice bath for 30 min. After subsequent centrifugation (1,600 x g at 4 C for 30 min), the supernatant was removed with a Pasteur pipet. Cholesterol in the supernatant after precipitation and in the ultracentrifugal fraction d>1.063 g/ml was measured by the Technicon AA-II (11) and gas chromatographic methods (16). The AA-II method of the Lipid Research Clinics (11) uses a serum calibrator supplied by the Lipid Standardization Laboratory of the Center for Disease Control, Atlanta, GA, whose purpose is to adjust AA-II results so that they are comparable with the manual Abell-Kendall reference method. Conditions for the GLC cholesterol method were the same as previously reported (16). C-HDL quantitated by GLC was essentially identical to C-HDL measured by AA-II, and, for consistency, results summarized in Table I are for the AA-II method.

Lipoprotein Electrophoresis

Thin agarose gel electrophoresis of the supernatant following precipitation of LDL and VLDL was carried out using a commercially available kit (ACI-Corning), which is a modification of the method of Noble (21). The agarose plates were stained with fat red 7B. Electrophoresis was also used to confirm proper gradient separation in the ultracentrifugal fractions of plasma. Ten mg/dl LDL cholesterol or less could be detected by the gel electrophoresis.

Immunodiffusion

Immunodiffusion studies were carried out in 1% agarose (Sigma A6877) in a pH 7.0 buffer, using a center well containing antibeta-lipoprotein antibody (Behring Diagnostics 14-512-301). Diffusion was carried out for 48 hr in a moist chamber at 10 C. Precipitation lines elicited by the unknown samples were compared to those formed by standards which were made by the serial dilution of sera with ultracentrifugally determined LDL concentrations. The sensitivity of the immunodiffusion was 5 mg/dl C-LDL was determined by use of preassayed standards.

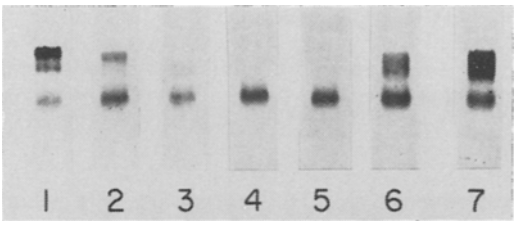

FIG. 4. Agarose gel electrophoresis of the total plasma (1) and the supernatant after the precipitation of LDL and VLDL using a final $MnCl_2$ concentration of 0.046 *M* and heparin in USP units/ml: (2) 46, (3) 92, (4) 184, (5) 642, (6) 734, and (7) 1468.

RESULTS

Comparison of C-HDL Quantitation by Precipitation and Ultracentrifugation

In 65 samples of plasma, with triglycerides ranging from 42 to 312 and cholesterols from 137 to 325 mg/dl, ultracentrifugally quantitated C-HDL ($\bar{X} \pm$ SE) was 54.3 ± 1.8 mg/dl, while C-HDL measured by precipitation in parallel aliquots was 56.0 ± 1.9 mg/dl (Table I). C-HDL measured by ultracentrifugal and precipitation methods was highly correlated, r = 0.982, P<.001 (Table I). Comparison of the two methods for C-HDL quantitation was also carried out for 14 subjects with familial hyper-alpha-lipoproteinemia (7.8), whose C-HDL levels were ⩾70 mg/dl. In these 14 patients, C-HDL by ultracentrifuge was 74.6 ± 2.5, not significantly different from 77.4 ± 2.7 by precipitation, and the two methods were again highly correlated, r = 0.941, P<.001. Plasma samples from 16 subjects with triglycerides ranging from 150-312 mg/dl were also evaluated using the precipitation and ultracentrifugal methods to quantitate C-HDL. Mean (± SE) C-HDL by ultracentrifugation and precipitation were, respectively, 41.4 ± 2.6 and 43.4 ± 2.8 mg/dl, and were closely correlated, r = 0.97, P<.001 (Table I).

Effects of Varying $MnCl_2$ Concentration with Heparin Constant

A constant amount of cholesterol in the

supernatant was measured over a final Mn^{++} concentration range of 0.046 to 0.23 *M* using both AA-II and GLC methodologies (Fig. 1). When final Mn^{++} concentrations were <0.046 *M*, however, the amount of cholesterol in the supernatant increased, with sharp increments observed as final $MnCl_2$ concentration was reduced from 0.042 to 0.02 *M* (Fig. 1). Agarose gel electrophoresis of the supernatant in samples where Mn^{++} concentrations were ≥ 0.046 *M* revealed HDL as the sole identifiable lipoprotein (Fig. 2). Immunodiffusion studies of these same supernatants revealed no remaining LDL when Mn^{++} was ≥ 0.046 *M*.

Two-factor analysis of variance (22) (using the least squares solution of the general linear hypotheses, Method I) confirmed that cholesterol in the supernatant (C-HDL) reached a constant level and that LDL and VLDL were completely precipitated at final Mn^{++} concentrations ≥ 0.046 *M*. Cholesterol levels in the supernatant varied significantly over final concentrations of 0.03-0.055 *M* Mn^{++} for both the colorimetric method ($F = 16.98$, $df = 4/30$, $P<0.0001$) and for GLC ($F = 9.68$, $df = 4/30$, $P<0.0001$). A *t*-test probe of the data indicated that cholesterol values in the supernatant containing Mn^{++} concentrations of 0.046, 0.05, and 0.055 *M* did not differ significantly from each other for both cholesterol assay methods. However, one sided *t*-test of the cholesterol level of the supernatant containing 0.042 *M* Mn^{++} differed significantly from the concentrations of 0.046, 0.05, and 0.055 *M* for both AA-II ($t = 8.24$, $df = 50$, $\alpha < 0.001$) and GLC ($t = 5.28$, $df = 50$, $\alpha < 0.001$). Five aliquots of plasma were analyzed for C-HDL at the final Mn^{++} concentrations of 0.034, 0.042, 0.046, and 0.048 *M*. Mean (± SE) C-HDL levels were, respectively, 54.4 ± 1.7, 45.6 ± 0.9, 44.8 ±0.5, and 46.0 ± 0.0 mg/dl, indicating reproducibility of C-HDL quantitation even at final $MnCl_2$ concentrations (0.034 *M*) which did not provide complete precipitation of VLDL and LDL.

Effects of Varying Heparin Concentration with $MnCl_2$ Held Constant

A constant amount of cholesterol in the supernatant was measured over a heparin concentration range of 92-734 USP units/ml (Fig. 3). At the final heparin concentrations <92 and >734 USP units/ml, precipitation of LDL and VLDL was incomplete; consequently, the amount of cholesterol in the supernatant was increased. Agarose gel electrophoresis of the supernatant obtained after the addition of various heparin concentrations revealed only HDL when the concentration was between 92 and 642 USP units/ml (Fig. 4). For heparin concentrations <92 and >734 USP units/ml, electrophoresis revealed unprecipitated LDL and VLDL (Fig. 4).

DISCUSSION

As initially shown by Burstein and Samaille (9) and confirmed by Fredrickson et al. (10), a final concentration of $MnCl_2$ >0.046 *M* is required for complete precipitation of VLDL and LDL for subsequent accurate measurement of C-HDL. The current report indicates, as did a previous pilot study (14), that this final $MnCl_2$ concentration is very near the minimum concentration required for accurate measurement of C-HDL (0.042 *M*). Minute inaccuracies in preparation of the $MnCl_2$ solution (often caused by hygroscopic qualities of $MnCl_2$ tetrahydrate) which reduce final $MnCl_2$ concentration to <0.046 *M* result in incomplete precipitation of LDL and VLDL (Figs. 1 and 2). At final Mn^{++} concentrations ≤ 0.042 *M*, cholesterol in the supernatant is reproducibly quantitated but is systematically and inaccurately elevated above levels obtained by the use of Mn^{++} final concentrations ≥ 0.046 *M*. When the final concentration of $MnCl_2$ is increased over a range from 0.046 to 0.23 *M*, a constant amount of C-HDL is measured. In sharp contrast, twofold increases and decreases of the heparin concentration often used (9) do not adversely affect precipitation of LDL and VLDL. An increase of the final $MnCl_2$ concentration (>0.06 *M*) should be considered to minimize the effects of minor inaccuracies in the preparation of $MnCl_2$ solution or in volumetric measurements which might affect the final Mn^{++} concentration and thereby the C-HDL determination.

Srinivasan et al. (13) have reported that in the presence of 0.025-0.075 *M* Mn^{++}, over 60% of HDL is associated with heparin which is attached to Sepharose. This report (13) further indicates that 25% of the total HDL produces an insoluble complex with free heparin in the presence of manganese. This would lead to a systematic underestimation of C-HDL by the precipitation method as compared to the ultracentrifugal method (which also includes V-HDL cholesterol). In the present study, however, the comparison of C-HDL in 65 plasma samples quantitated by the two methods revealed not only a highly significant correlation but means which were very close for the two procedures. Similar results were previously obtained by Wilson et al. (18).

Another potential problem involves plasma samples containing "sinking prebeta-lipoprotein," the Lp(a) antigen, an antigenic variant of

LDL with a density range overlapping that of HDL (23,24). A portion of Lp(a) would be recovered in the ultracentrifugally fractionated plasma at density >1.063 g/ml. In the occasional plasma samples where Lp(a) concentration is high, ultracentrifugally determined C-HDL might be consistently higher than C-HDL determined by precipitation. Although the presence or absence of Lp(a) was not determined in the 65 samples in this study, the notable similarity between C-HDL (ultracentrifuge) and C-HDL (precipitation) suggests that Lp(a) probably does not play a major role which would render measurement of C-HDL inaccurate.

This study confirms the adequacy of the final heparin and Mn^{++} concentrations suggested for precipitation of LDL and VLDL (9,10) and indicates that a conservative increment in final Mn^{++} concentration would give a greater margin of safety for accurate measurements. Accurate determination of C-HDL is important, not only for estimation of C-LDL (12), but also in evaluation of the role of C-HDL in familial hyperalpha-lipoproteinemia (6,7) and as an "anti-risk" factor in atherosclerosis (6-8).

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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 *Ochromonas malhamensis*[1]

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ABSTRACT

Tritium-labeled 14α-methyl-5α-ergost-8-enol and 5α-ergosta-8,14-dienol were converted to brassicasterol and poriferasterol in *Ochromonas malhamensis*. This indicates that the organism can rearrange these sterol structures so that they contain the naturally occurring 5(6) double bond, and suggests that *O. malhamensis* is able to introduce a 24(28) double bond into a nine-carbon saturated side chain which is alkylated to produce a C-29 sterol.

INTRODUCTION

Recent developments in sterol chemistry have led to the utilization of proposed intermediates as a means of analysis of an organism's biosynthetic capabilities. The isolation of 14α-methyl-5α-ergost-8-enol and 5α-ergosta-8,14-dienol from *Chlorella* treated with sterol biosynthetic inhibitors has led to speculation about the role of such compounds in the biosynthetic scheme (1,2). Cholesta-8,14-dienol has been enzymatically converted into cholesterol with animal systems (3-8). The biosynthetic fate of these compounds in the green alga *Chlorella ellipsoidea* is highly specific. They are converted only into ergost-5-enol (9). This is due to the apparent inability of the organism to alkylate or to introduce unsaturation into the saturated side chain, thus limiting the organism to rearranging the nucleus producing the 5-ene sterol.

In *Ochromonas malhamensis*, the ability to introduce the double bond at C-24(25) must exist, since the organism can convert 24-dihydrolanosterol and cholesterol into poriferasterol (10,11). Our purpose was to determine the ability of *Ochromonas* to metabolize sterols with an 9-carbon saturated side chain and the 14α-methyl Δ^8- or the 8,14-diene nucleus.

EXPERIMENTAL PROCEDURES

2,4-^{3}H-14α-methyl-5α-ergost-8-enol and 2,4-^{3}H-5α-ergosta-8,14-dienol were synthesized chemically from ergosterol (12,13) and labeled with tritium at the C-2 and C-4 positions by the method of Thompson et al. (14). Purity was established by gas liquid chromatography (GLC), thin layer chromatography (TLC), and infrared (IR).

The desired amount of sterol was dissolved in 0.25 ml of 95% ethanol and added to a 3-day old culture of *O. malhamensis* Pringsheim, American Type Culture Collection No. 11532. Growth conditions were as previously described (11). Cells were harvested on the 7th day, and sterols were extracted from freeze-dried cells with chloroform:methanol (2:1, v/v).

After saponification, the total sterols were acetylated and separated on Grade II neutral alumina. The ergosta-8,14-dienol was separated from the normally occurring sterols (brassicasterol 1%, poriferasterol 98%), using the $AgNO_3$ TLC method of Vroman and Cohen (15). The sterol acetates were separated by Anasil B column chromatography and lipophilic Sephadex column chromatography as previously described (9). The 14α-methyl-ergost-8-enol was separated from the *Ochromonas* diene sterols by Anasil B column chromatography. The brassicasterol and poriferasterol were then separated by lipophilic Sephadex chromatography. Quantitation and identification of sterols and acetates in all experiments were made by GLC on a 3% SE-30 column and a 3% OV-17 column. Relative retention times (RRT) with respect to cholesterol and its acetate were compared with authentic standards.

RESULTS AND DISCUSSION

The results of the incubation with 2,4-^{3}H-5α-ergosta-8,14-dienol and 2,4-^{3}H-14α-methyl-5α-ergost-8-enol are presented in Tables I and II. Both sterols are converted into brassicasterol and poriferasterol. The rearrangement of the nucleus of the diene appears to be a readily accomplished task, since a small quantity of highly labeled ergost-5-enol was identified. This compound had not been observed in this organism previously and may indicate that reintroduction of the C-24(28) double bond is a relatively difficult step. Upon identification of the labeled ergost-5-enol, 3.7 mg of authentic ergost-5-enol was added as carrier. Activity of the originally identified

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TABLE I

Incubation of *Ochromonas malhamensis* with [2,4-^{3}H] $\Delta^{8,14}$-ergostadien-3β-ol[a]

	Sterol wt (μg)	Radiation (dpm)	Sp. act. (dpm/μg)
Total ^{3}H sterol added	600	2.62×10^7	43,695
Total sterol extracted	43,890	3.27×10^6	74.7
Total sterol less ergosta-8,14 dienol 1st TLC plate	45,030	9.76×10^5	-
Activity $\Delta^{8,14}$ band 1st TLC plate	-	5.04×10^5	-
Total sterol less $\Delta^{8,14}$ dienol 2nd TLC plate	-	1.03×10^6	-
Activity $\Delta^{8,14}$ band 2nd TLC plate	-	7.88×10^4	-
Brassicasterol	791	7.96×10^3	10.3[b]
Poriferasterol	43,100	3.05×10^5	7.1[b]
Δ^5-ergostenol	26	5.81×10^4	2,237

[a]Total cell dry weight: 4.40 g. Activity recovered in medium: 6.53×10^5 dpm.
[b]After recrystallizations.

TABLE II

Incubation of *Ochromonas malhamensis* with [2,4-^{3}H]-14α-methyl ergost-8-enol[a]

	Sterol wt (μg)	Radiation (dpm)	Sp. act. (dpm/μg)
Total ^{3}H-sterol added	2,600	7.98×10^7	30,700
Total sterol extracted	47,606	1.37×10^7	288.1
Sterol monenes fraction	-	7.99×10^6	-
Sterol dienes fraction	-	2.78×10^6	
Brassicasterol	346	8.33×10^4	241
Poriferasterol	46,557	2.48×10^6	53.3[b]
Chondrillasterol	584		

[a]Total cell dry weight: 4.98 g. Activity recovered in medium: 8.28×10^6 dpm.
[b]After recrystallization.

material is reported. The production of labeled ergost-5-enol should not be surprising since it only requires a rearrangement of the nucleus as already seen in *Chlorella* (9). Ergost-5-enol was also observed by GLC from the incubation of 14α-methyl-5α-ergost-8-enol. Although a determination of activity was not made, it is presumed that this ergost-5-enol was radioactive also. A sterol with no activity and an RRT of 1.60 on SE-30 was isolated from the incubation of 14α-methyl-5α-ergost-8-enol. It is believed to be chondrillasterol (RRT 1.58) as identified by GLC and mass spectroscopy. The lack of activity may indicate that it is a normally occurring precursor, and the alkylation occurs prior to nuclear rearrangement. The natural occurrence of brassicasterol in the organism and the appearance of ergost-5-enol may provide clues to the sequencing of naturally occurring reactions. The main route to poriferasterol may be alkylation prior to complete nuclear rearrangement as evidenced by the natural occurrence of 24-methylene cycloartanol in the organism (16). Brassicasterol and sterols containing 9-carbon saturated side chains (ergost-5-enol) may be more slowly alkylated to produce poriferasterol.

14α-Methyl-5α-ergost-8-enol and 5α-ergosta-8,14-dienol are incorporated into poriferasterol, the major sterol of *Ochromonas*, demonstrating the capacity of the organism to remove the 14α-methyl group, convert the 8,14 double bond system to a Δ^5, and presumably introduce a 24(28) double bond into the saturated side chain to allow for a second alkylation.

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SHORT COMMUNICATION

Fecal Long Chain Fatty Acids and Colon Cancer Risk

ABSTRACT

To determine whether excretion of high concentrations of long chain fatty acids might be associated with high colon cancer risk, we compared concentrations of major long chain fatty acids in the feces of four populations at different risk for colon cancer. Concentrations of C18:1 were found to be significantly higher ($P<0.05$) in the feces of the two high risk populations than in the feces of the two low risk populations.

INTRODUCTION

Colon cancer incidence varies widely among different population groups. The main environmental factor associated with this variation appears to be diet. Diets high in meat and animal fat are positively correlated with a high risk of colon cancer (1,2). Moreover, colon cancer occurs primarily in people over the age of 50 (2). Thus, it is likely that the carcinogenic event takes place only after years of exposure of the colon mucosa to a chemical environment which is influenced by diet.

Because of the long period of exposure, even small differences in the colonic environment produced by a high meat-high fat diet could be important in determining colon cancer risk. Previous surveys of populations at different risk for colon cancer have shown that people eating such a high risk diet have higher concentrations of neutral steroids and bile acids in their feces than people from lower risk populations (3,4). Since long chain fatty acids (LFA) might also be expected to be present at higher concentrations in the colons of people eating a high meat-high fat diet, we have compared concentrations of LFA in the feces of people from four populations having different incidences of colon cancer.

MATERIALS AND METHODS

Collection of fecal specimens has been described in an earlier publication (5). The two low risk populations were: 29 rural native South Africans (collected by N.J. Richardson, South African Institute for Medical Research, Johannesburg, South Africa) and 18 rural Japanese (collected by Dr. A. Shimada, Akita University School of Medicine, Akita, Japan). The two high risk populations were: 49 Hawaii-Japanese, i.e., men of Japanese ancestry who had lived in Hawaii for at least 20 years, (collected by G.N. Stemmerman and G. Glober, Kuakini Hospital and Home, Honolulu, HI) and 44 North Americans (24 collected by S. Finegold, Wadsworth Hospital, Los Angeles, CA, and 20 collected by T.D. Wilkins, Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, VA). Specimens were frozen within 1 hr after collection. Specimens from South Africa, Japan, Hawaii, and California were shipped to the Anaerobe Laboratory in liquid nitrogen.

Freeze-dried fecal specimens were saponified with 1 N KOH in 90% ethanol at 90 C for 1 hr, then extracted twice with hexane to remove neutral steroids. The ethanol phase was acidified to pH 2 with concentrated HCl and the fatty acids were extracted with hexane. After evaporation to dryness under nitrogen, the hexane-extracted LFA were methylated with 14% BF_3-methanol (100 C/2 min). Methylated LFA were extracted into hexane and quantitated by gas liquid chromatography (GLC) on a Hewlett-Packard 5380 with a flame ionization detector, using a 2 mm ID, 2 m long column of 10% SP 216PS, 80/100 mesh on Supelcoport (Supelco Inc., Bellefonte, PA). The temperature of the injection port and detector was 200 C; column temperature was 145 C for 10 min and was then increased to 160 C at 10 C/min. Carrier gas (N_2) flow was 30 cc/min. Methyltricosanoate (Applied Science Laboratory, Inc., State College, PA) was used as an internal standard. [Carboxyl-^{14}C] tripalmitin was added to each dried fecal specimen as a recovery standard. Recovery was 97 ± 2%.

To determine the chemical state of the fecal LFA, dry fecal specimens from five subjects in each population were extracted with chloroform:methanol (2:1 v/v). Free LFA, mono-, di-, and triglycerides and neutral steroid esters were separated by silicic acid chromatography (6). LFA concentrations in each fraction were quantitated by the method outlined above.

RESULTS AND DISCUSSION

Median concentrations of the major LFA

TABLE I

Median Concentrations of Long Chain Fatty Acids (LFA) in the Feces of Four Populations at Different Risk for Colon Cancer

	Concentrations of LFA (mg/g dry feces)				P-values				
	High risk		Low risk		High risk vs. low risk			High risk vs. High risk	Low risk vs. Low risk
	North Americans (A)	Hawaii Japanese (B)	Rural Japanese (C)	Rural Africans (D)	(A vs. C)	(A vs. D)	(B vs. C)	(B vs. D)	(A vs. B)	(C vs. D)
Number of subjects	44	48	18	29						
Saturated LFA										
C14	1.3	0.5	0.4	0.7	<0.02	0.02	NS	NS	0.001	NS
C16	23.6	10.0	7.4	14.2	<0.001	<0.01	NS	NS	<0.001	0.01
C18	21.2	8.2	5.8	25.5	<0.001	NS	NS	<0.001	<0.001	<0.001
C20	0.5	0.3	0.6	0.9	NS	NS	NS	NS	NS	NS
Unsaturated LFA										
C18:1	22.4	12.5	7.0	6.0	<0.001	<0.001	0.05	<0.01	0.02	NS
C18:2	4.6	4.0	2.4	2.2	<0.05	<0.05	NS	0.05	NS	NS
C18:3	0.6	0.6	0.8	0.6	0.05	NS	0.05	NS	NS	0.05

found in the feces of the four populations studied and the statistical significance of differences in LFA distributions are given in Table I. Within each population, there was considerable variation in fecal LFA concentrations, and in many cases the data did not follow a normal distribution. Thus, the statistical significance of differences in LFA concentrations among different populations was determined by comparing LFA distributions using a chi-squared test. Similar levels of statistical significance were also obtained when populations were compared using the Mann-Whitney U-test (another nonparametric test) instead of the chi-squared test.

When concentrations of saturated LFA in the feces of the different populations were compared, significant differences were found but these differences did not appear to be associated with risk. For example, concentrations of C18 were significantly higher ($P<0.001$) in the feces of both the North Americans (high risk) and rural Africans (low risk) than in the feces of either the Hawaii-Japanese (high risk) or rural Japanese (low risk).

In the case of the unsaturated LFA, however, some risk-associated differences were observed. Concentrations of C18:1 were higher in the feces of the two high risk populations than in the feces of the two low risk populations, and these differences were statistically significant ($P<0.05$). Concentrations of C18:2 were also higher in the feces of the two high risk populations. This difference, however, was not significant at the $P = 0.05$ level when the high risk Hawaii-Japanese and low risk rural Japanese were compared, although it was significant for all of the other comparisons of high risk vs. low risk populations. No risk-associated differences in concentrations of fecal C18:3 were found.

Frequency distributions of fecal C18:1 for the four populations studied are shown in Table II. Because the four populations contained different number of subjects, frequencies for each population are expressed as percentages of the total number of subjects in that population. In the high risk populations, the range of C18:1 concentrations was much wider and the percentage of subjects with high concentrations of C18:1 was much greater than in the low risk populations.

In all four populations, the LFA occurred primarily in the form of free fatty acids or soaps (74-90%). The remainder were triglycerides (2-10%), diglycerides (3-6%), monoglycerides (1-4%), or esters of neutral steroids (4-6%).

The finding that high risk populations have

TABLE II

Frequency Distributions of Concentrations of C18:1 in the Feces of Four Populations at Different Risk for Colon Cancer

LFA concentration (mg/g dry weight)	Percentage of subjects in each concentration range			
	North Americans	Hawaii Japanese	Rural Japanese	Rural Africans
Number of subjects	44	48	18	29
C18:1				
0-5	5	14	45	28
6-10	14	27	34	52
11-15	18	27	0	14
16-20	11	11	11	3
21-25	18	4	5	0
26-30	9	4	0	0
> 30	25	13	5	0

higher concentrations of C18:1 in their feces than low risk populations does not necessarily indicate that C18:1 is directly involved in colon carcinogenesis. However, it has been reported that C18:1 can increase the number of tumors obtained in skin painting experiments in rats when applied daily after treatment with a carcinogen (7). Thus, it is possible that the presence of high concentrations of C18:1 in the colons of some individuals might affect the probability of tumor formation by a carcinogen ingested in very small amounts.

Whatever the meaning of the difference in fecal C18:1 (and possibly C18:2) concentrations between high and low risk populations, it is one of the few risk-associated differences which have been reported to date. Further investigation of this association between fecal C18:1 concentrations and colon cancer risk is needed.

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Effect of Ethanol Ingestion on Choline Phosphotransferase and Phosphatidyl Ethanolamine Methyltransferase Activities in Liver Microsomes

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ABSTRACT

The effect of ethanol ingestion on choline phosphotransferase and phosphatidyl ethanolamine methyltransferase activities, the two enzymes involved in phosphatidyl choline biosynthesis in liver microsomes, has been investigated. Female rats were fed a 5% ethanol-liquid diet containing amino acids, minerals, vitamins, with and without choline, for 2, 6, and 10 weeks. Control animals were pair-fed the same isocaloric diet with 5% sucrose with and without choline. Ethanol administration with or without dietary choline stimulated significantly ($P<0.001$) the specific activities of phosphatidyl ethanolamine methyltransferase in liver microsomes in the animals fed 5% ethanol for 2, 6, and 10 weeks, when compared to those control animals pair-fed the isocaloric diet with or without choline. Ethanol administration with or without dietary choline for 2 weeks stimulated significantly ($P<0.02$) the specific activities of choline phosphotransferase. The specific activities of phosphatidyl ethanolamine methyltransferase continued to increase in the liver microsomes from the animals in which dietary choline was omitted for 2, 6, and 10 weeks in the sucrose controls and alcohol-fed animals. Ethanol administration stimulates significantly ($P<0.001$) the phosphatidyl ethanolamine methyltransferase specific activities in liver microsomes of animals fed the liquid diet with dietary omission of choline and methionine for 2 weeks.

INTRODUCTION

Phosphatidyl choline biosynthesis in liver microsomes is known to occur by two major different pathways. The Kennedy (1) pathway involves choline phosphotransferase, which catalyzes the following reaction: cytidine diphosphocholine + 1,2-diglyceride to form phosphatidyl choline + CMP. The Bremer-Greenberg (2) pathway involves phosphatidyl ethanolamine methyltransferase, which catalyzes the following reaction: phosphatidyl ethanolamine + S-adenosyl methionine to form phosphatidyl choline. In this report, the enzymatic activity of choline phosphotransferase and phosphatidyl ethanolamine methyltransferase has been determined in liver microsomes of female rats fed 5% ethanol liquid diet with and without dietary choline. Control animals were pair-fed the same isocaloric diet with 5% sucrose with and without choline.

MATERIALS AND METHODS

Female albino rats of Sprague-Dawley strain weighing 152.1 ± 13.5 g were divided into two groups. Group I was fed the 5% ethanol liquid diet (3) with and without choline for 2, 6, and 10 weeks. The animals of Group II served as controls. They were pair-fed the isocaloric liquid diet containing sucrose instead of 5% ethanol with and without choline.

A liquid-alcohol diet, expressed as grams per 100 ml, was as follows (g): essential amino acids mixture, 2.307; nonessential amino acids, 2.422; salt mixture (Hegsted IV); 1; vitamin mixture, 1.250; Mazola corn oil, 1.250; cod liver oil, 0.375; Pastene olive oil, 3.000; sodium carrageenate (Viscarin), 0.400; ethanol (95% laboratory alcohol), 5.000; distilled water, up to 100 ml.

The composition of the essential amino acid mixture in grams per 100 g was as follows (g): L-lysine HCl, 13.434; L-arginine HCl, 8.125; DL-tryptophan, 2.166; DL-phenylalanine, 9.750; DL-leucine, 17.334; DL-isoleucine, 10.834; DL-valine, 15.167; L-histidine HCl, 5.850; DL-methionine, 6.500; DL-threonine, 10.834.

Nonessential amino acid mixture, grams per 100 g, was as follows (g): L-glutamic acid, 20.639; DL-serine, 5.159; glycine, 7.223; DL-tryosine, 28.895; L-cystine, 2.063; L-proline, 9.287; L-asparagine monohydrate, 14.344; DL-alanine, 12.383.

Composition of the vitamin mixture in milligrams per 100 g was as follows (mg): thiamine HCl, 5; riboflavin, 10; pyridoxine HCl, 5; cal-

TABLE I

Effects of Ethanol Administration at Various Time Intervals on Choline Phosphotransferase and Phosphatidyl Ethanolamine Methyltransferase Activity in Liver Microsome from Female Rats Fed 5% Alcohol Liquid Diets With and Without Choline[a]

Time on diet (weeks)	Material added to diet	Specific activities (nmol/min/mg protein) x 10			
		Choline phosphotransferase		Phosphatidyl ethanolamine methyltransferase	
		Control	Ethanol	Control	Ethanol
2	+ choline	(6)1.15 ± 0.19	(6)1.42 ± 0.14^{+}	(8) 3.47 ± 1.23	(8) 5.78 ± 0.52*
	- choline	(8)3.05 ± 0.48	(8)5.50 ± 1.23*	(6) 2.53 ± 1.22	(8) 9.88 ± 1.99*
6	+ choline			(3) 3.00 ± 1.29	(3)10.36 ± 2.31**
	- choline	(6)4.10 ± 1.96	(5)4.21 ± 1.28	(6) 7.29 ± 2.37	(5)13.77 ± 2.80*
10	+ choline	(4)2.38 ± 0.68	(4)3.29 ± 0.50	(6) 3.71 ± 2.23	(6) 6.92 ± 1.93^{++}
	- choline	(5)4.90 ± 2.12	(5)5.33 ± 0.64	(5)11.11 ± 1.61	(6)16.16 ± 1.41*

[a]The controls were pair-fed an isocaloric 5% sucrose diet. The concentration of choline chloride in the diet was 31.25 mg/100 ml. The values in parentheses indicate the number of animals. ± Values are standard deviations. The test of significance was applied between the mean values of control and ethanol fed animals. Probability for chance occurrence of this didfference was: *$P<0.001$, **$P<0.01$, $^{+}P<0.02$, $^{++}P<0.05$.

cium pantothenate, 40; nicotinamide, 30; choline chloride, 2500; biotin, 0.2; folic acid, 2; inositol, 200; 2-methyl-1,4-naphthoquinone, 2; vitamin B_{12}, 0.2; *p*-aminobenzoic acid, 100; sucrose, 97,610. The controls were fed similar isocaloric diet containing 8.75 g/100 ml of sucrose instead of the 5 g of ethanol. This diet was similar to that reported by Porta, et al. (3) and was purchased from General Biochemicals, Chagrin Falls, OH.

At the end of the dietary regimen, the control and the alcohol-fed rats were killed by decapitation, livers removed, rinsed with cold water, blotted and homogenized with ice-cold 0.25 M sucrose in a Potter-Elvehjem homogenizer with a Teflon pestle. The microsomal fraction was isolated by differential centrifugation (4). The nuclear and mitochondrial fractions were separated from the homogenate by centrifuging for 10 min at 14,500 g. The supernatant solution was centrifuged at 78,450 g for 45 min to sediment the microsomal pellet. Protein was determined by a modified Biuret method (5).

Choline Phosphotransferase Assay

The assay of the reaction catalyzed by the enzyme CDP-choline:1,2-diglyceride choline phosphotransferase (EC 2.7.8.2) was done by the method of Kennedy (1). The materials used were cytidine diphosphate-1,2-^{14}C-choline (ICN Tracerlab Chemical and Isotope Division, Irvine, CA) and Tween-20 (Sigma Chemical Co., St. Louis, MO). Diglycerides were prepared from egg lecithin by the method of Gurr et al. (7) and purified by the chromatography method of Barron and Hannahan (8). Each reaction mixture contained 50 μmol Tris-HCl (pH 8.0), 2 μmol 1,2-diglycerides emulsified in 0.1 ml of 1% Tween 20, 10 μmol $MgCl_2$, 0.5 μmol CDP-1,2-^{14}C-choline (specific activity, 4 x 10^5 cpm/μmol), and 10 mg microsomal protein. The final volume of the reaction mixture was 1.3 ml. The reaction time was 6 min.

Phosphatidyl Ethanolamine Methyltransferase Assay

The assay of the enzyme phosphatidyl ethanolamine S-adenosyl methionine methyltransferase (EC 2.1.1.c) was done by the method of Rehbinder and Greenberg (9) and used L-distearoyl-α-glyceryl phosphoryl-N,N-dimethyl-ethanolamine as substrate. Phosphatidyl ethanolamine methyltransferase is a specific methyltransferase (9). The materials used were ^{14}C-methyl-S-adenosyl methionine (New England Nuclear Corp., Boston, MA), unlabeled D-adenosyl methionine (Calbiochem, Los Angeles, CA), and L-distearoyl-α-glyceryl-phosphoryl-N,N-dimethylethanolamine (Schwarz-Mann, Orangeburg, NY). Each reaction mixture contained 1 μmol L-distearoyl-α-glycerylphosphoryl-N,N-dimethylethanolamine emulsified in 1 ml of 0.2 M Tris-HCl (pH 8.6) containing 0.4% deoxycholate, 0.2 μmol S-adenosyl-L-methionine-methyl-^{14}C (specific activity, 2.3 x 10^5 cpm/μmol), and 6 mg of microsomal protein. The final volume of the reaction mixture was 1.7 ml. The reaction time was 10 min.

RESULTS AND DISCUSSION

The enzymatic activity of choline phosphotransferase and phosphatidyl ethanolamine methyltransferase was shown to be linear with

TABLE II

Effect of Ethanol Administration for 2 Weeks Time Intervals on Choline Phosphotransferase and Phosphatidyl Ethanolamine Methyltransferase Activity in Liver Microsomes from Female Rats Fed 5% Alcohol Liquid Diets With and Without Choline or Methionine[a]

Material added to diet	Specific activities (nmol/min/mg protein) x 10			
	Choline phosphotransferase activity		Phosphatidyl ethanolamine methyltransferase	
	Control	Ethanol	Control	Ethanol
+ Choline + Methionine	(4)3.47 ± 0.40	(6)5.06 ± 0.45*	(4) 4.24 ± 2.33	(6)14.29 ± 0.76*
- Choline - Methionine	(6)4.31 ± 1.48	(6)5.74 ± 1.64	(6)11.25 ± 4.39	(6)16.48 ± 3.70+
+ Choline - Methionine	(6)6.07 ± 1.27	(5)6.65 ± 0.83	(6)13.17 ± 3.04	(6)16.15 ± 1.71
- Choline + Methionine	(8)3.05 ± 0.98	(8)5.50 ± 1.33*	(6) 2.53 ± 1.22	(8) 9.88 ± 1.99*

[a]The controls were pair-fed an isocaloric 5% sucrose diet. The concentration of choline chloride in the diet was 31.25 mg/100 ml, and DL-methionine was 0.15 g/100 ml diet. The values in parentheses indicate the number of animals. ± Values are standard deviations. The test of significance was applied between the mean values of control and ethanol-fed animals. Probability for chance occurrence of this difference was: *$P<0.001$, +$P<0.05$.

time and concentration of enzyme (10). The liquid diet fed to the animal represents 36% of total calories given as ethanol or sucrose. Lieber et al. (11) have observed an increase in total phospholipids concentration in male rats fed 5% ethanol and pair-fed isocaloric sucrose-supplemented controls after 10 and 24 days, respectively. French (12) reports a significant increase in the concentration of liver phospholipids in male and female rats by chronic ethanol feeding. The increase was observed in phosphatidyl ethanolamine and lysolecithin fractions. It has been demonstrated that ethanol administration increases the dietary choline requirement in the rat (6,13). Rats fed ethanol for a 24-day period were shown to have livers which on perfusion took up higher quantities of choline than control livers (14,15). These authors suggested that ethanol may increase the requirement of choline in the liver by stimulating the oxidative degradation of choline and may lower the concentration of adenine nucleotides. It has been shown that in choline deficiency a decrease in the concentration of ATP in the liver occurs (16). This decreased concentration of choline coupled with lowered level in ATP concentration in the liver following ethanol administration could account for the decreased incorporation of choline 1,2-^{14}C into phosphatidyl choline fractions 3 and 4 at 1, 2, 3, and 4 hr after administration of the isotopic compound that was observed in rats fed 5% ethanol liquid diet for 2 weeks (17). One of the first enzymatic reactions involved in the introduction of the phosphate in the biosynthesis of phosphatidyl choline utilized ATP to form phosphatidyl choline.

It is apparent from the data in Table I that 2, 6, and 10 weeks of ethanol administration with and without dietary choline increases signficiantly ($P<0.001$) the specific activity of phosphatidyl ethanolamine methyltransferase in liver microsomes in female rats when compared to those control animals pair-fed an isocaloric diet containing sucrose with or without choline. There was a significant ($P<0.001$) stimulation of the specific activities of choline phosphotransferase in the animals fed ethanol for 2 weeks and without dietary choline. This stimulatory effect of ethanol administration for 2 weeks on the phosphatidyl ethanolamine methyltransferase is still present if methionine is omitted from the diet (Table II). Dietary methionine can provide methyl groups for the biosynthesis of choline. The specific activities of the phosphatidyl ethanolamine methyltransferase in the control animals were 2.53, 7.29, and 11.11 at 2, 6, and 10 weeks, respectively, in the animals in which dietary choline was omitted (Table I). Controls receiving dietary choline were 3.47, 3.00,and 3.71, respectively. Intraperitoneal injection of 30 mg choline decreases significantly ($P<0.01$) the specific activity of phosphatidyl ethanolamine methyltransferase (18). This decrease in activity of the phosphatidyl ethanolamine methyltransferase following injection of additional cellular choline (18) may be expected since this phosphatidyl ethanolamine methyltransferase pathway has been suggested to provide the major

free choline of the cell (19,20). Other studies have demonstrated that methylation of phosphatidyl ethanolamine is the only pathway for choline biosynthesis de novo (21,22). The administration of ethanol stimulated significantly ($P<0.001$) the specific activity of phosphatidyl ethanolamine methyltransferase pathways in the biosynthesis of phosphatidyl choline in liver microsomes of animals with or without dietary choline (Table I). The ethanol stimulation of the phosphatidyl ethanolamine pathway of phosphatidyl choline biosynthesis is in agreement with the data of Fallon et al. (23), who report that isolated liver slices from male rats fed ethanol in drinking water for 7 days incorporate more methyl-^{14}C-methionine into phosphatidyl choline than controls. Fallon et al. (24) observed increased methylation of phosphatidyl ethanolamine in liver homogenates and microsomal fractions in ethanol-fed rats for 1 month and 1 year.

Phosphatidyl choline is the major phospholipid of membranes of the cell. It has been found in the liver cell that this lipid represents 45% of the total lipid phosphorus of mitochondria (25), 48.5% of the total lipid phosphorus of endoplasmic reticulum (25), and 37.4% of total lipid phosphorus of the plasma membrane (26). Iseri et al. (27) have shown that ethanol administration increases the proliferation of hepatic smooth endoplasmic reticulum and stimulates enzymatic activity of a number of enzymes found in endoplasmic reticulum of the liver (27-29). Ethanol may act like the drug phenobarbital, which stimulates enzymes in the endoplasmic reticulum (30) and phospholipids biosynthesis (31). This stimulation of the phosphatidyl ethanolamine methyltransferase activity by ethanol administration may be a direct drug effect of ethanol or a cellular response for the proliferation of the hepatic smooth endoplasmic reticulum, since this biosynthetic pathway would provide the necessary phosphatidyl choline inasmuch as it is the major lipid in this cellular component.

ACKNOWLEDGMENT

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Binding of Squalene, Lanosterol, Desmosterol, and Cholesterol to Proteins in Brain and Liver 105,000 g Supernatant Fractions: Evidence for Specific Binding Sites

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ABSTRACT

The binding of squalene, lanosterol, desmosterol, and cholesterol to proteins in 105,000 g supernatant fraction (S_{105}) from brain and liver of rats was investigated. The S_{105} fractions from both tissues contain specific binding sites for sterols, which are sensitive to trypsin. The dissociation constants for squalene and sterol protein complexes were in the range of 10^{-6} M and were not appreciably different for proteins in brain and liver S_{105}. Competition studies revealed that both brain and liver S_{105} contain one receptor protein which binds lanosterol and is specific for methyl sterols, and a second receptor which binds both desmosterol and cholesterol. Binding of 7-dehydrocholesterol reported by others must occur at a third independent site since this compound does not interfere with the binding of lanosterol, desmosterol, or cholesterol. Although binding of squalene to proteins in brain and liver S_{105} does occur, we were unable to show the specificity of squalene binding. The concentration of desmosterol and cholesterol binding sites, which ranged from 6 to 10 nmol/mg protein, was 3- to 5-fold higher than the concentration of squalene and lanosterol binding sites (1.6-2.3 nmol/mg protein). The brain S_{105} from suckling rats contained fewer binding sites for desmosterol and cholesterol than the brain S_{105} from weaned rats. However, the concentration of lanosterol binding sites in brain S_{105} did not show an age-dependent change. The receptor proteins in brain and liver appear to be identical.

INTRODUCTION

Microsomal synthesis of cholesterol from squalene and sterol precursors of cholesterol requires noncatalytic proteins present in the 105,000 g supernatant fractions (S_{105}) (1-4). It is generally believed that these substrates must be bound to the S_{105} proteins in order to be converted to cholesterol by microsomal enzymes. Recent evidence suggests that the liver S_{105} contains several substrate-specific proteins (5). If, as believed, substrate binding to S_{105} protein is necessary for the conversion, then, on the basis of this recent evidence (5), one would expect the S_{105} to contain protein receptor sites which will exhibit specific binding of each of the substrates. Although binding of these substrates to proteins in liver S_{105} has been demonstrated (6-8), information regarding the specificity of binding is lacking. In the present study, we have investigated the binding of squalene, lanosterol, desmosterol, and cholesterol to proteins in liver S_{105} in order to determine whether the S_{105} contains independent high affinity receptor sites for these substrates. Since we have already shown (9) that the brain S_{105} also contains the noncatalytic proteins, we investigated the binding of these substrates to proteins in brain S_{105} as well.

EXPERIMENTAL PROCEDURES

Materials

Mevalonate-2[^{14}C] sp. act. 4.4 μC/μmol, desmosterol-26[^{14}C] sp. act. 5.5 μC/μmol, cholesterol-4[^{14}C] sp. act. 56 μC/μmol, triolein [^{14}C] sp. act. 63 μC/μmol, and oleic acid-1[^{14}C] sp. act. 82 μC/μmol were purchased from New England Nuclear Corporation, Boston, MA. [^{14}C] Labeled squalene and lanosterol were prepared biosynthetically from mevalonate-2[^{14}C] by the method of Tchen (10). The labeled squalene and lanosterol were purified on an alumina column and checked for radiochemical purity by thin layer chromatography. Specific activity of labeled squalene and lanosterol was 1.1 μC/μmol. Unlabeled lipids were purchased from Applied Sciences Laboratory, State College, PA. The unlabeled and labeled lipids obtained commercially were also checked for purity pior to their use.

Methods

Liver and brain tissues obtained from

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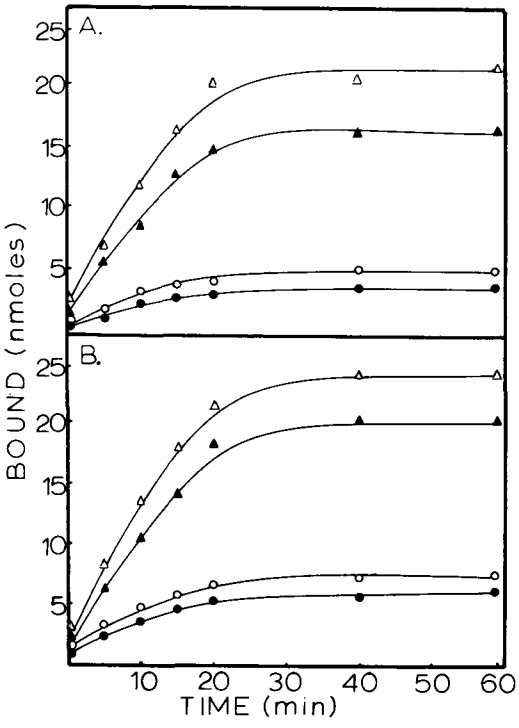

FIG. 1. Effect of incubation time on squalene and sterol binding to proteins in S_{105}. The S_{105} fractions were prepared as described in the text. [^{14}C] Labeled substrate (50 nmol) were incubated with brain and liver S_{105} (2.0 mg protein) at 37 C. After the respective incubation intervals, protein-bound substrate was separated from the unbound substrate by gel filtration on Sephadex G-25 as described in the text. Brain, A; liver, B; squalene (●- - -●); lanosterol (○- - -○; desmosterol (▲- - -▲); cholesterol (△- - -△).

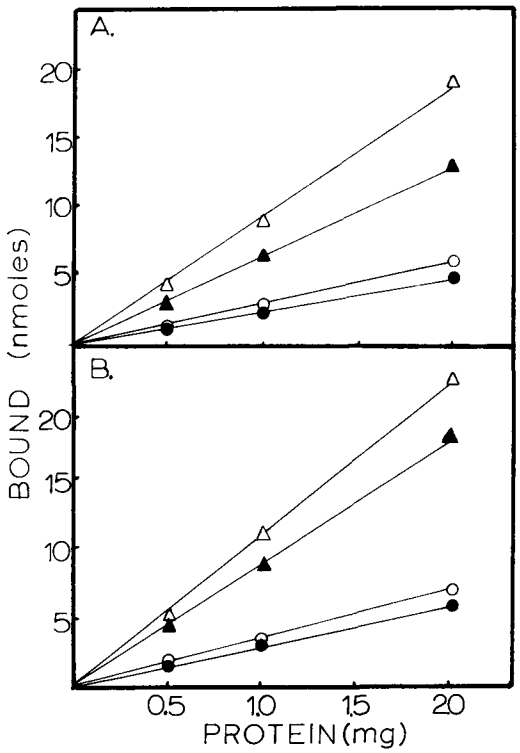

FIG. 2. Effect of protein concentration on binding of squalene, lanosterol, desmosterol, and cholesterol. Varying amounts of S_{105} fraction were incubated with [^{14}C] labeled substrate (50 nmol) for 1 hr. The incubation mixtures were then assayed for bound and unbound substrates by gel filtration as described in the text. Squalene (●- - -●); lanosterol (○- - -○); desmosterol (▲- - -▲); cholesterol (△- - -△).

Sprague-Dawley rats (females weighing ca. 200 g) were homogenized in 2.5 volume of 0.1 M potassium phosphate buffer (pH 7.4) containing 0.5 mM DTT. The homogenates were centrifuged at 105,000 g for 60 min. The floating layer at the top was drawn off and discarded, and the upper 2/3 portion of the supernatant fraction was collected and recentrifuged at 105,000 g for 60 min to remove microsomal contamination. The resulting supernatant (S_{105}) was then used for the binding studies. An aliquot of the S_{105} fraction was incubated with [^{14}C] labeled substrates for 1 hr at 37 C in an atmosphere of nitrogen. The substrates were added to 10 μl of propylene glycol:dioxane (2:1) solution, and the final volume of the incubation mixture was brought to 1 ml with phosphate buffer. After incubation, the contents of incubation tubes were transferred to a Sephadex G-25 column (1 x 10 cm) which had previously been equilibrated at 4 C with phosphate buffer. Radioactivity associated with the proteins which were eluted at the void volume was estimated by liquid scintillation counting. Control samples incubated in the absence of S_{105} were passed through a Sephadex column, and the small amount of radioactivity eluted at the void volume was subtracted to estimate the radioactivity associated with the proteins. The amount of bound substrate was calculated from the specific activity of the added substrate and the amount of radioactivity recovered in association with the eluted protein. The amount of unbound substrate was estimated by extracting the entire contents of the column with ethyl ether and assaying the extract for radioactivity. Protein was determined by the method of Lowry et al. (11).

RESULTS

Properties of Binding

The data in Figure 1 show the binding of squalene, lanosterol, desmosterol, and choles-

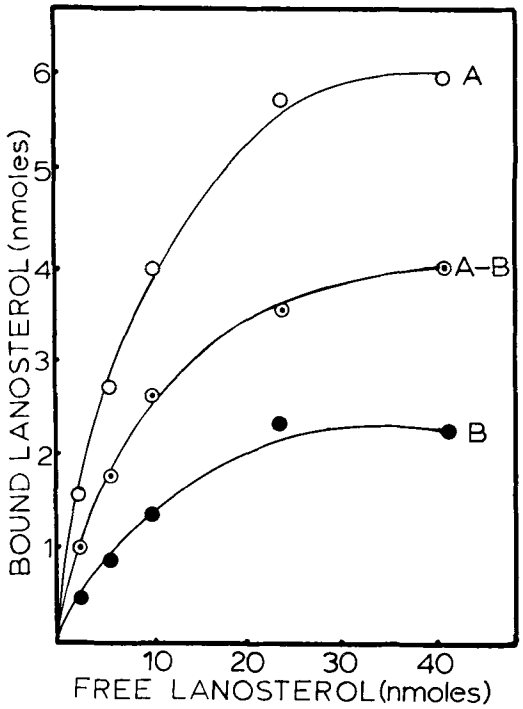

FIG. 3. Binding of [14C] lanosterol to proteins in liver S_{105}. Varying amounts of labeled lanosterol were incubated with 2.0 mg S_{105} protein at 37 C for 1 hr. In another pair of incubations, samples contained the same varying concentrations of labeled lanosterol plus 250 nmol on unlabeled lanosterol. The amount of bound and unbound lanosterol was determined by gel filtration of Sephadex G-25 as described in the text. A: S_{105} plus [14C] lanosterol (3-30 nmol), total binding; B: S_{105} plus [14C] lanosterol (3-30 nmol plus 250 nmol unlabeled lanosterol), low affinity binding; A-B: high affinity binding, curve obtained by subtracting low affinity binding from total binding.

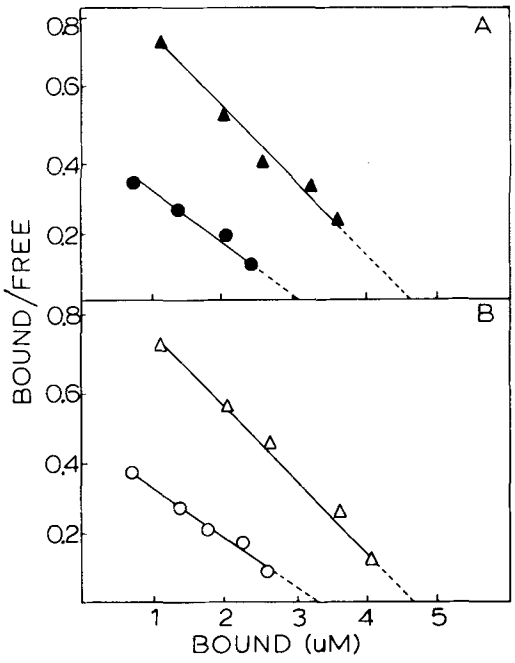

FIG. 4. Scatchard plots of high affinity binding of squalene and lanosterol. The data for high affinity binding of squalene to brain S_{105}, and of lanosterol to brain and liver S_{105}, were obtained in the same manner as described for lanosterol binding to brain S_{105} in Figure 3. The regression lines were computed by the method of least squares. Brain, A; squalene (●- - -●); lanosterol (▲- - -▲). Liver, B; squalene (○- - -○); lanosterol (△- - -△).

terol to proteins in brain and liver S_{105} as a function of incubation time. It can be seen that desmosterol and cholesterol are bound at a higher rate and in greater amount than squalene and lanosterol. Results in Figure 2 show that the amount of substrate bound increases in proportion to an increase in protein concentration. Treatment of S_{105} fraction with trypsin for 30 min caused almost complete loss of lanosterol and desmosterol binding.

Experiments were carried out to differentiate high and low affinity binding. In these experiments, various amounts of labeled substrates were incubated with and without 10- to 50-fold molar excess of unlabeled substrates, and the amount of bound substrate was then plotted against the amount of unbound substrate. Results obtained with lanosterol are typical and are shown in Figure 3. Low affinity binding represented by the lower curve accounted for ca. 30% of the total binding for all substrates studied.

Figures 4 and 5 show Scatchard plots of the high affinity binding data for squalene, lanosterol, desmosterol, and cholesterol. The high affinity binding data were derived by subtracting the low affinity binding from total binding. The values for the number of binding sites and dissociation constants obtained from these plots are presented in Table I. The apparent dissociation constants were of the order of 10^{-6} M for both brain and liver S_{105} protein-sterol complexes. (It should be pointed out that the specific activity of the substrates used would preclude the detection of binding sites with a dissociation constant in the range of 10^{-7} M or lower.) The dissociation constants for squalene, lanosterol, cholesterol, and desmosterol were not appreciably different. In the case of both tissues, however, the concentration of binding sites for cholesterol and desmosterol is 3- to 5-fold higher than for squalene and lanosterol. The concentration of lanosterol binding sites is somewhat higher than the concentration of squalene binding sites.

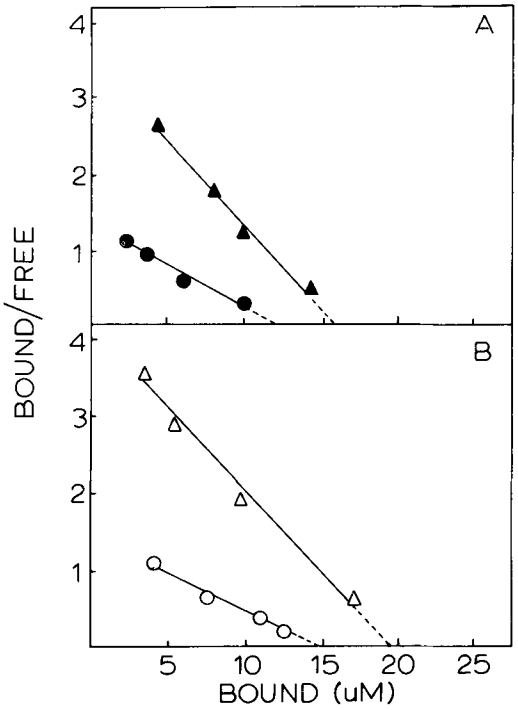

FIG. 5. High affinity binding of desmosterol and cholesterol. The data for high affinity binding of desmosterol and cholesterol were obtained as described in Figure 3. Brain, A; desmosterol (●- - -●); cholesterol (▲- - -▲). Liver, B; desmosterol (○- - -○); cholesterol (△- - -△).

Specificity of Binding

Competition studies were carried out in order to determine the degree of specificity of the binding for each compound. The results of 85% in the presence of excess unlabeled lanosterol, desmosterol, and cholesterol. The binding of [^{14}C] lanosterol was inhibited by 80% in the presence of excess unlabeled lanosterol and lanostenol. Lanosterol binding was unaffected by squalene and other sterols. Binding of [^{14}C] desmosterol was inhibited by 90% in the presence of added cholesterol and vice versa. Binding of desmosterol and cholesterol was inhibited strongly (80%) by zymosterol but was unaffected by 7-dehydrocholesterol, lanosterol, or squalene.

The presence in liver (12) and brain (13) S_{105} of a high affinity receptor specific for coritsol has been reported. We, therefore, examined the effect of cortisol on binding of squalene and the sterols. Cortisol inhibited the binding of [^{14}C] squalene almost completely but had no effect on binding of lanosterol, desmosterol, or cholesterol. Since the binding of triolein (7) and oleic acid (7,8) to proteins from liver S_{105} has also been reported by others, we examined the effect of triolein and oleic acid on the binding of squalene and sterols. Both compounds inhibited the binding of squalene by 75% but had no effect on the binding of lanosterol, desmosterol, or cholesterol. Binding of labeled triolein and oleic acid was not affected by sterols, squalene, or cortisol (Table IV).

Developmental Changes in Sterol Binding Sites in Brain S_{105}

It is known that the synthesis of cerebral cholesterol changes during development. In view of the possibility that this change may be related to a change in the sterol binding capacity of the protein in the S_{105} fraction, we studied sterol binding in S_{105} fractions prepared from the brain tissue of rats at different stages of development. Scatchard plots of the data obtained with these fractions are shown in Figures 6-8. The concentration of binding sites for both desmosterol and cholesterol increased 2- to 3-fold during development (Figs. 6 and 7). By comparison, the concentration of binding sites for lanosterol did not show an appreciable age-dependent change (Fig. 8). There was,

TABLE I

High Affinity Binding Parameters for Cholesterol, Desmosterol, Lanosterol, and Squalene in Brain and Liver S_{105}[a]

	Brain		Liver	
Substrate	Dissociation constant	Maximum binding capacity (nmol/mg) protein	Dissociation constant	Maximum binding capacity (nmol/mg) protein
Cholesterol	4.02 x 10^{-6} M	7.92	4.76 x 10^{-6} M	10.00
Desmosterol	8.64 x 10^{-6} M	6.04	9.09 x 10^{-6} M	7.02
Lanosterol	5.45 x 10^{-6} M	2.24	5.02 x 10^{-6} M	2.32
Squalene	6.27 x 10^{-6} M	1.60	7.02 x 10^{-6} M	1.59

[a]Dissociation constants were calculated from the slope of the Scatchard plots, Figures 4 and 5, and the amount of substrate bound from the intercept on the abscissa.

TABLE II

Inhibition of [^{14}C] Cholesterol, Desmosterol, Lanosterol, and Squalene Binding to Liver S_{105} by Unlabeled Compounds[a]

Additions	Percentage inhibition of binding			
	Cholesterol	Desmosterol	Lanosterol	Squalene
None	0	0	0	0
Cholesterol	89.5	86.2	7.31	83.4
Zymosterol	80.6	80.9	7.82	---
Desmosterol	89.5	87.3	2.32	82.2
7-Dehydrocholesterol	0.52	0.14	8.92	77.6
Lanosterol	5.34	17.3	82.6	81.3
Cortisol	7.30	13.2	20.1	76.0
Squalene	8.92	1.63	12.9	85.3
Triolein	9.12	8.42	4.21	77.5
Oleic acid	1.34	6.81	17.3	76.8

[a]Effect of various unlabeled compounds on binding of [^{14}C] squalene, lanosterol, desmosterol, and cholesterol to liver S_{105}. Radioactive substrates (3-30 nmol) were incubated in the presence and absence of added unlabeled competitor (300 nmol) with 2.0 mg S_{105} protein for 1 hr at 37 C. The extent of binding was determined in the same manner as described in Figure 1 and the text. The percent inhibition was calculated from the equation:

$$100 \times \frac{[\text{binding (cpm) with no additions}] - [\text{binding (cpm) with competitors}]}{[\text{binding (cpm) with no additions}]}$$

TABLE III

Inhibition of [^{14}C] Cholesterol, Desmosterol, Lanosterol, and Squalene Binding to Brain S_{105} by Unlabeled Compounds[a]

Additions	Percentage inhibition of binding			
	Cholesterol	Desmosterol	Lanosterol	Squalene
None	0	0	0	0
Cholesterol	85.6	84.6	0.28	81.6
Zymosterol	83.3	90.2	24.5	85.4
Desmosterol	87.6	96.5	4.9	86.2
7-Dehydrocholesterol	12.5	12.7	0.33	--
Lanosterol	8.1	10.1	87.1	79.2
Lanostenol	11.9	--	68.9	--
Cortisol	0.63	11.5	0.20	78.8
Squalene	4.7	10.0	16.5	89.1
Triolein	10.9	14.7	3.7	84.0
Oleic acid	4.2	12.6	0.22	84.0

[a]For experimental details see Table II.

TABLE IV

Inhibition of [^{14}C]Oleic Acid and [^{14}C] Triolein Binding to Brain and Liver S_{105} by Unlabeled Compounds[a]

Additions	Percentage inhibition of binding			
	Brain		Liver	
	Oleate	Triolein	Oleate	Triolein
None	---	---	---	---
Oleate	92.8	---	89.8	---
Cholesterol	8.23	4.62	4.68	6.74
Desmosterol	10.0	6.88	---	9.36
Lanosterol	13.4	7.07	14.1	10.0
Squalene	30.0	6.90	11.6	8.48
Triolein	34.8	86.6	8.16	81.5
Cortisol	21.4	---	17.3	---

[a]For experimental deatils see Table II.

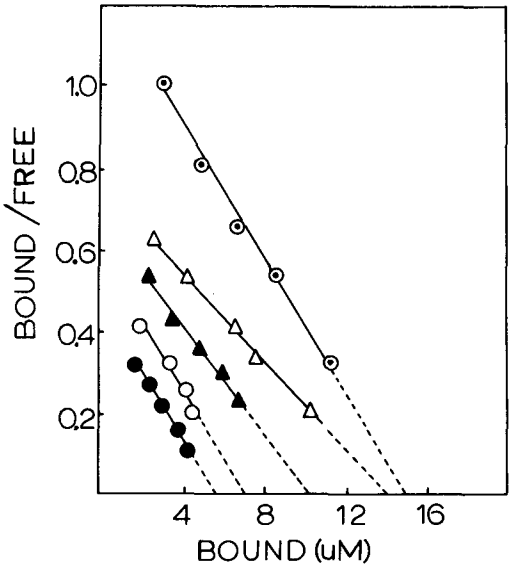

FIG. 6. Developmental changes in the high affinity binding of desmosterol to brain S_{105}. Brain S_{105} from rats of various age groups were prepared as described in the text. Binding was determined as described in Figure 3. 5 day (●- - -●); 10 day (○- - -○); 15 day (▲- - -▲); 20 day (△- - -△); 25 day (◉- - -◉).

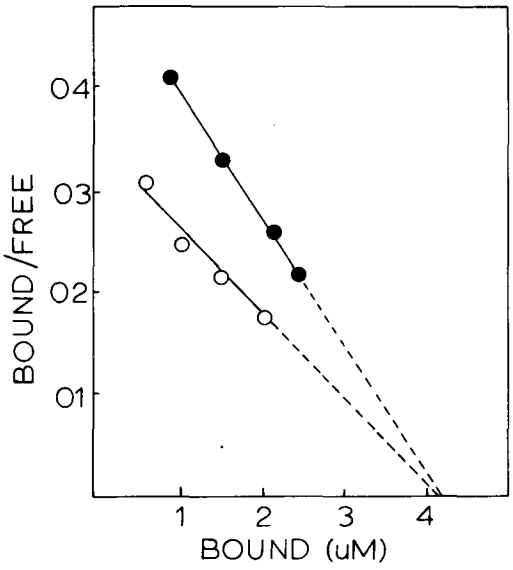

FIG. 8. Developmental changes in the high affinity binding of lanosterol to brain S_{105}. Brain S_{105} from rats of various age groups were prepared as described in the text. Binding was determined in the same manner as described in Figure 3. 5 day (○- - -○); 27 day (●- - -●).

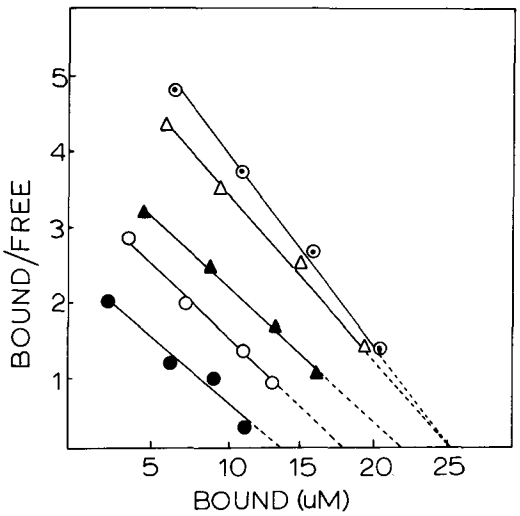

FIG. 7. Developmental changes in the high affinity binding of cholesterol to brain S_{105}. Brain S_{105} from rats of various age groups were prepared as described in the text. Binding was determined as described in Figure 3. 5 day (●- - -●); 10 day (○- - -○); 15 day (▲- - -▲); 20 day (△- - -△); 25 day (◉- - -◉).

moreover, no appreciable change in the apparent dissociation constant for binding of these sterols.

DISCUSSION

The results of the present investigation show that the S_{105} fractions from brain and liver contain trypsin-sensitive proteins which exhibit binding of squalene, lanosterol, desmosterol, and cholesterol. The fact that dissociation constants for substrate-protein complexes in brain and liver S_{105} were not much different indicates that the squalene and sterol receptors in the two tissues are identical. This is in accord with our earlier findings (9) that the S_{105} proteins from extrahepatic tissues which are required for the conversion of squalene to cholesterol are similar to those present in liver S_{105}.

The results of the competition studies indicate the presence of at least three sterol-specific binding sites in the S_{105} fraction from both brain and liver. One site, which binds lanosterol, appears to be specific for methyl sterols since lanostenol inhibited the binding of lanosterol while none of the C_{27} sterols interfered with the binding of lanosterol. Specificity of this receptor with respect to mono and di methyl sterols, however, remains to be determined. The presence of a second receptor site, which is shared by desmosterol and cholesterol, is indicated by the fact that desmosterol and cholesterol inhibit the binding of each other and lanosterol does not interfere with the binding of either desmosterol or cholesterol. Because zymosterol inhibits the binding of desmosterol and cholesterol, it is likely that this

second receptor site also binds zymosterol. 7-Dehydrocholesterol, which is known to bind to the proteins in liver S_{105}, however, does not affect the binding of desmosterol and cholesterol. There must, therefore, be a third receptor site which is different from the receptor site for desmosterol and cholesterol and which binds 7-dehydrocholesterol. That these three binding sites are specific for sterols is clear from our observation that neither triolein, oleic acid, cortisol, nor squalene interfere with the binding of these sterols.

Although proteins in S_{105} exhibit binding of squalene, we cannot, from the present data, ascertain the specificity of the squalene binding site. The inhibition of squalene binding by all the compounds tested appears to indicate at first sight that the binding of squalene is of nonspecific nature. However, since the addition of excess squalene during incubation has no effect on the binding of sterols, triolein, oleic acid, or cortisol. This suggests that squalene must bind at the site different from the receptor sites for sterols, triolein, oleic acid, and cortisol. It is possible that the inhibition of squalene binding is due to a change in the protein configuration which occurs in the presence of excess of other lipid components and results in the masking of squalene binding sites.

The binding of triolein and oleic acid to partially purified preparation of sterol carrier protein from liver S_{105} has been reported by Scallen et al. (7). Ritter and Dempsey (8) have also demonstrated the binding of fatty acids to their sterol carrier protein. However, it was not clear from those studies whether the binding of triolein and oleic acid occurred at the same receptor sites which bind squalene and sterols. It is apparent from the results of our studies that binding of triolein and oleic acid occurs at the sites other than the sterol binding sites.

Our data indicate that the concentration of lanosterol binding sites in brain S_{105} did not change during development. However, the concentration of desmosterol and cholesterol binding sites in brain S_{105} from suckling rats is approximately half that in S_{105} from adult rats. This is interesting in light of the fact that the rate of cholesterol synthesis is high in brains of suckling rats and is low in brains from young adult rats, a trend which is in the reverse direction of the change in the concentration of desmosterol binding sites. The developmental change in the reduction of desmosterol by preparations from rat brain (14), therefore, probably is not related to a change in the concentration of binding sites in S_{105} fraction. A lack of such a relationship is consistent with our earlier observations (15) that the change in the conversion of squalene to cholesterol by microsomal preparations from livers of suckling and weaned rats was associated with a change in the activity of microsomal enzymes and not with the factors in S_{105} fraction.

We cannot at present be certain that the sterol binding which we have observed involves S_{105} proteins which function as activator proteins. It is generally believed that sterol precursors must be bound to S_{105} protein in order to be converted to cholesterol by microsomal enzymes, and one would expect that such binding is characteristically specific in nature. The specificity in the binding of lanosterol, desmosterol, and 7-dehydrocholesterol observed by us is consistent with the recent finding of Scallen et al. (5) that S_{105} contains specific activator proteins, but the final conclusion that sterol binding proteins are identical to the activator proteins must await further experimentation.

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Lipid Absorption in the Young of Protein-deficient Rats

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ABSTRACT

The effect of reduced protein in the diet during pregnancy on the subsequent absorption of triolein and of oleic acid which were infused into the intestine of the young was studied. Pregnant rats were fed diets containing either 24% or 4% casein as the sole source of protein. Control and prenatally protein-deprived (PPD) young were studied at birth, before and after suckling, and at 4, 8, and 12 days. Both body weight and the weight and length of intestine were reduced in PPD young. Uptake of triolein from the lumen and retention in enterocytes increased on suckling in newborn control pups, but the amount transferred from or metabolized by the cells did not change. In suckled PPD young, transfer of triolein increased through the enterocyte as well. Unsuckled PPD pups had reduced absorption and retention per enterocyte, per g body weight, per cm gut, and in total. In intestines of control and PPD suckled newborn and postnatal pups, absorption per cell did not differ. Blood lipid levels were increased markedly between 0 and 4 days and tended to decrease to newborn levels by 12 days in both diet groups. Oleic acid absorption in newborn and 12-day PPD pups were reduced in total, per g body weight and per cm of gut. The individual enterocytes were shown to be equally capable of absorption and transfer of triolein and oleic acid. Differences in absorption are related primarily to the numbers of absorptive cells.

INTRODUCTION

Previous work in our laboratory has demonstrated that feeding a diet deficient in protein to pregnant rats results in the production of young with lower body weights at birth and decreased survival rate (1,2). Morphological development of numerous organs and tissues including those of the gastrointestinal tract is retarded in these prenatally protein-deprived (PPD) young, and histochemical methods have demonstrated alterations in enzyme activity (3).

When emulsified lipid was administered to these young, histochemical methods showed minimal fat content in the enterocytes of PPD young compared to the controls, suggesting that lipid absorption was impaired (4). This method provided no quantitative data, nor did the experimental design provide any information on fatty acid absorption or the movement of lipid from the enterocyte into other tissues.

The present study was undertaken to determine the quantitative effect of prenatal protein deprivation on the uptake of lipid from the intestinal lumen and its transfer to other tissues.

MATERIALS AND METHODS

Female rats of the Sprague-Dawley strain, 175-190 g, were mated overnight with normal males. Throughout pregnancy, diets were fed containing either 24% or 4% casein as the sole source of protein, as previously described (5).

Young of control and protein-deprived dams weighing, at birth, 5.8-6.7 g and 2.8-3.7 g, respectively, were used as subjects for study of intestinal absorptive capability in unsuckled and suckled animals. Suckled animals were 6-12 hr old and were those in whose stomachs milk could be observed. These pups were included to investigate the possibility of a change in digestive or absorptive capability in response to suckling in either or both diet groups. To produce postnatal subjects, five control pups and five PPD pups from the weight groups given above were suckled by a stock diet (Purina rat chow) fed female whose newborn young were discarded. This procedure assured a generous postnatal milk supply.

At the appropriate ages, pups were anesthetized with 0.03 mg/g body wt sodium pentabarbital (Diabutal, Diamond Laboratories, Des Moines, IA). The body temperature of the pups was maintained before and during the experiments by warmth from an incandescent lamp.

The intestines were infused in vivo by means of a cannula inserted through incisions in the lateral body wall and into the wall of the forestomach. After being passed into the first mm of the duodenum, the cannula was fixed in place by a ligature tied around the pyloric sphincter. One of two ^{14}C-labeled infusates was used: 1) A micellar solution containing 1 mM oleic acid (analytical grade, Sigma Chemical Co., St. Louis, MO; 1-^{14}C-oleic acid, Amersham-Searle Corp., Arlington Heights, IL) for newborn, or 5 M mM for postnatal pups, and

TABLE I

Mean Body Weights (g) of Control and Prenatally Protein-deprived Young

Animal group	Maternal diet	
	24% Casein	4% Casein
Newborn:		
Triolein-infused		
Unsuckled	6.40 ± 0.06[a] (11)[b]	3.38 ± 0.07 (11)[c]
Suckled	6.25 ± 0.07 (10)	3.48 ± 0.06 (10)[c]
Oleic acid-infused	6.34 ± 0.07 (13)	3.35 ± 0.07 (8)[c]
4 day, Triolein-infused	10.12 ± 0.47 (8)	5.48 ± 0.21 (8)[c]
8 day, Triolein-infused	17.2 ± 0.9 (6)	10.3 ± 0.4 (6)[c]
12 day:		
Triolein-infused	25.6 ± 0.8 (7)	15.9 ± 0.6 (8)[c]
Oleic acid-infused	25.9 ± 0.9 (9)	15.2 ± 1.0 (11)[c]

[a]Mean ± SEM.

[b]Number of pups.

[c]Significance of difference from controls in same group ($P<0.001$).

15 mM sodium taurocholate (Polysciences, Inc., Rydal, PA) in 5% glucose in Krebs-Ringer buffer from which the calcium and magnesium salts had been omitted; or 2) 60 mM triolein emulsion (analytical grade, Sigma Chemical Co., St. Louis, MO; glycerol tri(1-^{14}C)oleate, Amersham-Searle Corp., Arlington Heights, IL) containing 3 mM sodium taurocholate, 5% glucose, and 4% lecithin (Sigma Chemical Co., St. Louis, MO) as an emulsifying agent and calcium- and magnesium-free Krebs-Ringer buffer. Oleic acid was administered to unsuckled newborn and 12-day old young; triolein absorption was studied in both unsuckled and suckled newborn, and in 4-, 8-, and 12-day old pups. The concentration of the micellar solution of oleic acid was increased for 12-day old pups when preliminary experiments indicated 100% absorption in less than the 15 min period used in this study.

It was determined in preliminary experiments that 50 µl of solution infused at a rate of 1 µl/sec would fill ca 2/3 of the length of the intestine of newborn young of protein-deprived dams. In control young, the same proportion of intestine was filled by 100 µl of solution when the infusion rate was kept constant. Doses for control and PPD young were 200 and 100 µl, respectively, for control and PPD 4-day old young; 300 and 200 µl at 8 days; and 400 and 300 µl at 12 days. Any sample which was found to have filled less than 60% of the length of the intestine was discarded.

After infusion of the test substance, absorption was allowed to proceed for 15 min. The thoracic cavity was then opened, and cardiac blood was collected in a microhematocrit tube for scintillation counting. The intestine and stomach were removed with the cannula still in situ, and the total small intestinal length and the length filled by the infusate were measured. The intestine was flushed through the cannula with 0.2 M sodium fluoride. This fluid, which removed unabsorbed infusate and inhibited further absorption, was collected and designated as the "initial flush." A "final flush" of physiological saline was then made via the cannula and was collected in a separate scintillation vial. This final flush was used to determine the efficiency of the removal of labeled material from the lumen. Values in excess of 5% of the radioactivity present in the intestinal tissue were considered unacceptable, and the samples were discarded.

Finally, the intestine was flushed with air, the cannula was removed from the duodenum, the intestine and stomach were separated, and the mesentery was stripped from the length of the small intestine. The stripped intestine was weighed and placed in a scintillation vial for counting.

All samples were solubilized in 5 ml of solubilizer (NCS, Amersham-Searle Corp., Arlington Heights, IL) to which a few drops of 0.1 M acetic acid were added. Ten ml toluene-based scintillation fluid (PPO and POPOP, New England Nuclear, Boston, MA) was added before counting. Samples were counted in a Packard Tri-Carb Model II refrigerated scintillation counter (Nuclear-Chicago Corp., Des Plaines, IL) using a ^{14}C-channels ratio method.

The amount of material present in the vial of dissolved intestine was designated as the amount "retained." The amount of material present in the initial and final flushes represented material present in the lumen at the end of the absorptive period and was therefore considered to be "unabsorbed." The amount

TABLE II

Intestinal Weights and Lengths

Age	Weight (cm)		Length infused (cm)	
	Control	Experimental	Control	Experimental
Newborn				
Unsuckled	--	--	13.27 ± 0.45[a]	8.39 ± 0.36[b]
Suckled	--	--	13.22 ± 0.37	8.40 ± 0.26[b]
4 day	0.3134 ± 0.0141	0.1837 ± 0.0117[b]	17.1 ± 0.5	14.0 ± 0.5[c]
8 day	0.4482 ± 0.0253	0.3024 ± 0.0091[b]	28.5 ± 1.0	25.9 ± 0.8
12 day	0.6760 ± 0.0388	0.4808 ± 0.0324[c]	29.6 ± 1.7	24.5 ± 1.4[d]

[a]Mean ± SEM.

[b]Significance of difference from controls, $P<0.001$.

[c]Significance of difference from controls, $P<0.01$.

[d]Significance of difference from controls, $P<0.05$.

TABLE III

Number of Enterocytes in Suckling Rats

Observations	Diet	
	24% Casein	4% Casein
Enterocytes x 10^6/cm		
Newborn unsuckled	4.36 ± 0.10[a]	2.65 ± 0.14[b]
Newborn suckled	4.36 ± 0.08	2.61 ± 0.10[b]
4 day	6.58 ± 0.08	4.43 ± 0.11[b]
8 day	9.15 ± 0.11	7.28 ± 0.11[b]
12 day	9.95 ± 0.17	8.50 ± 0.16[b]
Enterocytes x 10^6/g intestine		
4 day	357.15 ± 10.75	337.62 ± 12.90
8 day	581.82 ± 19.95	623.91 ± 19.81
12 day	436.26 ± 24.54	433.17 ± 25.52
Enterocytes x 10^6/g body wt		
Newborn unsuckled	8.23 ± 0.76	6.61 ± 0.31[c]
Newborn suckled	9.22 ± 0.24	6.29 ± 0.16[b]
4 day	11.14 ± 0.31	11.48 ± 0.76
8 day	14.71 ± 0.93	18.50 ± 0.82[c]
12 day	11.42 ± 0.64	14.19 ± 1.16

[a]Mean ± SEM.

[b]Significance of difference from control, $P<0.001$.

[c]Significance of difference from control, $P<0.05$.

actually taken up by the intestine was therefore calculated as the difference between the amount infused and the residual material in the flushes. This value has been designated as "absorbed," in the manner used by others (6,7). The amount "transferred" from the intestinal lumen through the enterocytes was calculated by difference between absorption and retention.

The quantity of materials absorbed, retained, and transferred was expressed as a total amount and on the bases of body weight, per g of intestinal tissue, and per cm of infusate-filled intestine. Calculations on a per cell basis were based on the number of absorptive cells per cm of intestine obtained as previously described (8). To obtain an estimate of the total number of absorptive cells exposed to the infusate, an average of the number of cells per cm of duodenum and jejunum was multiplied by the number of centimeters of intestine filled with the infusate. The number of enterocytes per cm and per g of intestine and per g of body weight were also calculated.

Data were analyzed using Student's *t*-test (9).

RESULTS

Mean body weights of unsuckled and

suckled control and PPD newborn young and postnatal young are shown in Table I. There were no significant differences in body weights of suckled and unsuckled newborn animals in the same diet group or in body weights of triolein-infused and oleic acid-infused pups in the same diet group. At each age, body weights of PPD animals were significantly less than controls ($P<0.001$).

Weights and lengths of that portion of the intestines used for absorption studies are given in Table II. Intestinal weights were significantly decreased ($P<0.01$) in PPD animals at 4, 8, and 12 days. Intestinal weights were not obtained from newborn young. The lengths of the intestine infused were significantly reduced in PPD young in newborn pups and at 4 and 12 days. Lengths were also decreased at 8 days, but the difference was not statistically significant ($P<0.1$).

The number of enterocytes per unit length and per unit weight of intestine, and per unit body weight are given in Table III. The numbers of enterocytes per unit length of intestine was significantly ($P<0.001$) decreased in PPD animals at all ages, but did not differ significantly when expressed on the basis of intestinal weight. The number of enterocytes per g body weight was significantly decreased in newborn animals, did not differ at 4 days, and was significantly increased at 8 days.

Triolein Absorption

New born Young

Intestinal absorption of triolein and blood triolein levels in unsuckled and suckled newborn rats are shown in Table IV.

Effect of suckling: Suckling resulted in somewhat different effects in control and PPD animals. In the suckled control group, retention, regardless of the basis on which it was expressed, was significantly increased ($P<0.01$) compared to unsuckled controls. Absorption was somewhat less significantly affected ($P<0.05$), while transfer was unaffected.

PPD animals responded to suckling with a significant increase ($P<0.001$) in absorption. However, PPD animals also showed a significant increase ($P<0.001$) in transfer, as well as in retention ($P<0.01$).

Effect of prenatal diet on suckled newborn young: Absorption in PPD young was significantly decreased per g body weight ($P<0.01$), per cm intestine, and in total ($P<0.001$) as compared with controls. In both control and PPD animals, absorption differed statistically from the pattern demonstrated in unsuckled animals only when expressed on a per absorptive cell basis, where it was slightly, but not significantly, reduced. Retention in suckled PPD pups was also decreased very significantly ($P<0.001$). As in unsuckled pups, transfer was not significantly altered.

Effect on blood levels: Triolein levels in the blood were not significantly different in unsuckled PPD animals when compared to the corresponding controls. In suckled animals, however, blood triolein levels were significantly increased ($P<0.05$) in PPD young.

Postnatal Young

Intestinal absorption of triolein and blood triolein levels in 4-, 8-, and 12-day old young are shown in Table V. Total absorption and absorption per cm of intestine were significantly reduced ($P<0.05$) in PPD young at 4 days and 8 days, but not at 12 days. There was no significant difference in absorption expressed on the other bases.

At 4 days, the retention of triolein by PPD young was decreased compared to controls regardless of the basis on which it was expressed. By 8 days, there was no longer any difference in retention between the two groups.

The total amount of triolein transferred in PPD young was significantly less than that in controls at 4 and 8 days. At 12 days, the mean values were markedly different, but the difference was not statistically significant in view of the large standard deviation. The data also indicated that, at 8 days, transfer of triolein per cm of intestine was significantly ($P<0.05$) reduced as compared with controls, while at 4 days and 12 days the data were extremely variable and the reduction was not statistically significant. There were no differences when data were expressed per g of body weight, per g of intestinal weight, or per cell.

There were no significant differences in concentrations of labeled lipid in the blood at any age.

Effect of Age

A comparison of values for suckled newborn young and the successive postnatal ages studied shows that total transfer and absorption increased sharply between birth and 4 days, reaching a peak at 8 days, then dropping precipitously almost to newborn levels by 12 days. Transfer and absorption, expressed on a body weight basis or per cm of intestine, rose sharply by 4 days. The values per g of intestine were not obtained in newborn pups. However, in postnatal animals, these values are also at a plateau between 4 and 8 days and drop sharply by 12 days. On a per cell basis, transfer and absorption in both groups peak at 4 days and

TABLE IV

Intestinal Absorption and Blood Levels of Triolein in Newborn Control and Prenatally Protein-deprived Rats

Observation	Maternal diet			
	24% Casein		4% Casein	
	Unsuckled	Suckled	Unsuckled	Suckled
Total ($\mu M \times 10^{-1}$)				
Retention	3.52 ± 0.38[a] (11)[b]	5.46 ± 0.47 (10)[c]	0.48 ± 0.08 (11)[d]	1.04 ± 0.13 (10)[c,e]
Transfer	1.96 ± 0.55	2.17 ± 0.65	0.91 ± 0.13	1.65 ± 0.24[f]
Absorption	5.48 ± 0.58	7.63 ± 0.70[f]	1.39 ± 0.17[d]	2.69 ± 0.25[e,g]
Per g body wt ($\mu M \times 10^{-2}$)				
Retention	5.50 ± 0.33	8.67 ± 0.76[c]	1.41 ± 0.20[d]	3.02 ± 0.41[c,e]
Transfer	3.07 ± 0.87	3.46 ± 1.03	2.70 ± 0.40	4.76 ± 0.70[f]
Absorption	8.58 ± 0.92	12.22 ± 1.09[f]	4.11 ± 0.49[d]	7.78 ± 0.75[g,h]
Per cm intestine ($\mu M \times 10^{-2}$)				
Retention	2.66 ± 0.28	4.23 ± 0.45[c]	0.58 ± 0.09[d]	1.25 ± 0.17[c,e]
Transfer	1.52 ± 0.47	1.59 ± 0.44	1.07 ± 0.50	1.97 ± 0.27[f]
Absorption	4.18 ± 0.51	5.82 ± 0.55[f]	1.65 ± 0.19[d]	3.22 ± 0.29[e,g]
Per absorptive cell ($\mu M \times 10^{-9}$)				
Retention	6.13 ± 0.65	9.70 ± 1.04[c]	2.17 ± 0.33[d]	4.73 ± 0.66[c,e]
Transfer	3.49 ± 1.09	3.65 ± 1.02	4.04 ± 0.57	7.42 ± 1.03[f,i]
Absorption	9.62 ± 1.18	13.35 ± 1.26[f]	6.21 ± 0.72[j]	12.15 ± 1.10[g]
Concentration in blood ($\mu M \times 10^{-5}/\mu l$)	1.18 ± 0.36	1.33 ± 0.40	1.39 ± 0.30	1.81 ± 0.46[i]

[a]Mean ± SEM.

[b]Number of pups.

[c]Significance of difference from unsuckled pups in same diet group ($P<0.01$).

[d]Significance of difference from unsuckled controls ($P<0.001$).

[e]Significance of difference from suckled controls ($P<0.001$).

[f]Significance of difference from unsuckled pups in same diet group ($P<0.05$).

[g]Significance of difference from unsuckled pups in same diet group ($P<0.001$).

[h]Significance of difference from suckled controls ($P<0.01$).

[i]Significance of difference from suckled controls ($P<0.05$).

[j]Significance of difference from unsuckled controls ($P<0.05$).

TABLE V

Intestinal Absorption and Blood Levels in Postnatal Control and Prenatally Protein-deprived Rats

Observation	Age of young					
	4 Day		8 Day		12 Day	
	Control	Experimental	Control	Experimental	Control	Experimental
Total ($\mu M \times 10^{-1}$)						
Retention	6.0 ± 1.0[a](8)[b]	1.8 ± 0.2 (8)[c]	4.29 ± 0.68 (6)	2.50 ± 0.53 (6)	6.84 ± 1.11	6.44 ± 1.69 (11)
Transfer	27.8 ± 3.0	17.0 ± 1.5[c]	48.21 ± 3.98	35.56 ± 1.71[d]	16.58 ± 0.31	8.67 ± 3.44
Absorption	33.8 ± 2.8	18.8 ± 1.4[e]	52.50 ± 3.94	38.06 ± 1.44[d]	23.42 ± 3.05	15.11 ± 3.38
Per g body weight ($\mu M \times 10^{-2}$)						
Retention	6.10 ± 1.14	3.25 ± 0.34[d]	2.57 ± 0.49	2.53 ± 0.62	2.64 ± 0.41	4.19 ± 1.09
Transfer	27.96 ± 3.42	31.84 ± 3.80	28.26 ± 2.26	34.78 ± 1.75	6.60 ± 1.40	5.25 ± 2.05
Absorption	34.06 ± 3.70	35.09 ± 3.61	30.83 ± 2.41	37.31 ± 1.77	9.24 ± 1.37	9.44 ± 1.90
Per cm intestine ($\mu M \times 10^{-2}$)						
Retention	3.61 ± 0.68	1.28 ± 0.15[c]	1.54 ± 0.29	0.99 ± 0.23	2.40 ± 0.46	2.68 ± 0.71
Transfer	16.52 ± 2.01	12.49 ± 1.68	16.91 ± 1.15	13.77 ± 0.57[d]	5.89 ± 1.45	3.14 ± 1.15
Absorption	20.13 ± 2.16	13.77 ± 1.61[d]	18.45 ± 1.15	14.76 ± 0.74[d]	8.29 ± 1.46	5.82 ± 1.11
Per g intestine (μM)						
Retention	1.92 ± 0.31	0.97 ± 0.09[d]	0.98 ± 0.18	0.81 ± 0.15	0.97 ± 0.11	1.22 ± 0.26
Transfer	9.18 ± 1.34	9.75 ± 1.38	10.76 ± 0.59	11.91 ± 0.90	2.56 ± 0.51	1.96 ± 0.84
Absorption	11.10 ± 1.37	10.72 ± 1.33	11.74 ± 0.62	12.72 ± 0.84	3.53 ± 0.48	3.18 ± 0.79
Per absorptive cell ($\mu M \times 10^{-9}$)						
Retention	5.51 ± 1.03	2.88 ± 0.33[d]	1.68 ± 0.32	1.37 ± 0.32	2.41 ± 0.46	3.18 ± 0.82
Transfer	25.17 ± 3.06	28.20 ± 3.81	18.48 ± 1.22	18.90 ± 0.90	5.92 ± 1.45	3.72 ± 1.34
Absorption	30.68 ± 3.30	31.08 ± 3.63	20.16 ± 1.35	20.27 ± 1.01	8.33 ± 1.47	6.90 ± 1.28
Blood concentration ($\mu M \times 10^{-5}$/ul)	1.51 ± 0.18	1.60 ± 0.15	1.11 ± 0.21	1.50 ± 0.31	0.48 ± 0.06	0.64 ± 0.05

[a]Mean ± SEM.

[b]Number of pups.

[c]Significance of difference from controls of same age, $P<0.01$.

[d]Significance of difference from controls of same age, $P<0.05$.

[e]Significance of difference from controls of same age, $P<0.001$.

TABLE VI

Intestinal Absorption and Blood Levels of Oleic Acid in Control and Prenatally Protein-deprived Rats

	Age of young			
	Newborn		12 days	
	Control	Experimental	Control	Experimental
Total ($\mu M \times 10^{-2}$)				
Retention	6.87 ± 0.20[a] (13)[b]	2.84 ± 0.15 (8)[c]	63.6 ± 3.0 (7)	58.6 ± 2.9 (8)
Transfer	2.40 ± 0.23	1.34 ± 0.19[d]	80.3 ± 3.9	58.8 ± 4.6[d]
Absorption	9.27 ± 0.11	4.18 ± 0.05[c]	143.9 ± 4.7	118.4 ± 3.0[c]
Per g body weight ($\mu M \times 10^{-2}$)				
Retention	1.608 ± 0.03	0.85 ± 0.04[c]	2.49 ± 1.1	3.76 ± 2.9[d]
Transfer	0.38 ± 0.04	0.40 ± 0.06	3.16 ± 1.9	3.76 ± 2.4
Absorption	1.46 ± 0.09	1.25 ± 0.04[c]	5.65 ± 2.3	7.52 ± 3.1[c]
Per cm intestine ($\mu M \times 10^{-2}$)				
Retention	0.520 ± 0.017	0.301 ± 0.013[c]	2.78 ± 0.26	2.51 ± 0.12
Transfer	0.183 ± 0.018	0.145 ± 0.022	3.45 ± 0.14	2.59 ± 0.25[e]
Absorption	0.703 ± 0.015	0.446 ± 0.012[c]	6.23 ± 0.35	5.10 ± 0.72[c]
Per g intestine ($\mu M \times 10^{-1}$)				
Retention	--	--	8.99 ± 0.68	10.84 ± 0.79
Transfer	--	--	11.19 ± 0.26	11.22 ± 1.38
Absorption	--	--	20.18 ± 0.77	22.06 ± 1.57
Per absorptive cell ($\mu M \times 10^{-9}$)				
Retention	1.19 ± 0.03	1.14 ± 0.04	2.80 ± 0.26	2.96 ± 0.14
Transfer	0.42 ± 0.03	0.55 ± 0.08	3.46 ± 0.14	3.05 ± 0.30
Absorption	1.61 ± 0.03	1.69 ± 0.04	6.26 ± 0.36	6.01 ± 0.23
Blood concentration ($\mu M \times 10^{-6}/\mu l$)	1.17 ± 0.06	0.86 ± 0.11[e]	6.02 ± 0.74	5.69 ± 1.03

[a]Mean ± SEM.

[b]Number of pups.

[c]Significantly different from controls of same age, $P<0.001$.

[d]Significantly different from controls of same age, $P<0.01$.

[e]Significantly different from controls of same age, $P<0.05$.

then decrease at a regular rate to approximately newborn levels by 12 days. Amounts retained by the absorptive cells change much less markedly during the period studied.

Oleic Acid

Data on intestinal absorption, retention, and transfer and on blood concentrations of oleic acid in newborn and 12-day old control and PPD young are given in Table VI. In newborn PPD young, total retention and absorption, as well as retention and absorption per g body weight and per cm intestine, were significantly reduced ($P<0.001$). There were no differences between control and PPD young in absorption or retention per cell. Total transfer of oleic acid was significantly reduced in PPD animals ($P<0.01$).

At 12 days of age in intestines of PPD young, total absorption and absorption per cm of intestine were significantly reduced ($P<0.001$). Absorption per cell and per g intestine did not differ from that in control pups. Absorption per g body weight was increased ($P<0.001$). Total retention and retention per cm or g of intestine did not differ significantly from that in controls. Retention per g body weight was significantly increased ($P<0.01$). Total transfer and transfer per cm of intestine were significantly decreased ($P<0.05$). Transfer per g of body weight, per g of tissue, or per cell were not significantly affected.

Oleic acid level in the blood was significantly reduced in newborn PPD young ($P<0.05$), but not at 12 days.

DISCUSSION

Methods

The various methods used to study intestinal absorption have recently been reviewed (6). Some technical limitations were imposed by the small size of the animals in this study and the extremely fragile intestine existing in the newborn rat. As a result, in vitro techniques with everted sacs were impractical. In addition, the results of Sylven (10) suggest that intestinal absorption of lipid cannot be adequately studied by conventional in vitro methods; therefore, a method was chosen for this study in which normal blood and lymph supplies were maintained.

Results of studies of intestinal absorption might be expressed as a total per intestine or may be based on weight, length, or surface area of gut, or per enterocyte. Dry weight as a basis for expressing results was not used in the present study since previous work (11) had shown that the proportion of water in control and PPD young did not differ. Intestinal length data is often somewhat inaccurate since tension can cause changes in length and since intestinal diameter is not considered. It is important to the present study that villus development in PPD animals was considerably reduced compared to controls (8). As a consequence, an equivalent length of intestine represents widely divergent amounts of absorptive surface area in the two groups. Expression of results on a per cell basis provides information on the comparative absorptive capabilities of the enterocytes of the two types of rat pups. Since none of the available parameters is ideal for expression of results, several were used in the study in order that one mode might compensate for the deficiencies of another.

Triolein Absorption

Since the absolute amount of triolein transferred by the enterocyte is not significantly different in unsuckled newborn control and PPD animals, PPD young must transfer a greater proportion of the material absorbed, indicating that metabolism and transport within the cell are not rate-limiting. The increased proportion transferred results in a decreased amount remaining in the cells. This suggests that the rate-limiting step in the individual cells is in the uptake of triolein from the lumen into the enterocytes.

In suckled PPD newborns and 4-day young, however, the deficit in absorption per cell no longer exists, and a larger proportion of the amount absorbed is transferred. As a result, the amount retained in the cell is significantly less ($P<0.05$) than in controls. It must be concluded that the adaptation to suckling is sufficiently greater in the PPD young compared to controls that it compensates for the effects of the prenatal diet seen in the unsuckled rat. The data, therefore, demonstrate that, following suckling, individual enterocytes in control and PPD young were equally capable of uptake and transfer of triolein.

The unsuckled and suckled PPD animals do not, however, represent exactly the same population. PPD animals had a 31% mortality rate in the first 24 hr (8). In previous studies of effects on intestinal morphology in the young of prenatal protein deprived rats (8), it was noted that studies of postnatal young do not include those most affected because of the low postnatal survival rate. It has been reported previously that most young who did not survive had not suckled (12). The survivors may be those which are less affected by the protein deficiency and which are capable of the adapta-

tion necessary for absorption of an adequate quantity of nutrients to maintain life. Recently a method has been developed which may make it possible to study in newborn suckled and postnatal animals the more affected young in these litters (13). Procedures for the application of this method to the present animal model are currently being developed.

Morphological studies have shown that the intestine of newborn PPD young was shorter and narrower and had reduced numbers of villi per unit length as compared with controls. The villi were also shorter. As a result, there were reduced numbers of absorptive cells in total, per cm of gut, and per g of body weight in newborn PPD young (8). The weight and length data obtained in the present study confirm these findings. These changes, separately or in combination, may explain the decrease in retention and absorption when expressed on these bases. Since transfer does not differ, however, it must also be assumed that transfer proceeds at a more rapid rate in each individual cell in PPD young in order to compensate for the reduced numbers of cells. The data on transfer per enterocyte tends to support this concept, although the differences are not statistically significant.

Enterocytes per g of gut are approximately equal in number at all postnatal ages. With the exception of retention at 4 days, there were no differences in absorption, retention, or transfer at the postnatal ages studied, suggesting that these activities are a function of the numbers of absorptive cells as in suckled newborn young. The reduction in numbers of enterocytes per cm of intestine is accompanied by a simultaneous reduction in absorption per cm, supporting in general the conclusion that absorptive capacity depends on numbers of cells.

When absorption per g of body weight is examined, a different relationship is seen to exist. At 4 days, the number of enterocytes per g body weight in PPD animals exceeds that in the controls, but absorption per g body weight does not increase in proportion.

The changes in rate of triolein absorption and transfer with age cannot be relative to numbers of cells since the numbers of cells do not simultaneously change in the same proportion or direction with age (8). It is obvious that a marked change has occurred in the absorptive or digestive processes or both of this organ. The sharp increase in absorption in the first half of the suckling period has been observed previously. Hahn and Koldovsky (14,15) report a high rate of absorption of olive oil in 10-day old suckling rats. These findings could be consistent with those of the present study. Triolein absorption in intestines of control and PPD young reached a peak at the same time. This suggests that the changes are age-related, not related to the degree of morphological development.

The mechanism by which the absorption of triglyceride occurs in the suckling rat and by which the rate of absorption changes is open to some question since the suckling rat apparently lacks the components required (16) for lipid digestion and absorption in the manner in which these take place in adults. It has been shown in rats that intestinal lipase remains at extremely low levels until the age of 15 days (17). The concentration of pancreatic lipase was reported to be increased between days 10 and 20 in pancreatic homogenates (15), but the amount secreted by the pancreas into the intestinal lumen rises only at weaning (18,19). Since an increase in lipid absorption was found to occur at an earlier age in this study, availability of lipase cannot account for the change. Also, a subsequent decrease in absorption seems to occur before lipase levels increase. Therefore, the presence or absence of lipase would not seem to account for the observed changes in absorption.

The availability of bile salts in the suckling rat is unknown. The amount in the human infant has been measured at half of adult values (20). If this is also true in the rat, a deficiency of bile salts might account for the drop in triolein absorption at 12 days since the critical micellar concentration of taurocholate (21) was not provided in the perfusate used.

It has been suggested that fat absorption in the neonatal rat occurs by a process of pinocytosis (18) which apparently requires neither lipase nor bile salts. If it is assumed that the suckling rat loses the ability to pinocytize lipids between 8 and 12 days and that the high fat level in rat milk must then be digested and absorbed as in the adult, then an increase in bile salt requirement would simultaneously occur. The high fat intake of the suckling rat along with a lack of lipase and a possible lack of bile salts has been noted previously. Koldovsky (15) refers to this "paradox" and suggests that the explanation may lie in a change in rate, rather than amount, of lipid absorption.

The present study does not indicate the form in which the triolein was absorbed. Previous work (4) has indicated the presence of large fat droplets in the enterocytes. However, the mucosal cells of the newborn rat have been shown to be able to esterify fatty acids from birth (15); therefore, the triglyceride could be formed intracellularly rather than result from pinocytosis.

Oleic Acid

The lack of significant difference between control and PPD animals in oleic acid absorption, retention, and transfer when expressed on a per cell basis suggests that the rates of uptake of fatty acid, of metabolic processes within the cell, and transport of fatty acid from the cell do not differ in the enterocytes of the control and PPD young.

In the newborn control rats, 75% of the absorbed material was retained in the cells. As in adult rats (21), the rate-limiting step is not in uptake from the lumen since the oleic acid accumulates in the enterocytes and is transported away at slower rates. In the PPD young, 68% of the oleic acid absorbed accumulated in the cells, slightly but not significantly less than in controls. As animals mature in either group, the rate of transfer seems to increase. At 12 days, retention is 45% of absorbed material in controls and 50% in PPD animals. It seems reasonable to assume that the reduced absorption and retention per g body weight and per cm intestine are the consequence of the reduced number of cells in relation to those parameters as was observed in triolein absorption.

These similarities might indicate that the mechanisms for absorption of triglyceride and of fatty acid are alike in the suckling rat. However, there is an important point of difference in the data on absorption of the two substances. While triglyceride absorption has returned almost to newborn levels by 12 days, the oleic acid absorption has increased sharply. If the concept of low bile salt and lipase levels with absorption by pinocytosis in very young rats is accepted, oleic acid absorption in the newborn may be of little physiological significance. The data do suggest that the suckling rat may lose its capacity for pinocytosis while the ability to absorb fatty acids is increasing. It seems reasonable to speculate that, under these circumstances, a simultaneous increase in the lipases and bile salts required for adult-type lipid digestion is occurring.

The rapid rise in triglyceride transfer is not followed by a concurrent rise in triglyceride concentration in the blood. It has been reported that in young rats lipids are rapidly removed from the blood (22). Lipoprotein lipase level has been found to be high in the perinatal period, and adipose tissue weight increases markedly. The significantly higher blood triolein content in newborn suckled PPD rats may indicate a deficit of lipoprotein lipase. Over the 12 day period studied, however, it appears that their capacity to remove triglycerides from the blood is adequate to maintain normal blood lipid concentration.

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Effect of Dietary Fats on Ovine Adipose Tissue Metabolism

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ABSTRACT

The effects of different dietary fats on ovine adipose tissue metabolism have been investigated. Six-month old sheep were fed for 6 weeks a control diet or diets supplemented with either tallow or a mixture of sunflower seed oil and soybean oil, treated to protect the fats from hydrolysis and hydrogenation in the rumen, or with maize oil. The rates of fatty acid, glyceride glycerol, and CO_2 formation were measured in perirenal and subcutaneous adipose tissue slices by following the incorporation of either ^{14}C from labeled acetate or glucose, or ^{3}H from tritiated water into the appropriate product. Feeding protected tallow or maize oil but not protected sunflower seed oil plus soybean oil resulted in reduced rates of fatty acid biosynthesis in both perirenal and subcutaneous adipose tissue slices and CO_2 formation in perirenal adipose tissue. Feeding the fat-supplemented diets had no effect on the rate of glyceride glycerol formation. The fat-supplemented diets also resulted in reduced activities of various enzymes, thought to be involved in lipogenesis, measured in 105,000 x g supernatant fractions from adipose tissue homogenates. The results suggested that ovine adipose tissue lipogenesis is sensitive to both the amount and the nature of dietary fat.

INTRODUCTION

Although there is interest at present in increasing the proportion of polyunsaturated fatty acids (PUFA) in the lipids of ruminant tissues, little is known about the effects of such increased levels of PUFA upon ruminant metabolism (1-5).

Studies with rodents have shown that dietary PUFA are much more potent inhibitors of hepatic lipogenesis than are dietary saturated or monounsaturated fatty acids (6-8). Effects of different dietary fats on adipose tissue lipogenesis are less clearly defined. Dietary methyl linoleate has been reported to be a more effective inhibitor of rat adipose tissue lipogenesis than dietary methyl oleate (9), whereas a recent study has shown that dietary tallow resulted in a greater degree of inhibition of adipose tissue lipogenesis in rats and pigs than did dietary safflower oil (8).

Administration of safflower oil to suckling lambs reduced the rate of adipose tissue lipogenesis; however, the amount of safflower oil given comprised only 10% of the total fat intake (10). Also, due to the rudimentary development of rumen function, the digestive processes in the neonatal lamb resemble those in simple stomached animals. Thus, the metabolic response to different dietary fats in the young lamb may be different from those in the adult sheep with a fully functioning rumen.

This paper describes the different effects of various dietary fats upon adipose tissue metabolism of 6-month old ruminating sheep, and shows that ovine adipose tissue lipogenesis is sensitive to both the amount and the nature of dietary fat.

MATERIALS AND METHODS

Materials

Insulin (24 units/mg), bovine serum albumin (fraction V), reduced glutathione, citric acid, ATP, L-malic acid, sodium acetate, glycylglycine, NADP, D-glucose 6-phosphate, D-glucose, 6-phosphogluconate, DL-isocitrate, coenzyme A (CoA), acetyl CoA, malonyl CoA, NADPH, glucose 6-phosphate dehydrogenase (type VII, 300 units/mg), and 6-phosphogluconate dehydrogenase (type IV, 20 units/mg) were purchased from Sigma (London) Chemical Co. Ltd., Surrey, U.K. NAD, malate dehydrogenase (1100 units/mg), and citrate synthetase (110 units/mg) were purchased from Boehringer Corp. (London) Ltd., U.K. (1-^{14}C)-Acetic acid, 59 mCi/mmol, D-(U-^{14}C)-glucose, 3 mCi/mmol, (^{14}C)-$NaHCO_3$, 0.1 mCi/mmol, and tritiated water, 5 Ci/ml, were obtained from the Radiochemical Centre, Amersham, U.K., and Unisolve I was from Koch-Light Ltd., Colnbrook, U.K.

Albumin was defatted and dialyzed before use (11).

Animals and Diets

Four groups each of five 6-month old sheep of the Cheviot breed were given either a control diet of 500 g of hay and 300 g of concentrates (a cereal mixture) per day or diets in which a proportion of the concentrates was replaced by "protected" tallow, "protected" PUFA (ca. 70% sunflower seed oil, 30% soybean oil), or

TABLE I

Fatty Acid Composition of Dietary Constituents

Fatty acid (wt %)	Hay	Concentrate	Protected[a] tallow	Protected PUFA[b]	Maize oil
14:0	-	-	2.8[c]	-	-
16:0	20.7	27.6	28.3	7.5	9.9
16:1	2.2	0.5	3.2	-	-
18:0	5.6	0.6	17.9	4.5	2.1
18:1	18.9	38.3	41.0	16.5	22.9
18:2	25.0	32.8	4.8	69.9	64.4
18:3	17.9	-	1.5	1.6	0.7

[a]Protected with formaldehyde-treated protein.

[b]70% sunflower seed oil; 30% soybean oil.

[c]Results are means of two determinations.

maize oil. The dietary supplements of tallow and soybean oil were protected with formaldehyde-treated protein against hydrolysis and hydrogenation in the rumen (12); these dietary supplements were the commercial preparations Hi-Energy feed and Polyunsaturated Supplement 147, respectively, and were kindly donated by Alta Lipids U.K. Ltd. The maize oil was obtained from Mazola Products Ltd., Esher, U.K. The total fat intake of the sheep on the control diet was 32 g per day (20 g derived from the hay and 12 g from the cereal mixture), whereas sheep receiving the fat-supplemented diet had a fat intake of 60 g per day (31 g derived from the fat supplement, 9 g from the cereal mixture, and 20 g from the hay). The fatty acid compositions of the dietary constituents are given in Table I. The linoleic acid content of the maize oil was outside the standard range (34-62%) (13); the reason for this is not known, but the purpose of this dietary supplement was to provide a source of unprotected PUFA.

During the first 2 weeks of the experiment, the sheep given the fat-supplemented diets consumed only a proportion of the rations offered. Hence, during this period the food given to the sheep on the control diet was reduced by an appropriate amount. As a result of reduced food intake, there was a loss of body weight by all animals during the first 2 weeks. However, at the end of the 2nd week, all of the sheep were eating their full rations, and, during the subsequent 4 week period, the animals in all groups regained most of the body weight lost during the first 2 weeks of the experiment. After the 6th week on the experimental diets, the sheep were killed with a captive-bolt humane killer.

Tissue Incubations

Immediately after the sheep were killed, samples of adipose tissue were taken from both the perirenal and subcutaneous fat pads and were kept in isotonic saline at 37 C until used for preparation of tissue slices and homogenates (less than 5 min after slaughter). Adipose tissue slices were prepared free-hand. About 100 mg samples of tissue slices were incubated in 2.5 ml Krebs-Ringer bicarbonate buffer with half of original calcium concentration (1.27 mM) containing 2 mM acetate, 5 mM glucose, 0.1 IU/ml insulin, and 40 mg/ml bovine serum albumin, for 2 hr at 37 C in an atmosphere of O_2:CO_2 (95:5 v/v). The incubation medium also contained either (1-^{14}C)-acetate, 0.1 μCi/ml, or (U-^{14}C)-glucose, 0.1 μCi/ml, or ^3H_2O, 1 mCi/ml. When the rate of incorporation of (^{14}C) or (^{3}H) into fatty acids or glyceride glycerol was being measured, the incubations were stopped by removal of the tissue slices with fine forceps followed by homogenization of the tissue in chloroform:methanol:0.15 M KCl (1:1:1, by vol). Lipids were extracted from the homogenates by the Folch procedure (14). Total lipid content of samples was determined gravimetrically, and the radioactivity content of the lipids was measured following solution in a toluene:methanol (7:3 v/v) based scintillation fluid (15). Radioactivity was measured with an efficiency of 85% using an external standard. The radioactivity of the fatty acid fraction of the lipid samples was measured as described previously (10). The radioactivity of the glyceride glycerol fraction was taken as the difference between the radioactivity of the total lipid and fatty acid fractions. The rate of incorporation of (^{14}C) into CO_2 was measured as described previously (10).

Enzyme Assays

Sheep adipose tissue was homogenized at room temperature in 3 vol of a medium comprising 300 mM sucrose, 30 mM tris, 1 mM EDTA, and 1 mM GSH, pH 7.4. Homogeniza-

TABLE II

Effect of Dietary Fats on the Rate of Incorporation of Labeled Precursors into Fatty Acids, Glyceride Glycerol, and CO_2 in Ovine Perirenal Adipose Tissue Slices

Rate of incorporation (nmol/2 hr/mg protein)	Diet			
	Control	Protected tallow	Protected PUFA[a]	Maize oil
(1-^{14}C)-Acetate into fatty acids	737.0 ± 294.2[b]	93.2 ± 32.1[c,d]	472.5 ± 92.9	159.1 ± 46.6[c,d]
$^{3}H_2O$ into fatty acids	523.1 ± 110.3	86.5 ± 29.2[c,d]	469.4 ± 91.4	165.5 ± 36.9[c,d]
(U-^{14}C)-Glucose into glyceride glycerol	40.8 ± 8.7	46.5 ± 5.4	53.4 ± 5.1	45.6 ± 6.9
$^{3}H_2O$ into glyceride glycerol	275.8 ± 63.7	196.3 ± 20.8	213.6 ± 47.3	186.9 ± 22.1
(U-^{14}C)-Glucose into CO_2	101.6 ± 23.5	45.3 ± 6.4[c,d]	81.7 ± 13.9	52.0 ± 4.4[c,d]
(1-^{14}C)-Acetate into CO_2	300.0 ± 40.3	157.1 ± 33.9[c,d]	286.6 ± 35.1	122.2 ± 28.1[c,d]

[a]70% sunflower seed oil; 30% soybean oil.
[b]Results are mean ± SEM of five observations.
[c]Value significantly different ($P<0.05$) from control value.
[d]Value significantly different ($P<0.05$) from value obtained with sheep fed protected PUFA.

tion involved 20 strokes of a teflon pestle, rotating at 300 rpm in a glass body with a clearance of 0.3 mm. The resulting homogenate was filtered through cheesecloth, cooled in ice, and centrifuged at 105,000 x g for 60 min at 4 C. The resulting supernatant fraction was used for enzyme assays.

Acetyl CoA carboxylase (EC 6.4.1.2) was assayed as described by Ingle et al. (16) except that samples were incubated for 6 min at 37 C instead of 10 min and that the removal of unreacted (^{14}C) bicarbonate was performed by heating the vials at 60 C for 60 min prior to the addition of crushed dry-ice. Radioactivity was measured using Unisolve I as scintillation fluid.

The fatty acid synthetase assay was that used by Ingle et al. (16) except that 1 mM EDTA and 1 mM dithiothreitol were used and the final pH was 7.0.

The acetyl CoA synthetase (EC 6.2.1.1) assay was that used by Hanson and Ballard (17).

Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) were assayed by the method of Glock and McLean (18), except that 25 mM glycylglycine and 0.5 mM NADP were used. NADP-isocitrate dehydrogenase (EC 1.1.1.42) was assayed by the method of Ochoa (19) but using 1 mM DL-isocitrate and 0.5 mM NADP. NADP-malate dehydrogenase (EC 1.1.1.40) was assayed by the method of Ochoa (20), except that 0.5 mM NADP was used. Hexokinase (EC 2.7.1.1) assay was based on that of Walker and Holland (21), but using 5 mM glucose and also 1 mM dithiothreitol.

All assays were performed at 37 C using a Gilford model 240 recording spectrophotometer.

Protein concentration of samples was determined by the Lowry method (22).

Lipid Extraction and Analysis

Diet constituents were finely ground or chopped (hay), and refluxed with chloroform: methanol (2:1 v/v) for 3 hr. The lipids were extracted by the Folch procedure (14). Fatty acids were transesterified using methanolic HCl (23) and the relative concentrations of the fatty acids were determined by gas liquid chromatography using columns of 15% ethylene glycol succinate methyl silicone polymer (EGSS-X) on Gas-Chrome P (100-120 mesh; Applied Science Laboratories, State College, PA) at 180 C in a Pye 104 chromatograph.

Presentation of Results

Results are expressed as mean ± standard error of the mean (SEM). Significance was

calculated on the basis of the Mann-Whitney U test (24).

RESULTS

The changes in body weight during the experiment were similar for each dietary treatment, and the mean body weights (± SEM) for the 20 sheep at the beginning of the experiment and after 2 and 6 weeks on the experimental diets were 27.0 ± 1.0, 25.2 ± 0.8, and 26.7 ± 0.9 kg, respectively.

There was considerable variation in the rate of fatty acid biosynthesis, measured by the rates of incorporation of ^{14}C from acetate and ^{3}H from tritiated water into fatty acids, as indicated by the size of the SEM (Tables II and III). Thus, although all three fat supplements appeared to decrease the incorporation of ^{14}C and ^{3}H into fatty acids by both perirenal and subcutaneous adipose tissue slices (Table II and III), the decreased rate of incorporation was significant only in the sheep given the diet supplemented with protected tallow ($P<0.01$) and, apart from the incorporation of ^{14}C into fatty acids by subcutaneous adipose tissue slices, in those animals given the diet supplemented with maize oil ($P<0.05$). A comparison of the effects of the different fat supplements on the rate of incorporation of ^{14}C and ^{3}H into fatty acids showed that sheep fed the protected PUFA-supplemented diet had significantly greater rates of fatty acid biosynthesis in both perirenal and subcutaneous adipose tissue slices ($P<0.05$) than did sheep fed the protected tallow-supplemented diet, and also a greater rate of perirenal adipose tissue fatty acid biosynthesis than animals given the diet supplemented with maize oil ($P<0.05$) (Tables II and III). The results presented in Tables II and III show that supplementing the diet with either protected tallow or protected PUFA (but not with maize oil) gave lower rates of incorporation of ^{14}C and ^{3}H into fatty acids in subcutaneous than in perirenal adipose tissue slices ($P<0.05$).

The ratio of acetate carbon to water hydrogen incorporated into fatty acids was not altered by the various diets and the mean values (± SEM) for the 20 sheep were 1.09 ± 0.11 and 1.13 ± 0.26 for perirenal and subcutaneous adipose tissue slices, respectively. The rates of incorporation of ^{14}C from (U-^{14}C)-glucose into fatty acids by both perirenal and subcutaneous adipose tissue slices was negligible in all groups of sheep (results not presented).

The rates of incorporation of ^{14}C from (U-^{14}C)-glucose or ^{3}H from water into glyceride glycerol by adipose tissue slices were not

TABLE III

Effect of Dietary Fats on the Rate of Incorporation of Labeled Precursors into Fatty Acids, Glyceride Glycerol, and CO_2 in Ovine Subcutaneous Adipose Tissue Slices

Rate of incorporation (nmol/2 hr/mg protein)	Diet			
	Control	Protected tallow	Protected PUFA[a]	Maize oil
(1-^{14}C)-Acetate into fatty acids	607.8 ± 348.5[b]	15.7 ± 4.9[c,d]	122.2 ± 47.5	118.7 ± 73.9
$^{3}H_2O$ into fatty acids	504.8 ± 274.8	16.9 ± 4.0[c,d]	135.1 ± 40.4	79.6 ± 63.4[c]
(U-^{14}C)-Glucose into glyceride glycerol	36.6 ± 4.5	30.6 ± 4.5	34.8 ± 6.9	40.8 ± 8.1
$^{3}H_2O$ into glyceride glycerol	166.8 ± 28.5	131.1 ± 15.9	142.2 ± 39.9	170.3 ± 33.0
(U-^{14}C)-Glucose into CO_2	116.0 ± 41.5	49.8 ± 7.2	47.8 ± 11.3	52.2 ± 6.6
(1-^{14}C)-Acetate into CO_2	252.1 ± 81.6	93.3 ± 30.7	140.0 ± 30.2	133.1 ± 48.6

[a] 70% sunflower seed oil; 30% soybean oil.
[b] Results are mean ± SEM of five observations.
[c] Value significantly different ($P<0.05$) from control value.
[d] Value significantly different ($P<0.05$) from value obtained with sheep fed protected PUFA.

TABLE IV

Effect of Dietary Fats on the Activity of Various Enzymes of Ovine Perirenal Adipose Tissue

Enzyme activity (nmol/min/mg protein)	Diet			
	Control	Protected tallow	Protected PUFA[a]	Maize oil
Acetyl CoA synthetase	44.6 ± 10.2[b]	18.6 ± 1.0[c]	26.2 ± 4.1[c]	24.4 ± 3.8[c]
Acetyl CoA carboxylase	10.7 ± 3.0	3.5 ± 0.4[c,d]	7.2 ± 1.0	6.5 ± 2.4
Fatty acid synthetase	26.8 ± 9.2	11.7 ± 2.2[c]	16.9 ± 1.8	11.4 ± 1.1[c]
Glucose 6-phosphate dehydrogenase	66.0 ± 11.2	36.9 ± 2.2[c]	42.6 ± 6.6[c]	32.7 ± 4.5[c]
6-Phosphogluconate dehydrogenase	83.6 ± 11.4	65.4 ± 2.3	66.1 ± 7.8	46.5 ± 4.5[c,d,e]
Malic enzyme	10.5 ± 0.8	9.1 ± 0.9	10.3 ± 1.0	7.6 ± 0.9[c,d]
NADP-Isocitrate dehydrogenase	429 ± 30	374 ± 18	350 ± 22[c]	342 ± 25[c]
Hexokinase	15.5 ± 0.6	15.7 ± 1.6	15.1 ± 1.5	14.2 ± 0.6

[a]70% sunflower seed oil; 30% soybean oil.
[b]Results are mean ± SEM of five observations.
[c]Value significantly different ($P<0.05$) from control value.
[d]Value significantly different ($P<0.05$) from value obtained with sheep fed protected PUFA.
[e]Value significantly different ($P<0.05$) from value obtained with sheep fed protected tallow.

altered by dietary treatment (Tables II and III). The rates of incorporation of ^{14}C from both (U-^{14}C)-glucose and (1-^{14}C)-acetate into CO_2 were significantly reduced ($P<0.05$) in the perirenal adipose tissue slices from the sheep given maize oil or protected tallow (Table II). However, in the perirenal adipose tissue slices of the sheep given protected PUFA, the rates of incorporation of ^{14}C from glucose and acetate into CO_2 were similar to the rates observed for the animals given the control diet (Table II). On the other hand, all three dietary fat supplements appeared to result in decreases in the incorporation of ^{14}C from glucose and acetate into CO_2 by subcutaneous adipose tissue slices, but these decreases were not statistically significant.

The effects of the fat-supplemented diets on the activities of perirenal adipose tissue enzyme were varied (Table IV). Thus, the activities of acetyl CoA synthetase and glucose 6-phosphate dehydrogenase were significantly ($P<0.05$) reduced by all three fat-supplemented diets, whereas the activity of acetyl CoA carboxylase was reduced only when protected tallow was included in the diet. Similarly, the activities of 6-phosphogluconate dehydrogenase and malic enzyme were significantly ($P<0.05$) reduced by only the maize oil-supplemented diet. Hexokinase activity was not altered by any of the fat-supplemented diets.

The activities of subcutaneous adipose tissue enzymes were less affected by the fat-supplemented diets than were the activities of perirenal adipose tissue enzymes (Tables IV and V). There was, in fact, a significant reduction in the activity of only fatty acid synthetase and glucose 6-phosphate dehydrogenase (all three diets).

The 105,000 x g supernatant protein concentrations of both perirenal and subcutaneous adipose tissues were not altered by the various diets; pooled values were 3.52 ± 0.19 and 3.01 ± 0.17 mg/g adipose tissue (mean ± SEM of 20 observations) for perirenal and subcutaneous adipose tissue, respectively.

The fat-supplemented diets had no effect on either perirenal or subcutaneous adipose tissue total lipid concentration, pooled values being 708 ± 9 and 681 ± 14 mg/g adipose tissue, respectively: values are mean ± SEM of 20 observations.

DISCUSSION

The reason for the great variation in the rate of fatty acid biosynthesis in particular and other metabolic and enzymic activities within groups is not obvious. The effect of this was to

reduce the sensitivity of the experiment in so much as very large changes in mean values were required to demonstrate a statistically significant difference, and it also suggested that a nonparametric statistical test such as the Mann-Whitney U test (24) would be the most appropriate method of statistical analysis. Even so, the results indicate that the different fat-supplemented diets had markedly different effects on the rate of adipose tissue fatty acid biosynthesis.

The rate of incorporation of ^3H from tritiated water into fatty acids is considered to give an estimate of the total rate of fatty acid biosynthesis from all precursors (25,26). Studies with rats have shown that the incorporation of 2 ^3H from tritiated water into fatty acid is equivalent to the incorporation of 1.15 acetyl units (25,27). The ratio of C to H incorporated into fatty acids found in this study, 1.09 ± 0.11 and 1.13 ± 0.26 for perirenal and subcutaneous adipose tissue, respectively, would suggest that acetate was the precursor for essentially all de novo fatty acid synthesis in the sheep adipose tissue. This confirms previous conclusions that acetate is the predominant source of de novo fatty acids in adult ruminants, based on the relative rates of incorporation of ^{14}C into fatty acids from various ^{14}C-labeled precursors (28-30). The effect of the fat-supplemented diets on the rate of incorporation of ^3H from tritiated water into fatty acids also showed that the reduced rates of incorporation of ^{14}C from acetate into fatty acids observed were not due to an increase in the size of the cytoplasmic acetyl CoA pool resulting in an isotope dilution effect. The reduced rates of incorporation of ^{14}C from isotopically-labeled glucose and acetate into CO_2 observed when feeding fat-supplemented diets, however, could have been due to an increase in the size of the mitochondrial acetyl CoA pool due to increased fatty acid oxidation. The lack of effect of the various dietary fats on the incorporation of ^{14}C from glucose into glyceride glycerol would suggest that dietary fats have no effect on the early steps of glycolysis in ovine adipose tissue. The lack of change in the activity of hexokinase would also suggest this.

There was no obvious relationship between the effects of the various fat-supplemented diets on the rate of fatty acid biosynthesis and on the activities of enzymes examined. In fact, the fat-supplemented diets produced reduced activities of more enzymes of perirenal adipose tissue than subcutaneous adipose tissue, whereas these diets resulted in greater decreases in the overall rate of subcutaneous adipose

TABLE V

Effect of Dietary Fats on the Activity of Various Enzymes of Ovine Subcutaneous Adipose Tissue

Enzyme activity (nmol/min/mg protein)	Diet			
	Control	Protected tallow	Protected PUFA[a]	Maize oil
Acetyl CoA synthetase	17.2 ± 4.7[b]	11.1 ± 1.6	12.4 ± 3.1	11.9 ± 2.8
Acetyl CoA carboxylase	5.2 ± 1.7	3.1 ± 1.0	2.8 ± 0.7	3.2 ± 1.6
Fatty acid synthetase	23.8 ± 5.4	8.5 ± 3.1[c]	10.0 ± 2.7[c]	8.2 ± 2.0[c]
Glucose 6-phosphate dehydrogenase	51.5 ± 11.9	30.3 ± 3.3[c]	29.5 ± 3.7[c]	29.4 ± 5.7[c]
6-Phosphogluconate dehydrogenase	58.2 ± 6.9	49.8 ± 2.0	42.1 ± 6.7	42.5 ± 6.9
Malic enzyme	7.7 ± 0.8	7.4 ± 0.8	6.9 ± 1.0	7.9 ± 1.1
NADP-Isocitrate dehydrogenase	276 ± 35	272 ± 27	240 ± 40	280 ± 36
Hexokinase	15.3 ± 1.8	14.6 ± 1.5	13.4 ± 1.6	16.5 ± 1.6

[a] 70% sunflower seed oil; 30% soybean oil.
[b] Results are mean ± SEM of five observations.
[c] Value significantly different ($P<0.05$) from control value.

tissue fatty acid biosynthesis. However, the enzymes were assayed under optimal conditions, whereas their activities in adipose tissue may have been modulated by many factors. Acetyl CoA carboxylase, for example, which is thought to have a crucial role in regulating the rate of fatty acid biosynthesis, occurs in both active and inactive forms (31), whereas the assay procedure used measured total activity (16). It is pertinent to note that the correlation between the rate of fatty acid biosynthesis and total acetyl CoA carboxylase activity found in fasted and re-fed sheep (16) was not observed in this study.

The reason for the different effects of the three fat-supplemented diets on the rate of fatty acid biosynthesis is not clear. All four groups of animals consumed the same quantity of food and showed similar gains in body weight, indicating a similar caloric intake. Feeding the three fat-supplemented diets had little effect on the concentration of plasma lipids of these sheep (Noble, unpublished observations). However, feeding the protected PUFA diet did result in a significant increase in the proportion of 18:2 in several lipid classes in plasma (Noble, unpublished observation) and also other tissues (32,33), indicating that the protection against biohydrogenation was effective and that increased quantities of 18:2 were being absorbed by the animals. The maize oil diet also resulted in an elevated 18:2 content of various plasma and tissue lipids but not to such a great extent as the protected PUFA diet, presumably due to biohydrogenation in the rumen.

The response of adult sheep adipose tissue fatty acid biosynthesis to dietary PUFA was markedly different from that of neonatal lamb adipose tissue (10). However, in the study with neonatal lambs, the amount of safflower oil administered amounted to less than 10% of the total fat intake of the animals, the other fat being derived from cows' milk and so mainly comprised palmitic, stearic, and oleic acids. Thus, it is probable that the mechanism by which dietary PUFA exerted their effects on neonatal lamb adipose tissue metabolism is distinct from that by which the different dietary fats affected adipose tissue metabolism in adult sheep.

In this study, only one level of dietary fat (apart from the control diet), one age of animal, and one time period were used. It is conceivable that different effects of dietary fats may be obtained if these parameters are altered, but the results obtained show that ruminating sheep adipose tissue metabolism, like that from rats and pigs (8,9), responds differently to dietary saturated and polyunsaturated fats.

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Effects of Rapeseed Oil on Fatty Acid Oxidation and Lipid Levels in Rat Heart and Liver

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ABSTRACT

The comparative rates of oxidation of erucic and oleic acids and of their CoA esters were studied in heart and liver mitochondria of rats fed a standard diet or semisynthetic diets containing 25% of the calories as either rapeseed oil (46.6% erucic and 10.4% eicosenoic acid) or olive oil, for a period of 5 months. The long exposure to the diet containing 25% rapeseed oil did not alter the oxidative activity of mitochondria and did not induce morphological changes in the heart. It is confirmed that erucic acid is oxidized in mitochondria at lower rates than other long chain fatty acids and that its activation as CoA derivative may be one of the rate limiting steps of the overall oxidation process. Total lipids and triglycerides do not significantly change in the heart whereas they increase in the liver of rats fed the diet containing rapeseed oil.

INTRODUCTION

The administration of diets containing high levels of rapeseed oil rich in erucic acid (*cis*-13-docosenoic) results in accumulation of triglycerides in the heart muscle during the 1st week of treatment. Triglyceride levels in myocardium subsequently return to normal values even when administration of rapeseed oil is continued (1). However, after several weeks necrotic lesions are observed in heart muscle (1). It has been proposed that accumulation of lipids in myocardium of animals fed diets containing rapeseed oil depends upon the oxidation rate of erucic acid, and a number of reports concerning the oxidation of erucic acid and of its CoA and carnitine derivatives by heart mitochondria have recently appeared (2-5). The authors agree that the oxidation rate of this long chain substrate is lower than that of other acids with 18 and 16 carbon atoms, although different steps of the process are considered rate limiting. In the present work, the oxidation of erucic and oleic acids by heart mitochondria of rats fed for 5 months a semisynthetic diet containing 25% of the calories as rapeseed oil (46.6% erucic acid) are studied comparatively. The effects of the diet on total lipids, triglycerides, and erucic and eicosenoic acid levels in heart and liver, and on the histological appearance of the hearts, are also evaluated comparing the results with those obtained in rats fed either a standard pellet diet or a semisynthetic diet containing 25% of the calories as olive oil. This oil was chosen as the reference oil since the monoenoic acid content and the linoleic to linolenic acid ratio are rather similar to those present in rapeseed oil when compared to other edible oils.

MATERIALS AND METHODS

Reagents

Radioactive fatty acids were obtained from C.I.S. (Gif-sur-Yvette, France). Nonradioactive acids from Fluka A.G.; ATP, CoA, NAD^+, and D,L-carnitine HCl were purchased from Boehringer GmbH (Mannheim, Germany), PPO (2,5-diphenyl-oxazol) and dimethyl-POPOP (2,2'-p-phenylenbis(4-methyl)-5-phenyloxazol) were from Packard Instruments (Downers Grove, IL).

Instruments

Instruments used for counting and radio-gas chromatography were those previously described (6). A Packard radiochromatoscanner system, model 7201, and Silica Gel HF layers (0.25 mm thickness) were used for purification of radioactive substrates by thin layer chromatography (TLC).

Substrates

Radioactive fatty acids were diluted with the corresponding nonlabeled acids to obtain a specific radioactivity of 5×10^6 dpm/μmol. After separation by TLC using benzene:diethyl ether:acetic acid (70:30:1; v:v:v), acids were chemically and radiochemically pure as determined both by TLC and by radio-GC of their methyl esters prepared with diazomethane in diethyl ether. Acyl-CoA were prepared and purified as previously described (6).

Animals and Diets

One-month old male rats of the Sprague-

TABLE I

Fatty Acid Composition (% by wt) of Dietary Lipids

Fatty acids[a]	Control	Olive oil	Rapeseed oil
14:0	1.5	0.2	-
16:0	17.9	12.2	3.9
16:1	2.5	1.2	-
18:0	5.6	2.9	1.5
18:1	28.0	74.6	20.0
18:2	32.6	6.3	15.7
20:0	0.3	0.4	-
18:3	4.1	1.1	7.9
20:1	-	-	10.4
20:4	0.4	-	-
22:1	-	-	46.6
20:5	1.8	-	-
22:6	2.2	-	-

[a]The first number indicates the chain length, whereas the second number indicates the degree of unsaturation.

Dawley strain were fed either one of the following diets: a) standard pellet diet (Charles River) for rats with 17% of the calories as lipids; b) semisynthetic diets containing 25% of the calories as either olive oil or rapeseed oil as the only lipid component (46.6% erucic acid and 10.4% eicosenoic acid). The basic percent composition of the semisynthetic diets was as follows: vitamin free casein 20, sucrose 50, starch 20, alpha cellulose 4, vitamin mixture 1, salt mixture 4, choline chloride 1. Animals were sacrificed after 5 months of administration of the experimental diets.

The fatty acid composition of the lipid fraction of the semisynthetic diets, analyzed by gas liquid chromatography (GLC) is shown in Table I as further described for tissue lipid analysis.

Oxidation Experiments

Heart mitochondria prepared from an homogenate in 0.25 M sucrose containing 1 mM EDTA as described by Fritz and Kaplan (7) and liver mitochondria obtained as described previously (6) were incubated in 10 ml Warburg vessels. Hyamine M (0.25 ml) absorbed on filter paper was placed in the central well of the vessel and 0.15 ml of sodium carbonate (30 mg/ml) in the side arm. The incubation mixture in the main compartment was as described in Table II. At the end of the incubation time, the flasks were cooled to 0-4 C, and the carbonate solution was poured into the mixture. Sulphuric acid (0.15 ml, 6N) was then added to the incubation mixture through the side arm, and the flasks were shaken in a Dubnoff incubator for 90 min at 40 C. The filter paper was transferred from the central well to a counting vial containing 10 ml of scintillating fluid (PPO 6.5g, dimethyl POPOP 130 mg, naphthalene 104 g, toluene 500 ml, dioxane 500 ml); methanol (2 ml) used for washing the central well was also added to the scintillating fluid and the ^{14}C content of each vial determined by liquid scintillation. The incubation mixture was saponified at 60 C for 90 min with 1.5 ml of 2N KOH in 60% methanol, and the acids were extracted in diethyl ether after acidification of the mixture. The ether phase was washed with NaCl saturated water and the washings were mixed with the aqueous phase. Portions of both the ethereal (prior to evaporation) and of the aqueous solutions were counted.

After evaporation of the ether, the nonvolatile residue was treated with diazomethane. Radio-GC analysis and radio-chromatoscanning of TLC plates (solvent benzene:ethyl acetate; 70:30; v:v) showed only one radioactivity peak with retention time and R_F, respectively, of the methyl ester of the substrate.

Tissue Lipid Analysis

Total lipid was extracted with chloroform: methanol (2:1) according to Folch et al. (8). After evaporation of the solvent to a known volume in tared glass stoppered tubes under a stream of nitrogen and aspiration, total lipid in samples was determined by weighing microaliquots of the sample on a microanalytical balance (Cahn Gram electrobalance) (9). Triglycerides in samples were determined according to the automated method of Noble and Campbell (10). Percent values of oleic, eicosenoic, and erucic acids in total lipids were determined after preparation of fatty acid methyl esters by transmethylation (11), purification of methyl esters by TLC, and GLC on sylanized glass columns packed with either SE-30 1% (programming temperature 150-220 C at 3 C/min) or DEGS 5% (programming temperature 140-120 C at 2.5 C/min) (12).

TABLE II

Oxidation of [10-^{14}C] Oleate and [14-^{14}C] Erucate by Heart Mitochondria[a,b]

Diet	Substrate	$^{14}CO_2$ (%)	Substrate transformation[c] (%)
Standard	Oleate	24.6(±1.9)	76.3(±3.7)
	Erucate	2.0(±0.2)	5.0(±2.4)
Olive oil	Oleate	23.1(±0.4)	69.9(±1.3)
	Erucate	2.5(±0.1)	4.3(±2.8)
Rapeseed oil	Oleate	22.4(±0.1)	68.9(±0.9)
	Erucate	2.0(±0.5)	4.5(±1.1)

[a]Incubation conditions: mitochondrial protein, 1.5 mg; substrate, 0.1 mM (sp. act. 5 x 10^6 dpm/μmol); ATP, 0.1 mM; CoA, 0.05 mM; NAD^+, 2.4 mM; D,L-carnitine HCl, 2 mM; $MgCl_2$, 4.8 mM; KCl, 80 mM; malate, 0.02 mM; phosphate, 55 mM; sucrose, 90 mM; EDTA, 0.4 mM; final volume, 1 ml; pH 7.4.

[b]The reported values (± S.E.) are the mean of duplicate determinations obtained in two different experiments. Mitochondria in each experiment were prepared from a pool of six organs. Values are corrected for blanks obtained with boiled enzymes.

[c]Percent transformation was calculated as the difference between the incubated radioactivity (100 %) and the radioactivity associated with the nonreacted substrate recovered at the end of the incubation.

Morphological Examination

Hearts from six animals of each dietary group were fixed in buffered 10% formic aldehyde. Several sections of the myocardium after inclusion in paraffin were dyed with the following dyes: ematoxiline-eoxine, Schiff's periodic acid, and Mallory. Sections were analyzed for lipid deposition using Sudan III.

RESULTS AND DISCUSSION

Oxidation of [14-^{14}C] Erucate and [10-^{14}C] Oleate

The oxidation of the substrates by mitochondria obtained from pools of hearts of six animals of each dietary group was evaluated by determining both the $^{14}CO_2$ formed during the incubation and the radioactivity associated with the nonreacted substrate. The results reported in Table II indicate that erucate transformation accounts for 6-12% of the transformation of oleate. The oxidation is not modified by the 5 months diets with both olive oil and rapeseed oil.

Oxidation of [14-^{14}C] Erucoyl-CoA and [10-^{14}C] Oleoyl-CoA

In Table III are shown the results of the oxidation of labeled erucoyl-CoA and oleoyl-CoA with either heart or liver mitochondria obtained from tissue pools of six animals of each dietary group. Oxidation of erucoyl-CoA by heart mitochondria is about 7 times higher when compared with the oxidation with erucate as the substrate (Table II). No significant differences are observed between the transformation of erucoyl-CoA by heart mitochondria of the three dietary groups. Concerning incubations with liver mitochondria, erucoyl-CoA appears to be oxidized at a lower rate than with heart mitochondria, whereas similar transformation values are observed with both enzyme systems using oleoyl-CoA as the substrate. Moreover, a comparison of the oxidation by liver mitochondria of the different dietary groups shows that enzyme activity is influenced by the diet in that the transformation is lowered when liver mitochondria from animals treated with both olive oil and rapeseed oil are used as the enzyme source. However, no significant difference is observed between olive oil and rapeseed oil-fed animals.

Total Lipids, Triglycerides, and Fatty Acids in Heart and Liver

Levels of total lipids and triglycerides (mg/g fresh weight) in hearts and livers of animals fed either the standard diet or the semisynthetic diets containing rapeseed oil or olive oil are shown in Table IV. Levels of heart total lipids in the group fed rapeseed oil are higher than those of the normal group, although they are not significantly different from those of the olive oil group. In the liver, total lipids are significantly higher ($P<0.05$) in the rapeseed oil group with respect of both the control and olive oil groups. Triglyceride levels in the heart are similar in all experimental groups of animals, whereas in the liver triglycerides are significantly higher ($P<0.05$) in the rapeseed oil group with respect to the others. The levels of oleic, eicosenoic, and erucic acid (as percentage of total fatty acids) are shown in Table V. Percent levels of oleic acid are similar in all groups

in the heart, whereas they are lower in the rapeseed oil group than in the other groups in the liver. Eicosenoic and erucic acids are virtually absent in the tissues of animals fed either the control or the olive oil diets, whereas they are detectable both in heart and liver of the rapeseed oil group. Heart lipids contain higher levels of both eicosenoic and erucic acids than liver lipids. Erucic acid levels are higher than eicosenoic acid levels in heart lipids, whereas the reverse is observed in liver lipids.

Histology

The morphological examination of sections of the heart muscle from the various groups of animals showed a normal configuration of the structure of the myocardium elements without any appearance of lipidosis or necrotic processes.

DISCUSSION

The appearance of lipidosis in the heart followed by histopathological lesions in the myocardium of animals fed diets with high levels of rapeseed oil has been described by several authors (1). The early lipidosis in the heart has been correlated to the erucic acid concentration in the diet, whereas it is not clear whether the late effects are related to erucic acid since they are observed also with diets containing rapeseed oil low in this fatty acid (13). The accumulation of triglycerides containing erucic acid in the heart has been attributed to a low rate of oxidation of this acid. Blond et al. (4) and Cheng et al. (5) have recently shown that the low oxidation rate of erucate by heart mitochondria depends upon a slow activation of the acid. Contrasting results are reported by various authors concerning a possible inhibitory effect of erucic acid upon the oxidation of various long chain fatty acids. Christophersen and Bremer (2) have shown that the oxidation of palmitoyl carnitine by heart mitochondria is inhibited in the presence of erucoyl-carnitine, whereas according to Swarttouw (3) the oxidation of palmitate and of oleate is not inhibited by erucate. Comparison of these results is difficult due to the different substrates used. Inhibition could occur either at the level of the transfer of fatty acids through the mitochondrial membrane or at the level of the β-oxidation inside the mitochondria. Our results concerning the comparative oxidation rates of oleic and erucic acid and of oleoyl- and erucoyl-CoA by heart mitocondria agree with the findings of other investigators (3-5).

The oxidation of [14-^{14}C] erucic acid determined both as radioactive carbon dioxide produced and as radioactivity associated with

TABLE III

Oxidation of [10-^{14}C] Oleoyl-CoA and [14-^{14}C] Erucoyl-CoA by Heart and Liver Mitochondria[a,b]

Diet	Substrate	Heart		Liver	
		CO_2 (%)	Substrate transformation[c] (%)	CO_2 (%)	Substrate transformation[c] (%)
Standard	Oleoyl-CoA	26.2(±2.3)	56.6(±3.2)	23.5(±0.7)	51.7(±0.2)
	Erucoyl-CoA	14.8(±2.4)	31.8(±0.8)	4.1(±0.1)	23.0(±2.9)
Olive oil	Oleoyl-CoA	28.6(±1.5)	28.6(±1.5)	5.5(±1.1)	24.6(±1.5)
	Erucoyl-CoA	13.7(±0.1)	30.6(±0.4)	1.0(±0.1)	1.2(±0.1)
Rapeseed oil	Oleoyl-CoA	31.6(±2.9)	59.6(±5.9)	8.7(±2.6)	19.8(±2.2)
	Erucoyl-CoA	14.7(±1.1)	27.2(±1.1)	2.9(±0.3)	8.1(±1.5)

[a]Incubation conditions: mitochondrial protein, 1.5 mg; substrate, 0.1 mM (sp. act. 5 x 10^6 dpm/μmol); ATP, 0.1 mM; CoA, 0.05 mM; NAD^+, 2.4 mM; D,L-carnitine HCl, 2 mM; $MgCl_2$, 4.8 mM; KCl, 80 mM; malate, 0.02 mM; phosphate, 55 mM; sucrose, 90 mM; EDTA, 0.4 mM; final volume, 1 ml; pH 7.4.

[b]The reported values (± S.E.) are the mean of duplicate determinations obtained in two different experiments. Mitochondria in each experiment were prepared from a pool of six organs. Values are corrected for blanks obtained with boiled enzymes.

[c]Percent transformation was calculated as the difference between the incubated radioactivity (100 %) and the radioactivity associated with the nonreacted substrate recovered at the end of the incubation.

TABLE IV

Total Lipid and Triglyceride Levels in Hearts and Livers[a]

Diet	Total lipids (mg/g fresh weight)		Triglycerides (mg/g fresh weight)	
	Heart	Liver	Heart	Liver
Standard	28.8(±0.9)	42.2(±1.5)	9.8(±1.6)	13.7(±0.5)
Olive oil	32.4(±1.9)	41.9(±1.0)	9.8(±0.6)	13.9(±0.4)
Rapeseed oil	35.3(±0.5)	46.3(±0.7)[b,c]	11.2(±0.6)	20.5(±2.3)[b,c]

[a]Values are the average (± S.E.) of determinations carried out on five animals.
[b,c]Statistical significance of the difference: [b]from the control group, $P<0.05$; [c]from the olive oil group, $P<0.05$.

TABLE V

Levels of Oleic (18:1), Eicosenoic (20:1), and Erucic (22:1) Acids as Weight Percentages of Total Fatty Acids in Hearts and Livers[a]

Diet	Heart			Liver		
	18:1	20:1	22:1	18:1	20:1	22:1
Standard	21.8(±0.3)	trace	-	19.3(±0.7)	trace	-
Olive oil	20.3(±0.8)	trace	-	18.7(±0.5)	trace	-
Rapeseed oil	22.0(±1.0)	2.2(±0.3)	4.3(±0.2)	16.8(±0.1)[b,c]	1.3(±0.2)	0.6(±0.09)

[a]Values are the average (± S.E.) of determinations carried out on five animals.
[b,c]Statistical significance of difference: [b]from the control group $P<0.05$; [c]from the olive oil group $P<0.01$.

the nonreacted substrate after incubation was markedly lower than that observed using [10-^{14}C] oleate as the substrate for the oxidation, whereas the conversion of erucoyl-CoA was up to 50% of that of oleoyl-CoA. This is in agreement with the results obtained by Cheng et al. (5), indicating that oxygen uptake by heart mitochondria in the presence of erucoyl-CoA is about one-third of that determined by equal concentrations of palmitoyl-CoA. The rates of oxidation of the various fatty acids and of their CoA derivatives by heart mitochondria was not modified by administration for 5 months of diets containing either rapeseed oil or olive oil. This result is also in agreement with data recently reported by Cheng et al. (5), who did not observe changes in the rate of palmitoyl-CoA oxidation by heart mitochondria in rats fed 3 days on a diet containing either rapeseed oil or olive oil at 50% level of total calories with respect to controls, in spite of an accumulation of triglycerides as indicated by a pale appearance of the tissue. Moreover, recent results by Dow-Walsh et al. (14) demonstrate that the oxidative and energy coupling activities of heart mitochondria of rats fed 3 days on a diet containing 20% rapeseed oil is not impaired. Histological observations of hearts of six animals out of 20 under treatment in each dietary group did not show any pathological alteration. The lower percentage of rapeseed oil in the diet used in our experiment may explain the discrepancy with respect to results obtained by various authors showing long-term lesions in rats fed diets with high rapeseed oil content (15). On the other side, the administration of the diet with rapeseed oil for 5 months resulted in a slight increase of heart total lipid content with respect to controls, without a significant difference from the values measured in the group fed olive oil. Both eicosenoic and erucic acid (4.3 and 2.2%, respectively) were present in heart lipids of these animals, whereas they were undetectable in hearts of control animals. Triglyceride levels did not differ appreciably in the heart of animals fed the various diets. The long-term administration of the oil rich in erucic acid at the level of 25% of the calories, thus, did not modify the morphology of myocardium, nor its ability to oxidize fatty acids, although a slight increase in its total lipid content was detected. Erucic and eicosenoic acids were detectable in myocardial fatty acids.

In the liver, a different situation was observed. In fact, the rates of oxidation of both oleoyl- and erucoyl-CoA were equally lower in liver mitochondria from animals fed the semi-synthetic diets containing olive or rapeseed oil than those from control animals. However, animals fed the diet containing rapeseed oil

showed higher levels of total lipids and triglycerides with respect to both the control and the olive oil-fed groups. Erucic and eicosenoic acid in total fatty acids were detectable in liver of animals fed rapeseed oil, but levels were lower than those measured in the heart.

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Double Bond Position Affects Metabolism of *cis*-Octadecenoates[1]

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ABSTRACT

The metabolic fate of *cis*-positional isomers of octadecenoates has been compared to that of naturally occurring oleic acid (*cis*-Δ9). Radioactive mixtures of tritium-labeled positional octadecenoate isomer and oleic acid-10-^{14}C were administered to laying hens, and their eggs were analyzed for the isotopic ratios (^{3}H/^{14}C) incorporated into total egg lipid, triglycerides, and phospholipids. Variations in the isotopic ratios indicated the comparative metabolic utilization of *cis*-positional isomers Δ8 through Δ12. Incorporation into egg lipid fractions is as follows: triglycerides: Δ9>Δ8, Δ9>Δ10, Δ9>Δ11, Δ9>12; phospholipid: Δ9>Δ8, Δ9>Δ10, Δ9<Δ11, Δ9<Δ12.

INTRODUCTION

Commercial products made from partially hydrogenated soybean oil, such as margarines, shortenings, and liquid salad and cooking oils, contain mixtures of geometric and positional isomers of fatty acids (1,2). Carpenter and Slover (2) analyzed the monoene fractions from the hard cube, whipped cube, and soft tube margarines and showed that the double bond position of the *cis*-octadecenoates was distributed from the Δ8 to the Δ12 position. Scholfield et al. (1) found the same distribution in the *cis*-monoene fractions of liquid oils and shortenings. In vitro investigations demonstrated that the position of the double bond in *cis*-octadecenoates affects the activity of such enzymatic reactions as glycerophosphate acyltransferase (EC 2.3.1.15) (3), acyl-CoA synthetase (EC 6.2.1.3) (4), cholesterol acyltransferase (EC 2.3.1.26) (5), and cholesterol esterase (EC 3.1.1.13) (6).

We have previously examined the comparative metabolism of elaidic and oleic acids using the laying hen. The egg served as a "biological trap" providing an automatic daily biopsy with which to examine the metabolism of isomers in the hen (7). In the latest research, the *cis* positional isomers present in edible oil products have been administered to laying hens in dual isotope-labeled mixtures with oleic acid. The comparative metabolic fates were determined.

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MATERIALS AND METHODS

^{14}C-Labeled Oleic Acid

Oleic acid-10-^{14}C was purchased from Schwarz-Mann (Orangeburg, NY), specific activity (SA) = 10 mCi/mmol. As received from the supplier, this compound was ca. 83% *cis*-9 octadecenoic acid-10-^{14}C. After methylation with diazomethane, the compound was isolated free of impurities by argentation column chromatography (8).

^{3}H-Labeled Positional Isomers

Octadecynoic acids with triple bonds in positions 8, 10, 11, 12 were synthesized by Chang et al. (9). After esterification with diazomethane, the tritium-labeled *cis*-octadecenoates were prepared by reduction of the acetylenic bond as described previously (7). The products of reduction were analyzed by thin layer chromatography (TLC) on silver nitrate-impregnated silica gel plates; benzene: petroleum ether (80:20) was the mobile solvent. The developed plates were scanned with a Packard 7201 radiochromatograph scanner. Two radiochemical peaks were detected—one at the origin and a second in the area of a methyl oleate standard. The impurity was removed by chromatographing the sample through a short column of silica gel with petroleum ether as the mobile solvent. Position of the double bond was verified by ozonolysis-pyrolysis gas liquid chromatography (GLC). Infrared (IR) analysis determined the presence of *trans* isomers. Final analysis of each compound prepared:

cis-8-octadecenoate-8(9)-^{3}H; SA = 15.8 mCi/mmol; purity 99%; *trans* = <1.0%

cis-10-octadecenoate-10(11)-^{3}H; SA = 3.4 mCi/mmol; purity 99%; *trans* = <1.0%

cis-11-octadecenoate-11(12)-^{3}H; SA = 11.4 mCi/mmol; purity 99%; *trans* = <1.0%

cis-12-octadecenoate-12(13)-^{3}H; SA = 14.9 mCi/mmol; purity 99%; *trans* = <1.0%

cis-9-octadecenoate-9(10)-^{3}H; SA = 13.8 mCi/mmol; purity 99%; *trans* = <1.0%.

Radiochemical Analysis

Dual isotope assay was performed in a Beck-

TABLE I

Incorporation of *cis*-Octadecenoate Positional Isomers into Egg Yolk Lipids

Mixture administered Δ9-10-^{14}C with:	Isotopic ratios (^{3}H/^{14}C)		
	Administered	Triglycerides	Phospholipid
Δ8-8(9)-^{3}H	1.22	0.81 ± 0.07	0.98 ± 0.12
Δ9-9(10-^{3}H	1.85	1.83 ± 0.05	1.80 ± 0.17
Δ10-10(11)-^{3}H	1.19	0.39 ± 0.04	0.89 ± 0.19
Δ11-11(12)-^{3}H	0.72	0.56 ± 0.05	1.40 ± 0.21
Δ12-12(13)-^{3}H	2.36	1.44 ± 0.04	3.24 ± 0.61

TABLE II

Acyl Position Analysis of Egg Yolk Triglycerides (TG)[a]

Isomers administered		Isotopic ratio (^{3}H/^{14}C)			
				Positions	
^{3}H	^{14}C	Administered	TG	1 and 3	2
Δ8 + Δ9		1.22	0.81	0.85 ± 0.10	0.39 ± 0.01
Δ9 + Δ9		1.85	1.84	1.83 ± 0.05	1.84 ± 0.03
Δ10 + Δ9		1.19	0.40	0.39 ± 0.02	0.38 ± 0.04
Δ11 + Δ9		0.72	0.57	0.61 ± 0.05	0.38 ± 0.02
Δ12 + Δ9		2.36	1.44	1.24 ± 0.06	1.35 ± 0.12

[a]Fatty acid composition of triglycerides (mol %) 16:0 (31); 16:1 (5); 18:0 (9); 18:1 (41); 18:2 (15).

TABLE III

Acyl Position Analysis of Egg Yolk Phospholipids (PL)[a]

Isomers administered		Isotopic ratio (^{3}H/^{14}C)			
				Positions	
^{3}H	^{14}C	Administered	PL	1	2
Δ8 + Δ9		1.22	0.98	2.39	0.71
Δ9 + Δ9		1.85	1.80	1.84	1.80
Δ10 + Δ9		1.19	0.89	2.15	0.97
Δ11 + Δ9		0.72	1.40	4.33	1.37
Δ12 + Δ9		2.36	3.24	5.19	1.73

[a]Fatty acid composition of phospholipids (mol %) 16:0 (21); 16:1 (2); 18:0 (35); 18:1 (21); 18:2 (11); 20:4 (10).

man three-channel LS-250 liquid scintillation counter. Samples were counted in 10 ml of toluene containing 7 g of 2,5-diphenyloxazole per liter. Discriminator window iso-sets were adjusted to give a counting efficiency of 80% for ^{14}C and 40% for ^{3}H with less than 8% overlap of the ^{14}C-channel into the ^{3}H-channel. The counter was interfaced to an IBM 1800 computer, and all calculations were performed automatically.

Procedures

Mixtures of each tritiated positional isomer with ^{14}C-labeled oleic acid were divided into equal portions and administered to three laying hens, and egg yolk lipids were analyzed as described previously (10). Fatty acids were administered as the methyl esters in all experiments. Triglycerides and phospholipids isolated from egg yolk were hydrolyzed to determine the relative incorporation of the isomers into the acyl positions. Triglycerides were hydrolyzed with pancreatic lipase (11). The monoglycerides obtained as products of this reaction were isolated from the free acids, di-, and triglycerides by preparative TLC on Silica Gel G, developed with benzene:petroleum ether solvent (80:20). The bands were scraped; the 2-monoglycerides (2 position) and the fatty acids, representing those originally esterified at the 1 and 3 positions, were eluted from the silica gel with ether into 20-ml scintillation

vials. The solvent was evaporated in a stream of nitrogen, 10 ml of scintillation solvent was added, the samples were counted as before, and isotopic ratios were determined. Quadruplicate analyses were performed.

Phospholipids were hydrolyzed with snake venom (12). The fatty acids (representing those originally esterified at the 2-position), diacyl glycerol phosphatides, and the monoacyl glycerol phosphatides (representing the 1 position) were separated on a column containing 5 g of silica gel. Compounds were eluted with the following solvents: 50 ml of 10% methanol in chloroform, 250 ml of 40% methanol in chloroform, followed by 50 ml of 100% methanol. Isotopic ratios were determined as described above.

RESULTS AND DISCUSSION

The isotopic ratios ($^3H/^{14}C$) determined for the administered mixture, triglycerides, and phospholipid are given in Table I. A decrease in the isotopic ratio of either isolated egg lipid fraction compared to that of the administered mixture indicates a greater incorporation of the carbon-labeled oleic acid. In each feeding experiment (Table I), the isotopic ratio of the triglycerides was decreased, except when dual-labeled oleic acid was administered. There is a distinct preference for incorporation of the isomer when the double bond is located at the 9 position. Two isomers, Δ9 and Δ11, were incorporated to a greater extent than their adjacent isomers. Lippel et al. (4) observed a similar alternating pattern while studying the effect of double bond position on the activity of acyl CoA synthetase. Their in vitro studies indicated that the Δ10 and Δ12 isomers enhanced activity to a greater extent than their adjacent isomers. The two observations appear to be compatible since I found that Δ10 and Δ12 isomers are less likely to be incorporated into the triglyceride than the other isomers.

The isotopic ratios determined for egg phospholipids (Table I) showed that while Δ9 isomer was preferentially incorporated compared to the Δ8 and Δ10 isomers, there was a greater relative incorporation of the Δ11 and Δ12 isomers. As the double bond position is shifted down the chain away from the carboxyl group, incorporation of the isomer increases. Reitz et al. (3) reported that studies in vitro showed that the acyl-CoA:2-acyl-GPC acyltransferase activity of the Δ12 isomer was greater than that of the Δ9 isomer. They suggested that different enzymes may exist for different acyl-CoA isomers.

Christie and Moore (13) reported that in egg yolk triglycerides 89% of the octadecenoic acid is esterified at positions 2 (43%) and 3 (46%). My hydrolysis procedure did not permit an analysis of the 1 position separate from the 3 position; however, isotopic ratios were determined for the primary positions and for the 2 position of the triglyceride (Table II). The analyses indicate that Δ9 isomer is incorporated preferentially into triglycerides relative to each isomer. In addition, Δ9 isomer also exhibits preferential incorporation into the 2 position relative to the Δ8 and Δ11 isomers. The isotopic ratios of the monoglycerides, the fatty acids, and the triglyceride are the same in experiments with Δ10 and Δ12 isomers, but the ratio of the monoglyceride is decreased compared to that of the fatty acid, the triglyceride, and the administered mixture in experiments with Δ8 and Δ11 isomers. Since the amount of Δ9 isomer found in positions 2 and 3 of egg yolk triglyceride is approximately equal, the difference between the fatty acid and the monoglyceride isotopic ratios may indicate that the Δ8 and Δ11 isomers are incorporated preferentially in the 1 position. Further structural analyses are required to confirm this hypothesis.

Hydrolysis of egg yolk phospholipids yields products directly representing the fatty acids acylated at the 1 and 2 positions. A radioassay was made of the monoacyl glycerol phosphatides (1 position) and enzyme-hydrolyzed fatty acids (2 position) (Table III). The isotopic ratios of the 2-positional fatty acids, generally, are the same as those determined for the diacylated phospholipids. An exception is the experiment with the Δ12 isomer. Because the ratio is decreased compared to that of the phospholipid and the administered mixture, a distinct preferential incorporation into the 2-position of the Δ9 isomer relative to the Δ12 isomer is indicated. Increase in the isotopic ratio of the monoacyl glycerol phosphatide isolated in each experiment shows a preferential incorporation into the 1 position of the other positional isomers relative to Δ9 isomer. The greatest preference is shown with Δ11 and Δ12 isomers. Similar observations have been reported by Reitz et al. (3) in studies with liver microsomal preparations from rats and pigs.

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Monoacyl-*sn*-glycerol 3-Phosphate Acyltransferase Specificity in Bovine Mammary Microsomes

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ABSTRACT

The acyl-CoA:acyl-*sn*-glycerol 3-phosphate acyltransferases located in the microsomal fraction of lactating bovine mammary tissue show a preference for palmityl-CoA particularly above the apparent Km values of the acyl acceptors. Using saturating levels of monopalmityl-*sn*-glycerol 3-phosphate, the order of acylation was palmityl- > myristyl- > oleyl- > stearyl- >linoleyl-CoA. Apparent Km values for monopalmityl- and monooleyl-*sn*-glycerol 3-phosphate with palmityl-CoA as donor were 16 and 13μM, respectively, while the Km values for palmityl-CoA with these two acyl acceptors were 5 and 5.2μM, respectively. The apparent Vmax values for the palmityl acceptor and donor were 25 and 30 nmol/min/mg protein. Phosphatidic acid was the principal product. The inclusion of magnesium in the assay depressed activity while the addition of ethylenediaminetetraacetate doubled the rate of acylation.

INTRODUCTION

Bovine milk triglycerides are very heterogenous and differ from other animal glycerides in composition and location of component fatty acids. Unlike most natural triglycerides where the fatty acids on position *sn*-2 tend to be unsaturated, the glycerides from ruminant mammary tissue are predominantly saturated. This nonrandom distribution of fatty acids (1,2) is most probably controlled by the specificity of the acyltransferases involved in glycerolipid synthesis and to some degree by the relative availability of the precursor acyl-CoA molecules.

Some selectivity in patterns of acylation of fatty acids was observed by Pyndath and Kumar (3) and Askew et al. (4) using homogenates of lactating caprine and bovine mammary glands, respectively. The specificity of acyl-CoA utilization observed with rat mammary microsomes was consistent with the distribution of fatty acids in rat milk triglycerides (5).

A marked preference for palmityl-CoA, exhibited by the acyltransferase of bovine mammary microsome in the acylation of *sn*-glycerol 3-phosphate (GP), was observed by Gross and Kinsella (6), However, because in the above experiments both positions *sn*-1 and *sn*-2 of the GP were acylated to produce 1,2-diacyl-*sn*-glycerol 3-phosphate (DAGP), it was not possible to estimate the relative preference of the acyltransferase (AT) enzyme acylating position *sn*-2 of the putative intermediate, 1-acyl-*sn*-glycerol 3-phosphate (AGP). Thus, the apparent specificity observed was a composite of the selectivity of the transferases acylating both positions. To determine if a relationship exists between the location of fatty acids in position *sn*-2 of milk glycerolipids and the specificity of the AGP acyltransferases, we studied the relative activity of this enzyme from lactating bovine mammary tissue toward several acyl-CoA species.

MATERIALS AND METHODS

Lipid standards were purchased from Applied Science Laboratories (State College, PA). The pure acyl-CoA substrates were purchased from P and L Biochemical (Milwaukee, WI), and the monoacyl-*sn*-glycerol 3-phosphates (AGP) were obtained from Serdary Research (London, Ontario, Canada). Radiochemicals were purchased from New England Nuclear (Boston, MA). Lipid "free" bovine serum albumin (BSA) was obtained from Miles Laboratories (Kankakee, IL). The 5,5'-dithiobis (2-nitrobenzoate) (DTNB) and ethylenediaminetetraacetate (EDTA) were purchased from Sigma Co. (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA), respectively. All other chemicals were reagent grade, and double distilled deionized water was used.

Enzyme Preparation and Assay

Mammary tissue was obtained from a cow in the third trimester of her first lactation. Secretory tissue devoid of adipose and connective tissue was carefully excised, homogenized, and fractionated as outlined previously (7). The final microsomal pellet was washed, freeze-dried, and stored in sealed vials at -20 C. Under these conditions, the acyltransferases are stable for at least 3 months (6). For enzyme assays, the microsomes were dispersed in Tris-HCl buffer (65 mM, pH 7.4). Protein was quantified by

TABLE I

Rates of Acylation of Increasing Concentration of Monopalmityl- and Monooleyl-*sn*-glycerol 3-Phosphate with Different Acyl-CoA Species by Microsomal Acyltransferase from Lactating Bovine Mammary Tissue (nmol/min/mg protein)

Substrate[a]	12 μM		18 μM		24 μM		48 μM		350 μM[b]	
(Donor)	P	O	P	O	P	O	P	O	P	O
Myristyl-CoA	7.1	2.7	10.7	3.0	13.0	3.6	15.0	4.2	45	23
Palmityl-CoA	7.0	3.0	10.1	3.2	15.0	4.2	20.3	5.1	55	27
Stearyl-CoA	5.7	2.2	7.0	2.6	8.7	3.1	10.0	3.8	36	15
Oleyl-CoA	6.5	3.6	9.8	4.0	11.4	4.5	9.3	5.3	36	16
Linoleyl-CoA			0.2		0.3		0.6			

[a]Rates are initial velocities measured in the presence of substrate acyl-CoA species (10 μM) and 0.1 mg microsomal protein at 31 C. P and O refer to monopalmityl and monooleyl species of acyl-*sn*-glycerol 3-phosphate, respectively.

[b]Rates measured under similar conditions with acyl-CoA concentrations at 25 μM.

the procedure of Lowry et al. (8) using bovine serum albumin as standard. Prior to enzyme assay, the microsomal dispersion was sonicated in an ultrasonic cleaner (Model 8845, Cole-Palmer, Chicago, IL) for 1 min at 4 C.

The spectrophotometric assay method of Lands and Hart (9), in which the release of CoASH from acyl-CoA upon esterification is continuously monitored at 412 nm by the formation of a colored product with DTNB, was used for measuring enzyme activity. Prior to assay, the enzyme solution was held for 3 min to attain 31 C. The reaction was initiated by adding the AGP, and the change in absorbance was recorded continuously for 5-7 min using a Perkin-Elmer Model 356 spectrophotometer attached to a 10mV Hitachi Perkin-Elmer Model 165 recorder. The activity of acyl-CoA (palmityl-CoA) thiolase in this system was less than 0.5 nmol/min/mg microsomal protein. The standard assay contained Tris-HCl buffer, 65 mM, pH 7.4; acyl-CoA, 2-25 μM; AGP, 5-50 μM; DTNB, 1.0 mM; and microsomal protein, 0.1 mg/ml.

Products of the reaction, formed in identical assay systems containing [1-^{14}C] acyl-CoA, were extracted, fractionated by thin layer chromatography (TLC), and the radioactivity in various lipid classes was quantified as previously described (6).

RESULTS

Linear reaction rates were obtained at protein concentrations between 0.05 and 1.0 mg/ml. Using a microsomal protein concentration of 0.1 mg/ml, product formation was linear for at least 5 min. This enzyme concentration was used in all assays to minimize substrate binding and complications arising from monomer/micelle phase transitions (10,11). By using radioactively labeled acyl-CoA species in assays and subsequent separation of products by TLC, it was confirmed that phosphatidic acid was the predominant product (>90%), with diglycerides containing the remainder of the radioactivity.

Based on the composition of milk glycerolipids (1,12) and previous enzymatic studies of acyltransferases (13), it was decided that monopalmityl-*sn*-glycerol 3-phosphate (PGP) and monooleyl-*sn*-glycerol 3-phosphate (OGP) were appropriate substrates for the scyltransferases of bovine mammary microsomes, hence these were used as acyl acceptors. At low acceptor concentrations, acyl-CoA specificity was not very marked (Table I). Above Km concentrations of the acceptors, palmityl-CoA became the preferred acyl donor for PGP, and this preference was accentuated at saturating levels of PGP. Myristyl-CoA, oleyl-CoA, stearyl-CoA, and linoleyl-CoA were incorporated into PGP at progressively lower rates (Table I). At high concentrations of acceptors (350 μM) and with donor acyl-CoA at 25μM, i.e., substrates above the critical micellar concentration of the amphilphilic mixture, the rates of acylation were markedly enhanced, three- to fivefold, for the various acyl-CoA species studies.

The rates of acylation of OGP were markedly lower than those observed with PGP, and the acyl specificity was less apparent. Oleyl-CoA was the preferred acyl donor at all concentrations of OGP up to 48 μM. At high concentrations, palmityl-CoA was the preferred donor for OGP, with myristyl-CoA also showing a high rate of acylation.

The observed preference of the acyltransferase for PGP was consistent with the marked specificity of the *sn*-glycerol 3-phosphate acyltransferase for palmityl-CoA (6). The data may also indicate that in lactating bovine

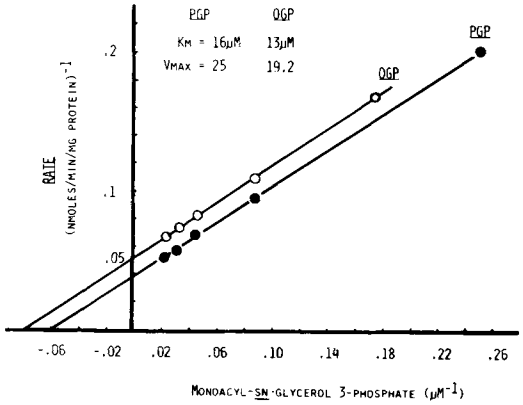

FIG. 1. Lineweaver-Burk plots showing rates of acylation at various concentrations of monopalmityl-*sn*-glycerol 3-phosphate (PGP) or monooleyl-*sn*-glycerol 3-phosphate (OGP) with palmityl-CoA by acyltransferase of microsomes from lactating bovine mammary tissue. Assays at 31 C contained palmityl-CoA (10 μM), 5,5'-dithiobis(2-nitrobenzoate) (1 mM), monoacyl-*sn*-glycerol 3-phosphate (varying levels), and 0.1 mg microsomal protein in 1 ml of Tris-HCl buffer (66 mM, pH 7.4). Vmax has units of nmoles/min/mg protein.

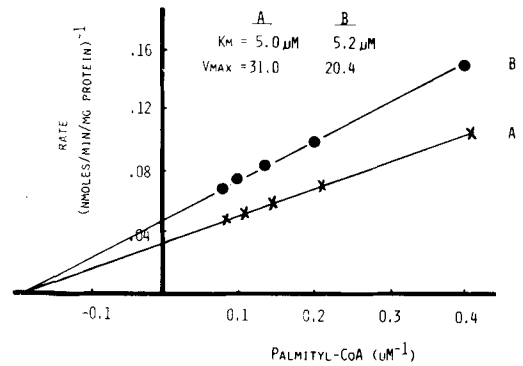

FIG. 2. Lineweaver-Burk plots showing the rates of acylation of fixed levels of monopalmityl-*sn*-glycerol 3-phosphate (A) and monoleyl-*sn*-glycerol 3-phosphate (B) by palmityl-CoA using acyltransferase of microsomes from lactating bovine mammary cells. Assays at 31 C contained palmityl-CoA (varying concentrations), monoacyl-*sn*-glycerol 3-phosphate (40 μM), (1 mM), and 0.1 mg microsomal protein in 1 ml of Tris-HCl buffer. Vmax has units of nmoles/min/mg protein.

mammary PGP is the preferred intermediate acyl acceptor in glycerolipid synthesis.

Using palmityl-CoA as donor, Km and Vmax values of 16 μM and 25 and 13 μM and 19.2 nmol/min/mg protein were determined for PGP and OGP, respectively (Fig. 1). When BSA (3 mg/ml) was included in similar assays, much higher levels of substrates were required and higher apparent Km values of 40 and 41 μM for PGP and OGP were obtained. This was probably caused by binding of the PGP and OGP to the BSA, thereby reducing the concentration of free acceptors (14).

At low substrate levels, in the absence of BSA, hyperbolic rate patterns were obtained with increasing levels of acyl-CoA from 2.5 to 10 μM. Lineweaver-Burk plots (Fig. 2) revealed apparent Km and Vmax values of 5 μM and 31 nmol/min/mg protein, and 5.2 μM and 20.4 nmol/min/mg protein for palmityl CoA with PGP and OGP, respectively. Comparable Km values were obtained with myristyl and oleyl-CoA, but Vmax data were lower than those obtained with palmityl-CoA.

In comparing data obtained with microsomes from lactating mammary tissue of different animals, marked variation in acylation rates was obtained (e.g., from 10 to 30 nmol palmityl-CoA acylated into PGP per min per mg protein). This may be attributed to differences in enzyme levels between animals, to the activity of acyl-CoA hydrolases, and to presence of varying amounts of inhibitory agents.

Because of the report of Jamdar and Fallon (15) that magnesium enhanced acylation and glycerolipid synthesis via stimulation of phosphatidate phosphohydrolase, the effect of this cation on acylation of PGP was examined. However, magnesium actually depressed acyltransferase activity in the present studies (Table II). This was consistent with the findings of Kuhn (16), using guinea pig mammary tissue, but contrary to observations with bovine tissue (6). Recently it was shown that the depression of PGP acyltransferase by magnesium in microsomes from lactating rabbit mammary was not caused by cation complexation of either of the substrates (11). It thus appeared possible that endogenous cations might be depressing acyltransferase activity, and conceivably variation in cation levels between different microsomal preparations could influence the acyltransferase rates observed experimentally. Since microsomes were obtained from lactating mammary tissue, it seemed likely that calcium was present in these preparations. The presence of inhibitory cations associated with the microsomes was indicated by the marked increase in acyltransferase activity when EDTA was included in the buffer used for suspending the microsomes (Table II).

DISCUSSION

The observed preference of the acyltransferase for palmityl-CoA was consistent with earlier observations obtained using caprine, bovine, rat, and guinea pig mammary tissue (3,5,6,16). This contrasts with many other

TABLE II

Effects of Magnesium and Ethylenediaminetetraacetate (EDTA) on the Palmityl-CoA:Monopalmityl-*sn*-glycerol 3-Phosphate Palmityltransferase Activity in Bovine Mammary Microsomes

Enzyme preparation	Rate of acylation (nmol/min/mg protein)
Microsomes alone	17.0
Microsomes + $MgCl_2$ (0.5mM)	14.0
Microsomes + $MgCl_2$ (1.0mM)	10.0
Microsomes + EDTA (1mM)	30.3
Microsomes + EDTA (2mM)	33.0
Microsomes + EDTA (3mM)	30.0

mammalian AGP acyltransferases which show a marked preference for unsaturated fatty acids (15,17-19). However, the validity of enzymatic data obtained with substrates that are anionic amphilphiles must be considered with reservation. Such substrates may exist as free monomers in solution, as micelles, or they may tightly bind to proteins in these systems. Hence, it is difficult to know the actual substrate concentration available to the enzyme. Some of the problems associated with such systems and the manner in which they affect kinetics and specificities have been studied and discussed (7,10,11,14,17,20,21).

In the present study, relatively low concentrations of donor substrates were employed to comply with the admonition of Tipton (22) that, in order to obtain reliable evidence of enzyme specificity, it is necessary to make initial velocity measurements at substrate levels in the vicinity of apparent Km values.

A marked difference in capacity of the mammary enzymes to acylate PGP compared to the OGP was apparent at all concentrations of substrates. Though Barden and Cleland (1969) observed some differences, they concluded that the nature of the fatty acids present in the 1-acyl-*sn*-glycerol 3-phosphate had a negligible effect on the rate of acylation and that the specificity resided in the selectivity of the acyltransferases for the donor fatty acids. In contrast to the present data, Okuyama and Lands (1972) reported that selective acylation of AGP occurred only when concentrations of this acyl acceptor was very low, i.e., below apparent Km values.

The affinity of the bovine mammary microsomal enzymes for PGP and OGP were quite similar (Fig. 1). These Km values are within the range reported by Barden and Cleland (14) and were below the critical micellar concentration (cmc) of these amphiphiles (14). In contrast to the observations of Okuyama and Lands (17) and Numa and Yamashita (19), little specificity in acylation of either AGP or OGP at low concentrations was obtained in the present study, and selectivity for acyl donor group only became apparent at comparatively high concentrations of acceptors. The specificity for acyl-CoA's (palmityl > oleyl > myristyl-CoA) was similar to that observed by Barden and Cleland (14) for acyl-CoA concentrations ranging from 6 to 9 μM.

The apparent Km values for the acyl-CoA species were around the cmc of these compounds, which ranges from 3 to 7 μM (14). These observed Km values may be somewhat high, being inflated by nonspecific binding (11). Acyltransferases apparently prefer substrates in true solution (14,17). However, recently the presence of isoenzymes of AGP acyltransferases in rabbit mammary has been reported (20). One isoenzyme utilizes substrate in monomeric form, whereas the other isoenzyme species function at much higher rates with substrate in micellar form (20,23). The marked increase in rate of acylation at high concentrations of PGP and OGP observed in the present study may also indicate isoenzymes in bovine mammary microsomes. Conceivably these facilitate the continued synthesis of glycerolipids in the presence of relatively large concentrations of amphilphilic lipids as may occur in lactating mammary tissue.

The observed selectivity in acylation of the different acyl-CoA species by the enzyme from bovine mammary microsomes was consistent with the relative concentrations of these fatty acids in position *sn*-2 of milk triglycerides (1).

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Occurrence of 2- and 3-Hydroxy Fatty Acids in High Concentrations in the Extractable and Bound Lipids of *Flavobacterium meningosepticum* and *Flavobacterium* IIb

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ABSTRACT

The major hydroxy fatty acids of cellular lipids in *Flavobacterium meningosepticum* and *Flavobacterium* sp. King's group IIb were identified as 2-hydroxy 13-methyltetradecanoic, 3-hydroxy 13-methyltetradecanoic, 3-hydroxy palmitic, and 3-hydroxy 15-methylhexadecanoic acids using gas chromatography-mass spectrometry and GC-mass fragmentography. The concentration of these hydroxy fatty acids comprised up to 30-40% of the total extractable and 20-30% of the bound lipid fatty acids, respectively. From the stability for mild alkaline hydrolysis, 2-hydroxy fatty acids seemed to be attached with ester linkage, and 3-hydroxy fatty acids with amide linkage.

INTRODUCTION

The occurrence of various hydroxy fatty acids in the lipids of many species of bacteria has been well established recently. In Gram-negative bacteria, 3-hydroxy fatty acids were known to associate mostly with the lipopolysaccharide molecule (1-3), and 2-hydroxy fatty acids with phospholipids in the limited genera of Gram-positive bacteria (4-6). On the other hand, the coexistence of both 2- and 3-hydroxy fatty acids as the components of lipoamino acids has also been reported in several genera of Gram-positive and -negative bacteria (7-9). However, the concentrations of hydroxy fatty acids in the total extractable lipids are usually several percent or less (8). *Flavobacterium meningosepticum* is a Gram-negative aerobic rod that is known as one of the etiologic agents of purulent meningitis in newborn infants (10,11). *Flavobacterium* sp. King's group IIb is a deep yellow organism thought to be closely related to *F. meningosepticum* (12). Their cellular lipid composition has not been reported yet. Recently, we found that a strikingly high concentration of branched-chain 2- and 3-hydroxy fatty acids occurred not only in the "bound" lipid fraction but also in the "extractable" lipid fraction of both strains. Present

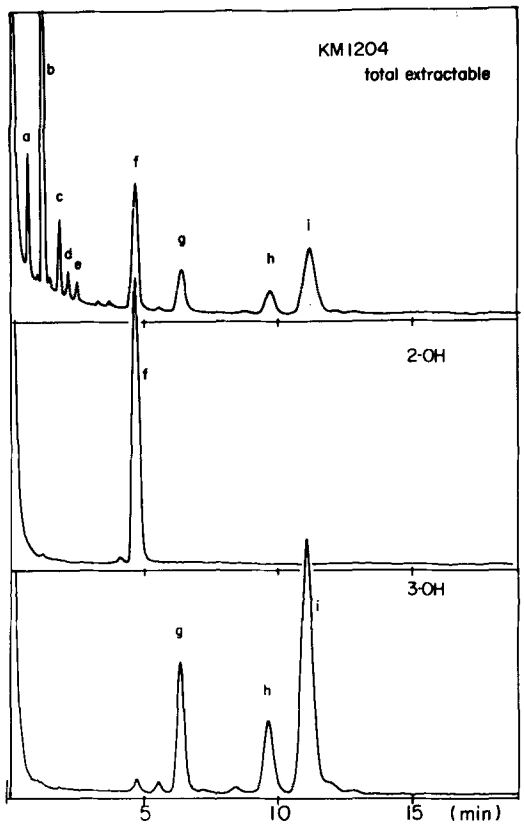

FIG. 1. Gas liquid chromatogram of the total extractable (upper), 2-hydroxy (middle), and 3-hydroxy (lower) fatty acid methyl esters of *Flavobacterium meningosepticum* (KM-1204). A polar column (15% EGS) was used. Nonpolar and hydroxy fatty acids were identified from the comparison of the retention times with standards and log-retention times vs. carbon numbers plot. The relative retention times (1.00 for n-16:0) are as follows: iso-13:0 (0.37 for a), iso-14:0 (0.50), n-14:0 (0.58), iso-15:0 (0.66 for b), anteiso-15:0 (0.70), n-15:0 (0.74), iso-16:0 (0.87), n-16:0 (1.00 for c), iso-17:0 (1.16 for d), anteiso-17:0 (1.25), n-17:0 (1.35 for e), 2-hydroxy iso-15:0 (2.55 for f), 3-hydroxy iso-15:0 (3.44 for g), 3-hydroxy n-16:0 (5.22 for h), 3-hydroxy iso-17:0 (6.04 for i), and 3-hydroxy anteiso-17:0 (6.48).

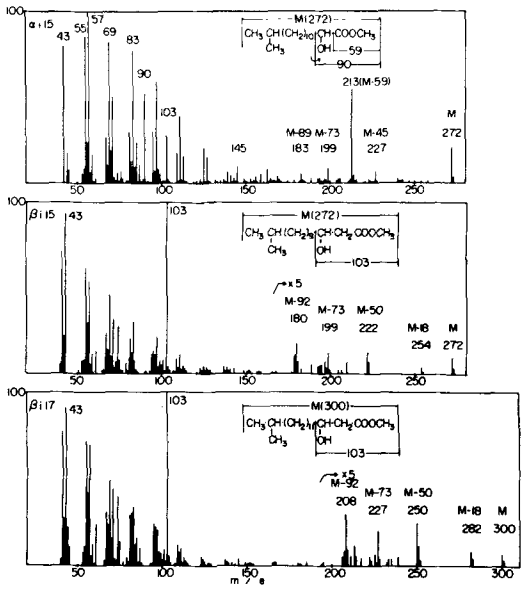

FIG. 2. Mass spectra of the 2-hydroxy 13-methyltetradecanoic (upper), 3-hydroxy 13-methyltetradecanoic (middle), and 3-hydroxy 15-methylhexadecanoic (lower) acid methyl esters of *Flavobacterium meningosepticum* (KM-1204). The conditions for mass spectrometry are described in the text.

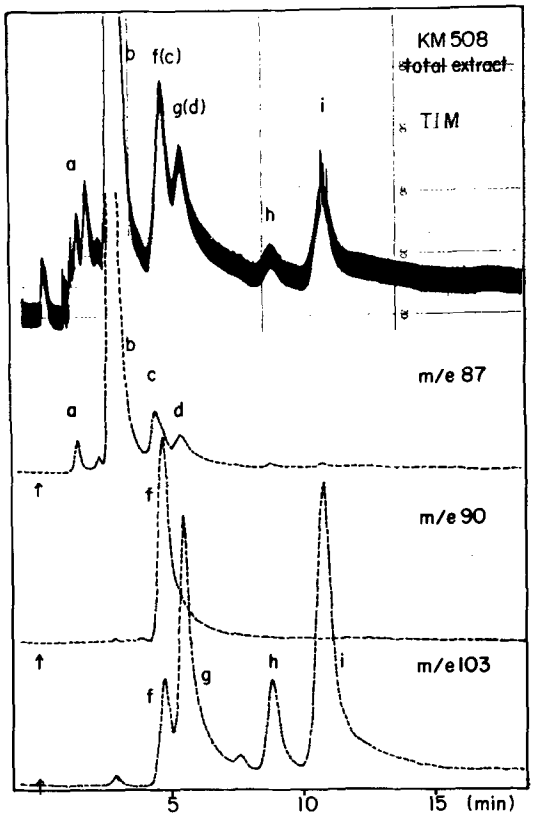

FIG. 3. Mass fragmentogram of the total fatty acid methyl esters of extractable lipids from *Flavobacterium meningosepticum* (KM-508). From upper, monitoring by the total ionic current (TIM), m/e 87, m/e 90, and m/e 103 ions, respectively. A nonpolar column (3% OV-17) was used. The detailed conditions for mass fragmentography are described in the text.

communication reports on the exact identification and determination of these hydroxy fatty acids by a newer technique of gas chromatography-mass fragmentography.

MATERIALS AND METHODS

F. meningosepticum and *Flavobacterium* IIb strain were grown on a solid medium containing 1% polypeptone (Daigo-Eiyo Chem. Co., Osaka), 0.5% yeast extract (Difco, Detroit, MI), 1% glucose, and 1.5% agar, pH 7.0. After 20 hr incubation at 30 C, the cells were harvested and the lipids were extracted twice with 20 vol of chloroform:methanol (2:1, by vol) and washed by the method of Folch et al. (13). The "extractable" lipid fraction was hydrolyzed with 3 N HCl for 2 hr under reflux. The resultant fatty acids were extracted with n-hexane and then methylated with benzene-methanol-H_2SO_4 (10:20:1, by vol) for 1 hr. On the other hand, the residue fraction containing tightly bound fatty acids was hydrolyzed directly with 3 N HCl, and the fatty acids extracted were methanolyzed. The fatty acid methyl esters were separated on a thin layer plate (0.5 mm thick) of Silica Gel G (Merck, Darmstadt, West Germany) with a solvent system of n-hexane:diethyl ether (80:20, by vol). The individual spots were visualized by short time exposure with iodine vapor, and the nonsubstituted, 2-hydroxy, and 3-hydroxy fatty acid methyl esters were located by comparing the R_f values with authentic standards. The individual methyl esters were analyzed using a Hitachi 063 type gas chromatograph with a 15% EGS column (2 m x 3 mm) at 200 C. The mass spectrometric analyses were carried out with a JMS-D 100 gas chromatograph-mass spectrometer (Nihon Denshi Co., Tokyo) with a column of 3% OV-17 (2 m x 3 mm). The energy of the bombarding electrons was 30 eV, and the temperature of the ion source was 200 C. The mass fragmentographic analyses were performed with three-channel multiple ion detector systems.

RESULTS AND DISCUSSION

Thin layer chromatographic (TLC) analysis of fatty acid methyl esters obtained from the "extractable" lipid fraction of *F. meningo-*

TABLE I

Fatty Acid Composition (%) of Extractable and Bound Lipids in *Flavobacterium meningosepticum* and *Flavobacterium* IIb

	Flavobacterium meningosepticum								*Flavobacterium* IIb	
	505[a]		1113		1204		1346		1390	
Fatty acid	Ext.[b]	Bound	Ext.	Bound	Ext.	Bound	Ext.	Bound	Ext.	Bound
iso-13:0	1.3	4.6	2.2	2.5	1.9	3.1	8.6	3.9	tr[d]	3.2
iso-14:0	tr	1.0	2.0	1.9	tr	0.8	1.7	tr	tr	tr
n-14:0	1.3	1.4	1.7	3.2	0.9	2.4	tr	tr	tr	tr
iso-15:0	46.2	60.8	39.5	44.7	40.8	64.2	49.6	56.1	37.6	57.3
iso-16:0	tr	tr	1.6	1.3	tr	0.8	1.2	tr	tr	tr
n-16:0	8.8	3.1	11.3	14.3	7.8	2.3	4.2	3.8	14.9	3.0
iso-17:0	5.3	1.6	4.9	3.5	3.9	2.3	3.0	1.0	6.9	2.5
n-17:0	3.6	0.8	2.2	1.1	2.8	1.5	2.1	tr	6.8	5.2
2-OHiso-15:0[c]	14.8	4.9	11.5	9.7	14.7	4.9	12.9	4.1	17.9	4.1
3-OHn-14:0	tr	0.3	0.3	0.3	tr	0.5	tr	tr	tr	tr
3-OHiso-15:0	4.0	10.9	3.4	3.1	5.6	8.0	4.1	11.8	0.2	11.4
3-OHn-16:0	1.4	4.7	5.8	5.0	3.9	4.4	1.8	5.2	0.2	tr
3-OHiso-17:0	13.3	5.7	13.5	9.3	17.4	6.7	8.2	13.6	17.6	13.3
3-OHanteiso-17:0	tr	tr	tr	tr	tr	0.2	tr	0.6	tr	tr
Total hydroxy	33.5	26.5	34.5	27.4	41.8	24.7	27.0	35.3	35.9	28.8

[a] KM (Kansai Medical University) strain number.

[b] Extractable lipids.

[c] Hydroxy fatty acid.

[d] tr = Trace amount (< 0.1%).

septicum showed three spots corresponding to nonsubstituted, 2-hydroxy, and 3-hydroxy fatty acids, respectively. As demonstrated in Figure 1, gas liquid chromatography (GLC) showed that nonsubstituted fatty acids consisted largely of iso-branched chain fatty acids ranging from C_{13} to C_{17}, with a smaller amount of straight chain fatty acids (peaks a to e). On the other hand, the fatty acid methyl esters corresponding with 2-hydroxy and 3-hydroxy esters on TLC gave peak f and peaks g, h, and i on GLC, respectively. The mass spectrum of peak f (Fig. 2) showing the parent ion at m/e 272 and ions at m/e 90 and m/e 103 due to C_2-C_3 and C_3-C_4 cleavages, respectively, indicates that peak f is to be 2-hydroxypentadecanoic acid methyl ester. However, the retention time of peak f did not coincide with 2-hydroxypentadecanoate but with 2-hydroxy-iso-pentadecanoate. Therefore, peak f was identified as 2-hydroxy 13-methyltetradecanoic acid. On the other hand, the mass spectrum of peaks g, h, and i commonly showed a very intense peak at m/e 103 and the parent ions at m/e 272 (hydroxy C_{15}), m/e 286 (hydroxy C_{16}), and m/e 300 (hydroxy C_{17}), respectively. From the comparison of their retention times on GLC and a plot of log retention times vs. chain length, peaks g, h, and i were identified as 3-hydroxy 13-methyltetradecanoic, 3-hydroxy palmitic, and 3-hydroxy 15-methylhexadecanoic acid, respectively. Since the mass fragment ions of m/e 87, m/e 90, and m/e 103 are stable and characteristic for nonsubstituted, 2-hydroxy, and 3-hydroxy fatty acid methyl esters (14), we have applied the mass fragmentographic techniques to the identification of total fatty acid methyl esters in mixtures. Figure 3 shows the mass fragmentogram of m/e 87, m/e 90, and m/e 103 ions, and the peaks of nonpolar, 2-hydroxy, and 3-hydroxy fatty acid methyl esters were detected separately; this also supported the above identification. The fatty acids of "bound" lipid fraction were also identified similarly. The results of fatty acid analysis on the selected four *F. meningosepticum* and one *Flavobacterium* IIb strain are summarized in Table I. Although the "extractable" lipid composition of above strains is not known, it is noted that the concentration of 2- and 3-hydroxy fatty acids in the "extractable" lipid of these five strains is extremely high and the alkaline hydrolysis (15) liberated only nonsubstituted and 2-hydroxy fatty acids. Therefore, it may be postulated that the 2-hydroxy fatty acids associated with specific lipid with ester bond, and 3-hydroxy fatty acids with amide linkage. Since Oshima and Yamakawa (16) have already reported the occurrence of a glucosamine-containing glycolipid in *Flavobacterium thermophilum* and we have found various types of lipoaminoacids in high concentrations in *F. meningosepticum* (unpublished data), the occurrence of hydroxy fatty acids may be related to these types of amino- or glycolipids. The similar fatty acid profiles were commonly observed for the other strains of *F. meningosepticum* and *Flavobacterium* IIb.

It was also suggested that the gas chromatography-mass fragmentography was simple and especially useful for the analysis of the mixtures of nonsubstituted and 2- and/or 3-hydroxy fatty acids in bacteria. Detailed lipid analyses of these organisms are now in progress.

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Biosynthesis of Polyunsaturated Fatty Acids in the Developing Brain: II. Metabolic Transformations of Intracranially Administered [3-^{14}C] Eicosatrienoic Acid, Evidence for Lack of Δ^8 Desaturase

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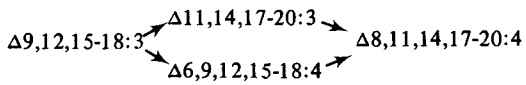
ABSTRACT

[3-^{14}C] Eicosatrienoic acid (Δ11,14,17) chemically synthesized from [1-^{14}C] linolenic acid was injected intracranially into 14-day old rats and sacrificed 8 hr later. The analysis of brain fatty acids by radio-gas liquid chromatography before and after ozonolysis showed that the tetraene fraction consisted of a desaturated product, Δ5,11,17-20:4, and its elongated product, Δ7,13,16,19-22:4. Both of these products, with a combined total of 61% of the total radioactivity recovered in the tetraene fraction, contain a nonmethylene interrupted double bond system and, therefore, are unsuitable for further desaturation. The other two components, Δ6,9,12,15-18:4 and Δ8,11,14,17-20:4, must have been formed from Δ9,12,15-18:3, formed by retroconversion of the starting material 20:3, followed by desaturation and elongation. These results suggest a lack of Δ^8 desaturase in the developing brain, leading to formation of Δ5,11,14,17-20:4 rather than Δ8,11,14,17-20:4. However, the nonmethylene interrupted double bond isomer does not restrict chain elongation.

INTRODUCTION

Earlier work from this laboratory (1) showed that, from intracranially injected [1-^{14}C] linolenic acid, eicosatrienoic acid Δ11,14,17-20:3 and also Δ8,11,14,17-20:4 were found to be radioactive. The pathways by which these can be formed are as follows:



However, Δ11,14,17-20:3 may not be a suitable substrate for desaturation, if Δ^8 desaturase is absent, to form Δ8,11,14,17-20:4. Sprecher and Lee (2) have postulated that the rat liver lacks Δ^8 desaturase and therefore cannot form Δ8,11,14,17-20:4 from Δ11,14,17-20:3. In our work on 1-^{14}C linolenic acid, both of these products were formed, but these could have been formed by way of independent elongation of Δ9,12,15-18:3 → Δ11,14,17-20:3 and by initial desaturation to Δ6,9,12,15-18:4 followed by elongation to Δ8,11,14,17-20:4. Thus, in order to determine which of the two possibilities occurs in the developing brain, we chemically synthesized [3-^{14}C]

TABLE I

Percent Distribution of Radioactivity and Specific Activity of Brain Lipids 8 hr Following Intracranial Injection of [3-14C] Eicosatrienoic Acid

Lipids	Specific activity (cpm/mg)	Percent of total radioactivity (TLC)
Total lipids	18,095	
Total polar lipids	16,353	53.9
Free cholesterol	4,484	5.7
Triglycerides	-	20.7
Free fatty acids	-	17.4
Total polar lipids		
Choline phosphoglycerides		53.0
Ethanolamine phosphoglycerides		18.7
Serine phosphoglycerides		13.1
Cerebroside		13.0
Sphingomyelin		1.4

TABLE II

Percent Distribution of Radioactivity in Brain Fatty Acid Methyl Esters 8 hr Following Intracranial Injection of [3-^{14}C] Eicosatrienoic Acid

Fatty acid (GLC)	Percent of total radioactivity	
	Hydrogenation	
	Before	After
16:0	7.6	6.2
18:0	3.0	7.1
18:3 (n-3)	5.3	-
20:0	-	83.8
20:3 (n-3)	75.8	-
20:4 (n-3)	4.8	-
20:5 (n-3)	3.4	-
22:0	-	2.9
22:5 (n-3)	0.4	-
22:6 (n-3)	trace	-

Δ11,14,17-20:3 and injected it directly into the brain. This paper characterizes the products formed and discusses the possible biosynthetic pathways.

MATERIALS AND METHODS

Animals

Seven 14-day old Wistar albino rats, male and female, weighing 31.5 ± 2.5 were used for intracranial injection.

Tracer

[3-^{14}C] Eicosatrienoic acid (Δ11,14,17) is not readily available commercially. Therefore, [1-^{14}C] octadecatrienoic acid (Δ9,12,15) was purchased (*cis*9,12,15 octadecatrienoic acid, 52.5 mc/mM; DHOM Products, Hollywood, CA) and elongated to give [3-^{14}C] Δ11,14,17-20:3 using the following reactions. Linolenic acid was reduced to the alcohol using $LiAlH_4$, followed by 20-hr reflux (under nitrogen atmosphere) in the presence of CBr_4 and triphenylphosphene to obtain the corresponding primary bromide. The bromide was condensed with sodium diethyl malonate to give the fatty acid diester. the diester was hydrolyzed and decarboxylated to give [3-^{14}C] eicosatrienoic acid (Δ11,14,17). At every stage, purity was checked by thin layer chromatography (TLC). Details of this procedure will be published elsewhere.

Each rat was given an intracerebral injection of 21 μl containing 4.64 μc of [3-^{14}C] eicosatrienoic acid and was sacrificed after 8 hr.

Lipid Extraction and Fractionation

The extraction of total lipids from pooled brains, fractionation into various components to determine distribution of radioactivity, radio-gas liquid chromatography (GLC) of fatty acids, reductive ozonolysis to characterize double bond position, and $AgNO_3$:SiO_2 TLC to determine the number of double bonds has been described earlier (1).

RESULTS

Table I shows the specific activity and the distribution of radioactivity in brain total lipids 8 hr following intracranial administration of [3-^{14}C] eicosatrienoic acid. Of the injected dose 10.3% was retained in brain lipids and only 0.8% was recovered in the liver. A little over half of the total lipid radioactivity was found in the polar lipids (glyco- and phospholipids). Surprisingly, triglycerides and free fatty acids contained appreciable amounts of radioactivity. When the polar lipid fraction was examined by TLC, a little over half of the total recovered radioactivity was found in choline phosphoglycerides. Ethanolamine phosphoglycerides contained ca. 19% whereas both serine phosphoglycerides and cerebrosides retained ca. 13% of the total radioactivity.

The brain total fatty acid methyl esters were analyzed by GLC before and after hydrogenation (Table II). The identification was based on comparison of retention times with authentic known standards and confirmation of the chain length by analysis of the hydrogenated samples. The injected tracer, 20:3 (n-3) retained the highest amount of radioactivity, ca. 76%, and the remaining activity was more or less equally distributed in 16:0, 18:0, 18:3, 20:4, and 20:5. The end product of the (n-3) family, 22:6, contained only trace amounts of radioactivity. These component fatty acids were further characterized by ozonolysis to determine double bond positions (Table III). The initial separation into fractions according to degree of unsaturation eliminates uncertainty about fatty acids with retention times either overlapping or very close to each other. Thus, the triene fraction consisted of two components, 18:3 and 20:3, and, since the ozonization gave C_9 and C_{11} aldehyde esters, their final structures must be Δ9,12,15-18:3 and Δ11,14,17-20:3. Similar analytical procedure established that the tetraene fraction consisted of Δ6,9,12,15-18:4; 20:4, however, consisted of two components, Δ5,11,14,17-20:4 and Δ8,11,14,17-20:4. From the recovered radioactivities in this fraction, it seems that the Δ5,11,14,17-20:4 with the nonmethylene-interrupted double bond system contained almost twice the amount of radioactivity as did the other component, Δ8,11,14,17-20:4. Another component in the tetraene fraction was Δ7,13,17,19-22:4, which

also has a nonmethylene-interrupted double bond system and contained about the same amount of radioactivity as the 18:4 component. The pentaene fraction was characterized as Δ5,8,11,14,17-20:5 and Δ7,10,13,16,19-22:5. This fraction did not contain much radioactivity, but, from the ozonized fraction, it seems that 22:5 contained a higher proportion of radioactivity than 20:5.

DISCUSSION

Brain lipids differ in composition from either adipose tissue fat or liver lipids. They contain only a very small amount of triglycerides, and the bulk is made up of cholesterol and polar lipids such as glycolipids and phospholipids (3). In earlier work (1,4), it was observed that, generally, the polar lipids of the brain incorporated a major portion of the radioactivity from administered fatty acid with only a minor amount of incorporation into neutral lipids such as triglycerides or free fatty acids. However, in the present work, it seems that radioactivity from injected [3-^{14}C] eicosatrienoic acid was incorproated to an appreciable amount into the brain neutral lipids (Table I). Part of the injected tracer remained unincorporated. Further, it was noted that the distribution of radioactivity into cholesterol or palmitate was also low compared to earlier work with [1-^{14}C] linolenic acid (1), a precursor of eicosatrienoic acid. Since the radioactivity of both palmitate and cholesterol would be derived from acetate formed by β oxidation of the injected tracer, one would conclude that the injected eicosatrienoic acid was not readily oxidized to acetate.

Earlier work using [1-^{14}C] linolenic acid (1) could not establish with certainty a lack of Δ^8 desaturase in the brain because both Δ11,14,17-20:3 and Δ8,11,14,17-20:4 were identified as products. However, both of these products could have formed independently. Δ11,14,17-20:3 could be a product of an elongation of Δ9,12,15-18:3, and Δ8,11,14,17-20:4 could be formed by the following pathway: Δ9,12,15-18:3 → Δ6,9,12,15-18:4 → Δ8,11,14,17-20:4. However, it was assumed that, if one starts with Δ11,14,17-20:3 and examines the desaturated product, tetraene component, the ambiguity in the pathways could be resolved. The tetraene fraction, isolated from the brain by $AgNO_3$:SiO_2 TLC following intracranial injection of [3-^{14}C] eicosatrienoic acid, was examined before and after ozonolysis. It was found to be composed of four components: a) Δ8,11,14,17-20:4, b) Δ5,11,14,17-20:4, c) Δ7,13,16,19-22:4, and d)

TABLE III

Identification and Characterization of Brain Polyunsaturated Fatty Acid Methyl Esters 8 hr Following Intracranial Injection of [3-^{14}C] Eicosatrienoic Acid

Identification by $AgNO_3SiO_2$ TLC	Identification by radio-GLC				
	GLC peak identity	Percent of recovered radioactivity	Aldehyde ester from ozonolysis	Percent of recovered radioactivity	Final structure
Triene fraction	18:3 (n-3)	2	C_9	2	Δ9,12,15-18:3
	20:3 (n-3)	98	C_{11}	98	Δ11,14,17-20:3
Tetraene fraction	18:4 (n-3)	12.8	C_6	15.5	Δ6,9,12,15-18:4
	20:4 (n-3)	69.2	C_5	45.3	Δ5,11,14,17-20:4[a]
	---	---	C_8	23.5	Δ8,11,14,17-20:4
	22:4 (n-3)	18:0	C_7	15.7	Δ7,13,16,19-22:4[a]
Pentaene fraction	20:5 (n-3)	---	C_5	35.4	Δ5,8,11,14,17-20:5
	22:5 (n-3)	---	C_7	64.6	Δ7,10,13,16,19-22:5

[a]Nonmethylene interrupted double bond system.

Δ6,9,12,15-18:4. Of the recovered total radioactivity in this tetraene fraction, components a, b, and c contained 23.5, 45.3, and 15.7% of the total radioactivity (Table III). Thus, a combined total of 61% of the total recovered radioactivity was found in the tetraene components b and c, both of which contain a nonmethylene interrupted double bond system. One can, therefore, conclude that the major desaturated products of Δ11,14,17-20:3 contained a double bond system incompatible with further desaturation and, hence, can be categorized as dead end products. It may be pointed out here that although Δ5,11,14,17-20:4 is unsuitable for a desaturation reaction, it seems to be capable of chain elongation. The presence of Δ6,9,12,15-18:4 and also Δ8,11,14,17-20:4 suggested a retroconversion, Δ11,14,17-20:3 → Δ9,12,15-18:3, followed by desaturation to 18:4 and elongation to 20:4. A careful analysis of the triene fraction revealed a small but definite peak of radioactive 18:3, which on ozonolysis gave a radioactive C_9 aldehyde ester peak. This reaction of retroconversion can now explain the existence of both Δ6,9,12,15-18:4 and its elongated product, Δ8,11,14,17-20:4. It was thus concluded that radioactive Δ8,11,14,17-20:4 was formed not so much by a direct Δ^8 desaturation of the injected tracer Δ11,14,17-20:3 but via an initial retroconversion to 18:3 followed by the usual desaturation-elongation process. The retroconversion reaction has been demonstrated in saturated (5) as well as polyunsaturated fatty acids (6,7) but may not be a major pathway (8,2), and thus the Δ8,11,14,17-20:4 formed by this pathway should have a relatively smaller amount of the total radioactivity. The experimental data confirms this assumption. In conclusion, the Δ^8 desaturase necessary for reactions in the optional pathway (9) for biosynthesis of polyunsaturated fatty acids seems to be absent in the brain just as it was found to be lacking in the liver system studied by Sprecher and Lee (2).

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In Vitro Biosynthesis of Prostaglandin E_2 by Kidney Medulla of Essential Fatty Acid Deficient Rats

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ABSTRACT

Weanling rats were fed either a semi-synthetic diet with no fat, with 28% by wt partially hydrogenated fish oil, or with 28% by wt arachis oil (control diet) for 6 or 7½ months. The in vitro conversion of arachidonic acid to prostaglandin E_2 by homogenates of the rat kidney medulla was measured by gaschromatography with electron capture detection. The kidney medulla of essential fatty acid deficient animals showed increased activity for the in vitro conversion of exogenous arachidonic acid to prostaglandin E_2 when compared to the controls. The change of the enzymatic activity in the essential fatty acid deficient animals was reversible, as shown by refeeding. Inhibition of the prostaglandin synthetase was found at exogenous substrate concentrations higher than 50-100 μM.

INTRODUCTION

In the literature, contradictory results are reported for the in vitro biosynthesis of prostaglandin E (PGE) by different tissues of essential fatty acid (EFA) deficient rats (1,2). Using spectrophotometric analysis, Tan and Privett (1) found absence of biosynthetic activity in the vesicula gland of EFA-deficient rats. Nugteren and Hazelhof have made a limited study of prostaglandin biosynthesis in EFA-deficient rats (2). By adding radioactive substrate to their preparations, they found a remarkably increased biosynthesis in rat lung and small intestine in EFA-deficiency. The amount of substrate for the prostaglandin synthetase is reduced in vivo in tissues of EFA-deficient rats (3). Thus, an increase in the in vitro conversion of exogenous substrate may be expected in the deficient animals if the enzyme is intact in the homogenate. In order to compare the biosynthetic activity in EFA-deficient and normal rats, it appears relevant to measure prostaglandin biosynthetized from both endogenous and exogenous substrate. In the present study, this was achieved by using gaschromatography (GC) with electron capture detection. Preliminary experiments showed that the kidney medulla was the enzymatically most active tissue of the rat. The effect of increasing concentrations of substrate on the in vitro biosynthesis of PGE_2 by rat kidney medulla was measured.

MATERIALS AND METHODS

Forty weanling male rats (21 days old) of the Wistar strain (K. Møllegård-Hansens Avls-lab., A/S; L1. Skensved, Denmark) were divided into three groups of 20, 10, and 10 animals, respectively. Groups 1 and 2 were fed on EFA-deficient diets. Group 1 was reared on a fat-free diet (FF) (4). Group 2 was reared on the fat-free diet in which 28% by wt of sucrose was replaced by 28% by wt of partially hydrogenated fish oil (PHFO). The oil contained 1% of linoleic acid. In the control diet (group 3), 28% by wt of sucrose in the fat-free diet was replaced by 28% by wt of arachis oil (AO), containing 39% linoleic acid. Diets and water were provided ad libitum. After 6 months, 1/3 of the animals of group 1 and half of the animals of groups 2 and 3 were killed by decapitation. The rest of the animals in group 1 (FF) were divided into two equally large subgroups. One subgroup continued on the fat-free diet while the other received the control diet (AO) for 6 weeks. The rest of the animals in group 2 (PHFO) were changed to the control diet (AO). After 6 weeks, all the animals were killed by decapitation. The kidneys were quickly excised and frozen in solid CO_2. The tissues were stored at −80 C. Before testing, the medulla was separated from the cortex immediately after thawing and instantly homogenized. After cutting the kidney, the medulla is seen as the lighter part containing the papilla.

Arachidonic acid, ω-nor PGE_2 and ω-homo PGE_1, was obtained from Dr. D.A. van Dorp, Unilever Research. Diazomethane was prepared according to the method of Boer and Bacher (5) and stored at −20 C until use. N,O-bis-(trimethylsilyl)-acetamid (BSA) was obtained from Pierce Chemical Co., Rockford, IL. Other reagents used were pro analysis grade from Merck, Darmstadt, West Germany.

The determination of PGE was based on the method described by Jouvenaz et al. (6) using GC with electron capture detection. A Perkin Elmer GLC model 3920 equipped with Ni-63 electron capture detector was used. The carrier gas used was argon/methane, 90:10. Flow rate was ca. 35 ml/min, detector temperature 250 C,

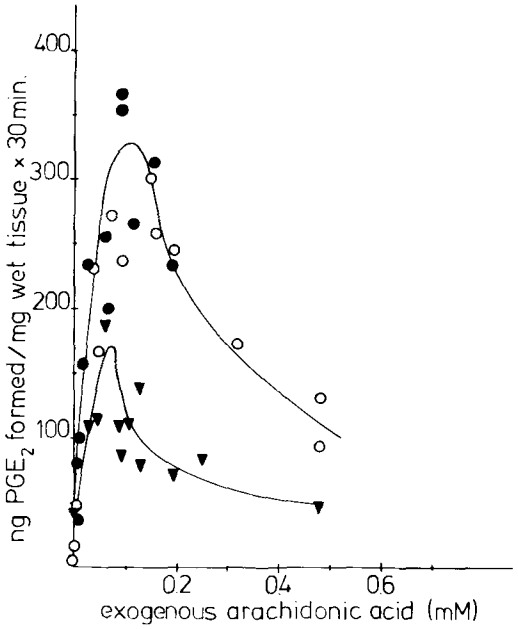

FIG. 1. Effect of increasing concentrations of exogenous arachidonic acid on the in vitro biosynthesis of prostaglandin E_2 by homogenate of kidney medulla from rats of **group 1** (FF). ○ Rats fed a fat-free diet for 6 months. ● Rats fed a fat-free diet for 7½ months. ▼ Rats fed a fat-free diet for 6 months and re-fed for 6 weeks on the control diet.

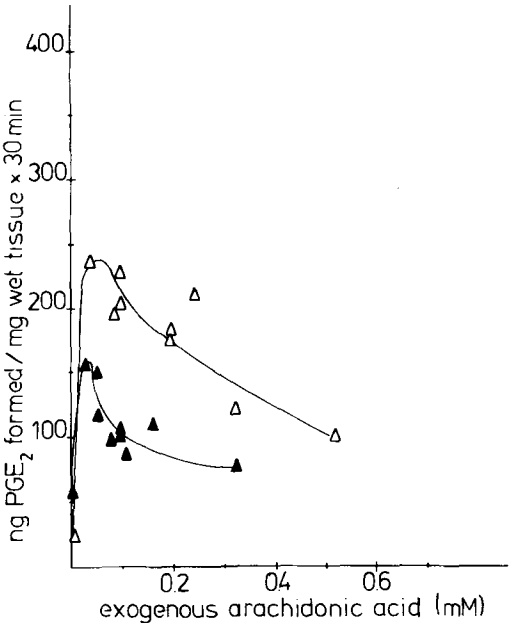

FIG. 2. Effect of increasing concentrations of exogenous arachidonic acid on the in vitro biosynthesis of prostaglandin E_2 by homogenate of kidney medulla from rats of **group 2** (PHFO). △ Rats fed a diet containing 28% by wt partially hydrogenated fish oil for 6 months. ▲ Rats fed a diet containing 28% by wt partially hydrogenated fish oil for 6 months and re-fed for 6 weeks on the control diet.

and column temperature 225 C. The dimensions of the column were 200 X 0.3 cm. Two percent OV-1 was used as stationary phase on Chromosorb W (HP) 100/120 mesh. As only one kidney medulla was applied in each experiment, the method was modified for the use of 20-50 mg of tissue only. The amount of prostaglandin biosynthetized was high in proportion to the small amount of tissue; thus, purification by column or thin layer chromatography after extraction was not needed.

After addition of internal standards (ω-nor PGE_2 and ω-homo PGE_1) and arachidonic acid, the kidney medulla was homogenized by hand at room temperature for 1 min in 750 μM phosphate buffer (pH 7.4) containing 1 mM glutathion and 20 mM EDTA. The preparation was incubated for 30 min at 37 C, and the reaction was stopped by adding 2 M citrate to reach pH 3. Four volumes of ethanol were added. After standing for 1 hr in ice, the preparation was centrifuged and the ethanol from the supernatant was evaporated off in vacuum, leaving an acid aqueous solution. The latter was extracted as described by Jouvenaz et al. (6). The last ether extraction was evaporated to dryness under nitrogen. To convert PGE to PGB, 0.1 ml 0.5 N 75% methanolic KOH was added and the solution was kept for 25 min at room temperature (7). After acidification of the preparation, the PGB was extracted with ether, esterified with diazomethane, and silylated with BAS for analysis by GC.

RESULTS AND DISCUSSION

Feeding of fat-free diet to experimental animals for several weeks results in characteristic symptoms of EFA deficiency (3). If the fat-free diet is supplemented with partially hydrogenated oils as the sole dietary fat, the symptoms of EFA deficiency appear earlier and are more pronounced than in animals on a fat-free diet (3). One of the many changes in severe EFA deficiency is necrosis of the kidney papilla (3). Several experimental rats fed on the EFA-deficient diets (FF and PHFO) exhibited dark red papillas indicating a beginning degeneration.

The effects of increasing concentration of exogenous arachidonic acid on the in vitro biosynthesis of prostaglandin E_2 are shown in Figures 1-3. The kidney medulla of EFA-deficient animals ([FF] Fig. 1 and [PHFO] Fig. 2) exhibited increased activity for the in vitro conversion of exogenous arachidonic acid to

prostaglandin E_2. In animals fed the fat-free diet (Fig. 1), the formation of PGE_2 was doubled in proportion to the control group ([AO] Fig. 3). The biosynthetic activity in animals fed partially hydrogenated fish oil (Fig. 2) also increased, although to a lesser degree than in group 1. Linoleic acid is known to be an inhibitor of the prostaglandin synthetase (8,9). As the partially hydrogenated oil contains 1% of linoleic acid, a possible inhibitory effect on the prostaglandin synthetase may explain the difference between group 1 and group 2. After feeding of group 1 (FF) and group 3 (AO) for 6 months, a continuation for another 6 weeks on the fat-free diet and the diet containing 28% by wt arachis oil, respectively, apparently did not influence the enzymatic activity. For the sake of simplicity, only one curve is drawn in Figures 1 and 3.

Refeeding the EFA-deficient animals for 6 weeks with the control diet (AO) resulted in a similar relation between substrate concentration and prostaglandin formation as in the control group (Figs. 1 and 2). This indicates that the change of biosynthetic activity in the EFA-deficient rats was reversible. Thus, the kidney medulla of rats fed EFA-deficient diets for several months returned relatively quickly to normal enzymatic activity when essential fatty acids were added to the diet.

The figures showing the relation between the prostaglandin formation and the substrate concentration exhibit a maximum at ca. 100 μM exogenous arachidonic acid in the fat-free group (Fig. 1) and a maximum at ca. 50 μM exogenous arachidonic acid in the control group (Fig. 3). Because of the content of endogenous substrate in the tissues of the control rats, a comparison of the values is not meaningful. The liberation of arachidonic acid from phospholipids during homogenization and incubation was expected to be minimal due to the inhibition of the Ca^{++} sensitive phospholipase by EDTA in the incubation buffer (10). However, in the very recent literature (11,12), it has been pointed out that the triglycerides of the rat renal medulla contain considerable amounts of arachidonic acid. A hydrolysis of these triglycerides should, therefore, be considered in future experiments.

Inhibition of the PGE formation has been found at concentrations of arachidonic acid higher than 0.3-0.5 mM using the synthetase from the microsomal fraction of bovine seminal vesicle (13-15). This phenomenon was not observed with rabbit kidney microsomes (16,17). In the present experiments using homogenates of rat kidney medulla, inhibition was observed at increasing concentrations of arachidonic acid for all experimental groups.

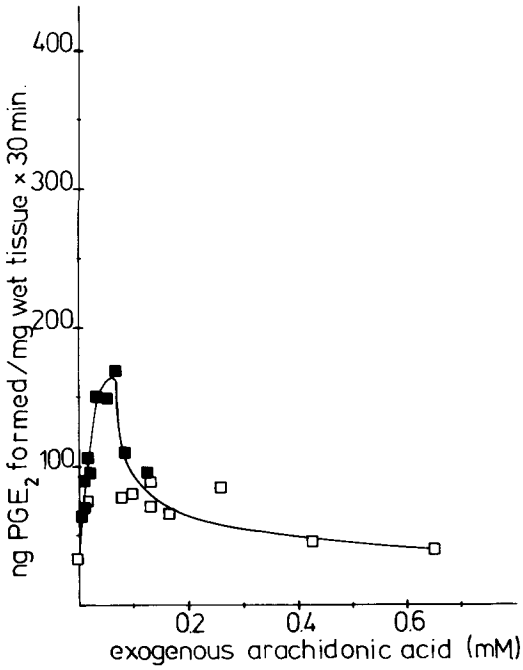

FIG. 3. Effect of increasing concentrations of exogenous arachidonic acid on the in vitro biosynthesis of prostaglandin E_2 by homogenate of kidney medulla from rats of group 3 (AO). □ Rate fed a diet containing 28% by wt arachis oil for 6 months. ■ Rats fed a diet containing 28% by wt arachis oil for 7½ months.

From the present experiments, it is not possible to conclude why the kidney medulla of EFA-deficient rats is able to convert more arachidonic acid than is the same tissue from the control rats. An explanation may be an increased amount of synthetase or perhaps a change in the kinetics of the prostaglandin synthetase during EFA deficiency. Deficiency in essential fatty acids gradually changes the composition of the phospholipids (3). This may influence the position and/or the activity of the enzyme in the membrane. A general change in the content of free fatty acids in the tissue may also influence the activity of the prostaglandin synthetase. Deficiency of linoleic acid in the EFA-deficient animals may cause increased activity of the synthetase as linoleic acid is an inhibitor of this enzyme (8,9). In EFA-deficient animals, trienoic acid (20:3 [n-9]) accumulates (3). The inhibitory effect of this fatty acid on the prostaglandin synthetase has been shown by Ziboh et al. (18). However, in the present experiments, an increase of the enzymatic activity was found regardless of the possible inhibitory effect of the eicosatrienoic acid.

Different synthetase systems may exist depending on the tissue under consideration (14,17). This may explain the difference between the present results and the results obtained by Tan and Privett (1).

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Phospholipid Composition of *Culex quinquefasciatus* and *Culex tritaeniorhynchus* Cells in Logarithmic and Stationary Growth Phases

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ABSTRACT

Culex quinquefasciatus and *Culex tritaeniorhynchus* cells were grown in spinner culture and harvested in logarithmic and stationary phases of growth. The phospholipids were extracted from the cells, and the fatty acid profiles of the phospholipid classes were determined and compared. The major components were phosphatidylcholine and phosphatidylethanolamine, constituting $\geq$ 80% of the phospholipid. The fatty acid profiles of lysophosphatidylcholine, phosphatidylinositol, and cardiolipin showed changes with aging of the Culex cells and between the species. In the lysophosphatidylcholine fraction, there was an increase in saturation of the fatty acids of the *C. quinquefasciatus* cells, and chain lengthening occurred in both species from the logarithmic to stationary phases of growth. In the phosphatidylinositiol fraction, both Culex species showed a decrease in monoenes and an increase in polyenes, while only the *C. tritaeniorhynchus* cells showed an increase in fatty acid chain length with aging. The *C. quinquefasciatus* cells had an increase in polyenes with aging in the cardiolipin fraction. Differences in the percentage composition of the fatty acids were shown in all the phospholipid fractions between the Culex species in the logarithmic phase of growth and all except the phosphatidylinositiol and cardiolipin fractions in the stationary phase.

INTRODUCTION

It has been reported that the lipid composition of Dipteran insects differs from most other insects and mammals (1). The phospholipids of Diptera generally contained $>$ 50% phosphatidylethanolamine and ca. 25% phosphatidylcholine in contrast to the phospholipids of mammals, which usually had 50% phosphatidylcholine and 25% phosphatidylethanolamine (1,2).

It has been shown that the composition of the lipids from cells grown in tissue culture is similar to the composition of those obtained from the analysis of whole insects (3). Also, the fatty acid profiles from lipids of whole insects and specific tissues have been shown to be similar (4).

Municio et al. (5), using homogenates of the Dipteran *Ceratitis capitata*, reported that each insect developmental stage possessed a different biosynthetic capability of producing fatty acids. Further evidence was presented that the rates of lipid synthesis were less in the egg and adult stages of the insect than the pupal and larval stages. In later studies, Municio and coworkers (6) showed that larval and pharate adult homogenates of *C. capitata* followed a different pattern of incorporation of labeled fatty acids into the main classes of lipids. Experiments were performed to determine changes in composition of the fatty acids at various stages of insect development by in vivo feeding of larvae of *C. capitata* with labeled fatty acids (7). The elongation of acids occurred both in in vivo and in vitro studies. However, the in vivo results of incorporation differed from the in vitro studies in the triacylglycerol-free fatty acid relationship.

Cells, from different developmental stages of insects, which were cultivated in tissue culture medium and harvested at selected phases of growth, could provide an additional tool and means of approach to understanding the metabolism of fatty acids and changes within insect cells.

In this study, the profiles of the fatty acids of phospholipids of *Culex quinquefasciatus* and *Culex tritaeniorhynchus* cells were compared after growth in spinner culture. The cells were harvested in logarithmic and stationary phases of growth in an attempt to determine if lipid differences existed between species and with aging of the cells.

MATERIALS AND METHODS

Cells

Dr. S.H. Hsu, U.S. Naval Medical Research Unit No. 2, Taipei, Taiwan, provided the *C. quinquefasciatus* and *C. tritaeniorhynchus* cells (8,9). The cells were cultivated in suspension at 28 C in nine 250 ml spinner flasks containing

TABLE I

Distribution of Phospholipids of *Culex quinquefasciatus* and *Culex tritaeniorhynchus* Cells in Late Logarithmic and Stationary Phases of Growth

Phospholipid classes	Leafhopper medium	*C. quinquefasciatus*		*C. tritaeniorhynchus*	
		Log[a]	Stat[b]	Log	Stat
Lysophosphatidylcholine	8.2[c] ± 1.2[d]	1.2 ± 0.1	1.1 ± 0.0	2.3 ± 0.3	1.1 ± 0.0
Sphingophospholipids	16.0 ± 1.2	6.7 ± 2.1	10.1 ± 2.4	5.2 ± 1.0	6.3 ± 0.3
Phosphatidylcholine	71.8 ± 0.8	43.0 ± 5.1	35.1 ± 1.4	37.2 ± 2.2	39.4 ± 2.6
Phosphatidylserine	0.3 ± 0.1	0.4 ± 0.0	2.5 ± 2.3	4.2 ± 0.0	2.2 ± 1.8
Phosphatidylinositol	2.0 ± 0.3	5.4 ± 0.1	3.4 ± 0.6	3.8 ± 0.8	7.3 ± 2.3
Phosphatidylethanolamine	1.7 ± 0.3	41.3 ± 3.1	46.9 ± 1.8	45.1 ± 1.1	42.7 ± 3.4
Cardiolipin		2.0 ± 0.2	0.9 ± 0.1	2.2 ± 1.2	1.0 ± 0.1

[a]Logarithmic phase of growth.

[b]Stationary phase of growth.

[c]Relative percentage of fatty acid.

[d]Mean ± differences from the mean.

150 ml leafhopper medium (10) supplemented with 20% heat-inactivated newborn calf serum. The cells were harvested after 3 days and 2 days in the late logarithmic phase of growth and 10 days and 8-10 days in the stationary phase of growth of *C. quinquefasciatus, C. tritaeniorhynchus* cells, respectively.

The cells of each species were washed 2 times in Hanks' balanced salt solution (11).

Lipid Analysis

The total lipids were extracted from the mosquito cells by the method described by Makino et al. (12) and fractionated into total neutral lipids and total phospholipids on a silicic acid column (13). The total phospholipids were separated into classes by thin layer chromatography using plates coated with Silica Gel H and the solvent system chloroform: methanol:acetic acid:water (25:15:4:2) (14). The positions of the bands of phospholipids on the plates were located by exposing the plates to iodine vapor (15). The bands were scraped from the plates. The lipid was extracted from the band containing phosphatidylinositol, phosphatidylserine, and ceramide phosphorylethanolamine. The extracted lipid was rechromatographed on plates spread with Silica Gel H containing magnesium acetate (1.5 g magnesium acetate and 20 g Silica Gel H suspended in 55 ml water), and the classes further separated with chloroform:methanol:28% aqueous ammonia (65:25:5) (16).

Methyl Ester Analysis

The bands of absorbent containing each of the phospholipid fractions were transferred to screw cap tubes for methylation. The lipids were transesterified with 5% HCl in methanol (17).

The methyl esters were analyzed by gas liquid chromatography on a Fisher Victoreen gas chromatograph, Model 4000. The column used was 8 ft x 1/8 in. ID (244 cm x 0.32 cm) packed with 15% EGSS-X on Gas Chrom P, 100-120 mesh (Applied Science Laboratories, State College, PA).

Standards

Phospholipid standards were obtained from the Lipids Preparation Laboratory, The Hormel Institute, Austin, MN; Applied Science Laboratory, State College, PA; Supelco, Inc., Bellefonte, PA; or prepared in our laboratory. The methyl ester standards were purchased from the Lipids Preparation Laboratory, The Hormel Institute, Austin, MN.

RESULTS

The results were obtained from two sets of independent experiments with each mosquito cell strain harvested at logarithmic and stationary phases of growth. Comparisons were made between the two Culex cells strains at both stages of development and the leafhopper medium which contained a mammalian source of lipid.

The relative percentages of the phospholipid classes are listed in Table I. Phosphatidylcholine and phosphatidylethanolamine represented the main components and were approximately equal in amount.

The sphingophospholipids, ceramide phosphorylcholine and ceramide phosphorylethanolamine, were isolated and purified as reported previously (18). According to our studies, the fatty acid composition of these two sphingophospholipids from the Culex cells were essentially the same as previously reported (18) (data not repeated here).

TABLE II

Constituent Fatty Acids of Phospholipids–Lysophosphatidylcholine Fraction

Fatty acid	Leafhopper medium	*Culex quinquefasciatus* Log[a]	*Culex quinquefasciatus* Stat[b]	*Culex tritaeniorhynchus* Log	*Culex tritaeniorhynchus* Stat
14:0[c]	0.6[d] ± 0.1[e]	0.7 ± 0.2	0.7 ± 0.2	0.8 ± 0.1	1.4 ± 0.6
15:0	0.5 ± 0.1				
16:0	28.6 ± 2.3	13.5 ± 2.7	12.6 ± 0.9	20.6 ± 3.0	18.1 ± 4.2
16:1	2.3 ± 1.3	3.9 ± 0.8	1.9 ± 0.0	4.2 ± 1.8	7.3 ± 0.0
17:0	1.6 ± 0.2			0.7 ± 0.1	
18:0	39.5 ± 0.5	13.5 ± 1.4	13.0 ± 3.1	18.5 ± 3.7	13.1 ± 1.1
18:1	19.5 ± 1.8	17.4 ± 0.0	6.4 ± 0.1	20.4 ± 5.7	17.4 ± 3.3
18:2	2.4 ± 0.5	2.8 ± 0.2	1.4 ± 0.1	2.2 ± 0.1	2.4 ± 0.4
18:3	2.9 ± 1.1				
20:0		25.2 ± 0.8	34.9 ± 0.2	15.4 ± 0.2	23.3 ± 0.2
20:3	0.5 ± 0.1				
20:4	1.0 ± 0.1	2.2 ± 0.8	0.8 ± 0.3	0.8 ± 0.8	tr[f]
20:5			1.3 ± 0.0	1.5 ± 1.5	1.3 ± 0.7
22:0		20.8 ± 3.8	26.5 ± 2.1	13.4 ± 2.5	16.3 ± 0.0
22:4				0.7 ± 0.7	
22:5	tr				
22:6	tr				
Saturates	70.8	73.7	87.7	69.4	72.2
Monoenes	21.8	21.3	8.3	24.6	24.7
Polyenes	6.3	5.0	3.5	5.2	3.7

[a]Logarithmic phase of growth.

[b]Stationary phase of growth.

[c]Number of carbon atoms in acid:number of double bonds.

[d]Relative percentage of fatty acid.

[e]Mean ± difference from the mean.

[f]tr = trace, < 0.5%.

TABLE III

Constituent Fatty Acids of Phospholipids–Phosphatidylcholine Fraction

Fatty acid	Leafhopper medium	*Culex quinquefasciatus* Log[a]	*Culex quinquefasciatus* Stat[b]	*Culex tritaeniorhynchus* Log	*Culex tritaeniorhynchus* Stat
14:0[c]	tr[d]	1.6[e] ± 0.1[f]	2.4 ± 0.0	1.4 ± 0.1	1.9 ± 0.1
15:0	0.9 ± 0.3				
16:0	15.4 ± 2.2	18.3 ± 0.3	21.7 ± 0.5	17.8 ± 0.0	17.1 ± 0.8
16:1	1.9 ± 0.7	11.7 ± 0.2	11.2 ± 0.1	10.1 ± 0.1	15.1 ± 0.3
17:0	0.9 ± 0.2	1.0 ± 0.1	0.6 ± 0.1	0.8 ± 0.0	0.6 ± 0.0
17:1	0.7 ± 0.3				
18:0	21.4 ± 0.3	10.4 ± 0.7	11.0 ± 0.1	13.3 ± 0.1	11.3 ± 0.2
18:1	31.2 ± 3.7	39.2 ± 1.3	34.4 ± 0.1	41.6 ± 0.5	34.9 ± 0.0
18:2	10.5 ± 0.9	8.6 ± 0.5	9.6 ± 0.2	6.4 ± 0.8	9.6 ± 0.7
18:3	0.9 ± 0.4	0.8 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.8 ± 0.0
20:0		0.8 ± 0.1	1.9 ± 0.3	0.6 ± 0.1	1.0 ± 0.2
20:2	0.9 ± 0.5				
20:3	4.1 ± 0.4	0.8 ± 0.1	1.0 ± 0.1	1.2 ± 0.0	1.2 ± 0.0
20:4	6.8 ± 0.5	4.2 ± 1.4	1.9 ± 0.0	2.7 ± 0.1	3.1 ± 0.3
20:5	0.6 ± 0.1	1.2 ± 0.5	tr	tr	0.5 ± 0.0
22:4		tr	0.7 ± 0.0	0.6 ± 0.0	0.5 ± 0.1
22:5	1.5 ± 1.0	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.0	1.2 ± 0.1
22:6	1.7 ± 0.1	tr	0.7 ± 0.2	0.5 ± 0.1	0.7 ± 0.1
Saturates	38.6	32.1	37.6	33.9	31.9
Monoenes	33.8	50.9	45.6	51.7	50.0
Polyenes	27.0	16.6	15.7	13.0	17.6

[a]Logarithmic phase of growth.

[b]Stationary phase of growth.

[c]Number of carbon atoms in acid:number of double bonds.

[d]Tr = trace, < 0.5%. Trace amounts of 12:0, 14:1, and 22:3 not listed.

[e]Relative percentage of fatty acid.

[f]Mean ± differences from the mean.

TABLE IV

Constituent Fatty Acids of Phospholipids—Phosphatidylserine Fraction

Fatty acid	Leafhopper medium	*Culex quinquefasciatus*		*Culex tritaeniorhynchus*	
		Log[a]	Stat[b]	Log	Stat
14:0[c]	1.0[d] ± 0.3[e]		0.6 ± 0.0	tr[f]	tr
15:0	0.7 ± 0.3		tr		tr
16:0	15.7 ± 2.4	2.7 ± 0.0	5.3 ± 2.3	4.9 ± 0.8	4.5 ± 1.4
16:1	1.1 ± 0.0	5.3 ± 0.9	7.2 ± 0.0	7.1 ± 0.4	11.9 ± 2.4
17:0	1.2 ± 0.0		tr	0.5 ± 0.1	tr
17:1	1.2 ± 1.2				
18:0	35.8 ± 2.5	22.1 ± 0.2	19.6 ± 0.0	22.2 ± 0.2	19.3 ± 3.6
18:1	20.9 ± 1.5	61.0 ± 0.5	53.5 ± 2.5	56.0 ± 1.6	51.5 ± 3.0
18:2	3.0 ± 0.2	1.8 ± 0.2	4.0 ± 0.3	4.5 ± 0.0	4.0 ± 1.5
18:3	0.6 ± 0.1	1.3 ± 0.3	1.2 ± 0.1	0.8 ± 0.0	0.7 ± 0.1
20:0	2.6 ± 0.8	2.8 ± 0.7	4.6 ± 0.0	1.2 ± 0.0	3.4 ± 0.8
20:3	4.7 ± 3.2	0.9 ± 0.1	0.7 ± 0.7	1.3 ± 0.0	0.8 ± 0.8
20:4	7.1 ± 0.7	1.0 ± 0.3	2.0 ± 0.2	1.2 ± 0.0	1.6 ± 0.3
22 series	5.0 ± 4.9		tr		
Saturates	57.0	27.6	30.1	28.3	27.2
Monoenes	23.2	66.3	60.7	63.1	63.4
Polyenes	20.4	5.0	7.9	7.8	7.1

[a]Logarithmic phase of growth.

[b]Stationary phase of growth.

[c]Number of carbon atoms in acid:number of double bonds.

[d]Relative percentage of fatty acid.

[e]Mean ± difference from the mean.

[f]Tr = trace, < 0.5%.

TABLE V

Constituent Fatty Acids of Phospholipids—Phosphatidylinositol Fraction

Fatty acid	Leafhopper medium	*Culex quinquefasciatus*		*Culex tritaeniorhynchus*	
		Log[a]	Stat[b]	Log	Stat
14:0[c]	tr[d]	0.5[e] ± 0.2[f]	0.5 ± 0.0	tr	tr
15:0		tr	tr	tr	
16:0	10.4 ± 0.0	10.0 ± 0.1	9.6 ± 1.0	12.7 ± 0.1	9.0 ± 3.0
16:1	1.5 ± 0.3	10.7 ± 1.0	6.9 ± 1.5	6.4 ± 0.7	7.2 ± 0.5
17:0	1.4 ± 0.0	0.5 ± 0.1	tr	tr	tr
18:0	33.5 ± 0.8	17.8 ± 0.7	19.3 ± 1.2	21.6 ± 0.2	21.3 ± 4.2
18:1	19.8 ± 2.1	45.4 ± 0.5	37.3 ± 0.4	48.0 ± 2.8	37.9 ± 0.8
18:2	3.0 ± 0.5	6.0 ± 1.1	9.8 ± 1.9	4.8 ± 2.5	9.0 ± 2.1
18:3	2.3 ± 1.8	1.1 ± 0.1	1.0 ± 0.1	0.8 ± 0.0	1.1 ± 0.1
20:0	tr	3.1 ± 0.3	4.1 ± 0.8	1.2 ± 0.3	2.6 ± 0.7
20:2	tr				
20:3	6.3 ± 0.3	1.7 ± 0.4	2.7 ± 0.8	1.2 ± 0.5	5.1 ± 1.1
20:4	16.9 ± 0.3	1.9 ± 0.2	4.8 ± 0.9	1.6 ± 1.1	5.1 ± 0.7
20:5	tr	tr	1.1 ± 0.0		1.0 ± 0.0
22:4	0.7 ± 0.3	tr	0.6 ± 0.3		
22:5	1.8 ± 1.0	tr	tr		
22:6	0.8 ± 0.1		0.7 ± 0.7		
Saturates	45.3	31.9	33.5	35.5	32.9
Monoenes	21.3	56.1	44.2	54.4	45.1
Polyenes	32.4	10.7	19.4	8.4	21.3

[a]Logarithmic phase of growth.

[b]Stationary phase of growth.

[c]Number of carbon atoms in acid:number of double bonds.

[d]Tr = trace, < 0.5%.

[e]Relative percentage of fatty acids.

[f]Mean ± differences from the mean.

TABLE VI

Constituent Fatty Acids of Phospholipids—Phosphatidylethanolamine Fraction

Fatty acid	Leafhopper medium	*Culex quinquefasciatus*		*Culex tritaeniorhynchus*	
		Log[a]	Stat[b]	Log	Stat
14:0[c]	0.7[d] ± 0.1[e]	1.8 ± 0.2	2.1 ± 0.2	0.5 ± 0.0	0.9 ± 0.2
15:0	2.9 ± 1.2				
16:0	7.7 ± 0.0	18.8 ± 0.1	18.5 ± 0.1	15.6 ± 0.6	14.3 ± 0.1
16:1	1.4 ± 0.0	13.2 ± 1.9	13.3 ± 0.0	9.4 ± 0.4	12.8 ± 0.1
17:0	1.1 ± 0.6	tr[f]	tr	0.5 ± 0.0	
17:1	1.0 ± 0.1				
18:0	19.8 ± 0.2	12.1 ± 0.9	10.7 ± 0.2	13.0 ± 0.2	13.6 ± 0.2
18:1	26.8 ± 1.1	44.8 ± 1.9	40.9 ± 1.5	47.0 ± 0.8	40.6 ± 0.8
18:2	9.7 ± 1.2	5.7 ± 0.0	8.6 ± 1.0	8.0 ± 0.9	10.8 ± 0.9
18:3	0.7 ± 0.5	0.5 ± 0.2	0.9 ± 0.4	0.9 ± 0.2	0.8 ± 0.1
20:0	0.8 ± 0.0	1.4 ± 0.2	2.2 ± 0.6	0.6 ± 0.1	1.5 ± 0.0
20:2	1.8 ± 1.4				
20:3	2.9 ± 0.9	0.6 ± 0.1	0.5 ± 0.1		0.5 ± 0.5
20:4	15.7 ± 1.1	0.8 ± 0.0	2.0 ± 0.4	3.1 ± 0.0	4.2 ± 0.2
20:5	0.9 ± 0.9	tr		0.5 ± 0.0	
22:4	1.4 ± 0.3				
22:5	4.1 ± 1.7				
22:6	1.2 ± 1.2				
Saturates	33.0	34.1	33.5	30.2	30.3
Monoenes	29.2	58.0	54.2	56.4	53.4
Polyenes	38.4	7.6	12.0	12.5	16.3

[a]Logarithmic phase of growth.

[b]Stationary phase of growth.

[c]Number of carbon atoms in acids:number of double bonds.

[d]Relative percentage of fatty acids.

[e]Mean ± differences from the mean.

[f]Tr = trace, < 0.5%.

C. quinquefasciatus Cells—Fatty Acid Profiles from the Logarithmic and Stationary Phases of Growth

The relative amounts of the phospholipid fractions (Table I) were similar from cells obtained in both stages of growth of *C. quinquefasciatus.* With aging of cells, the following observations were made relative to the fatty acids of various phospholipid fractions:

a. There was an increase in the amount of total saturated acids and a decrease in monoenes in the lysophosphatidylcholine fraction (Table II). The fatty acid profiles of lysophosphatidylcholine showed a decrease of 18:1 acid, an increase of 20:0 acid, and an increase in the average chain length of the acids.

b. The results from the data of the analysis of the phosphatidylcholine fraction (Table III) suggested an increase in saturated acids with a corresponding decrease in monoenes, and a tendency toward an increase in the 16:0 fatty acid and a decrease in the 18:1 fatty acid.

c. In the phosphatidylserine fraction (Table IV), the amounts of either the total saturated acids or polyenes were about the same in each phase of growth. In the fatty acid profile, there was a decrease in the 18:1 fatty acid.

d. There was an increase in the amount of polyenes and a decrease in the amount of monoenes in the phosphatidylinositol fraction (Table V). A decrease was observed in 18:1 fatty acid and an increase in the 20 carbon unsaturated fatty acids.

e. There was some increase in the polyenes and in the percentage of the 18:2 fatty acid in the phosphatidylethanolamine fraction (Table VI).

f. In the cardiolipin fraction (Table VII), the amount of saturated acids decreased and polyenes increased. There was a decrease in the relative percentage of 18:1 fatty acid and an increase in 18:2 fatty acid.

C. tritaeniorhynchus Cells—Fatty Acid Profiles from the Logarithmic and Stationary Phases of Growth

The relative percentages of the phospholipid classes were about the same in the *C. tritaeniorhynchus* cells from logarithmic to stationary phase (Table I). With the aging of cells, the following observations were made about the fatty acid profiles of each phospholipid class:

a. The 20:0 fatty acid and the average chain length of the fatty acids increased in the lysophosphatidylcholine class (Table II).

b. There was a suggested increase in polyenes in the phosphatidylcholine fraction (Table III). Changes in the fatty acid profile occurred with

TABLE VII

Constituent Fatty Acids of Phospholipids–Cardiolipin Fraction

Fatty acid	*Culex quinquefasciatus*		*Culex tritaeniorhynchus*	
	Log[a]	Stat[b]	Log	Stat
12:0[c]	1.1[d] ± 0.1[e]			
14:0	3.2 ± 0.6	2.8 ± 0.1	1.6 ± 0.6	2.4 ± 0.4
14:1	0.7 ± 0.2	1.5 ± 0.6	0.5 ± 0.0	tr[f]
15:0			0.7 ± 0.7	
16:0	15.2 ± 1.6	13.4 ± 2.4	12.4 ± 0.2	11.0 ± 0.2
16:1	12.5 ± 1.6	16.5 ± 3.4	12.8 ± 1.0	23.9 ± 3.3
17:0	0.7 ± 0.1	0.6 ± 0.6	1.2 ± 0.2	1.1 ± 0.2
18:0	13.4 ± 0.5	10.0 ± 1.8	13.5 ± 0.5	9.8 ± 0.3
18:1	31.2 ± 1.7	22.6 ± 3.9	31.7 ± 2.0	19.8 ± 6.3
18:2	9.9 ± 0.4	19.8 ± 1.5	15.9 ± 0.5	21.9 ± 1.3
18:3	1.2 ± 0.2	1.1 ± 1.1	1.9 ± 0.7	0.8 ± 0.8
20:0	4.4 ± 0.4	4.0 ± 0.3	1.4 ± 0.2	1.9 ± 0.3
20:3	3.8 ± 0.3	3.2 ± 1.4	2.4 ± 0.1	3.2 ± 0.8
20:4	2.1 ± 0.9	4.1 ± 0.6	3.9 ± 1.7	0.8 ± 0.8
20:5	0.7 ± 0.7	0.6 ± 0.6		2.4 ± 2.4
Saturates	38.0	30.8	30.8	26.2
Monoenes	44.4	40.6	45.0	43.7
Polyenes	17.7	28.8	24.1	29.1

[a]Logarithmic phase of growth.
[b]Stationary phase of growth.
[c]Number of carbon atoms in acid:number of double bonds
[d]Relative percentage of fatty acid.
[e]Mean ± differences from the mean.
[f]Tr = trace, < 0.5%.

an increase in the amount of 16:1 acid and a decrease in the amount of 18:1 acid.

c. The phosphatidylserine fraction (Table IV) showed an increase in the relative percentage of 16:1 acid in the fatty acid profile.

d. The phosphatidylinositol fraction (Table V) showed a decrease in the amount of monoenes and an increase in polyenes. A decrease was observed in the percentage of 18:1 acid and an increase in the amount of 20 carbon acids in the fatty acid profiles.

e. The fatty acid profile of the phosphatidylethanolamine fraction (Table VI) showed an increase in 16:1 acid and a decrease in 18:1 acid.

f. In the cardiolipin fraction (Table VII), there was a slight increase in polyenes, increases in the relative percentages of 16:1 and 18:2 fatty acids, and decreases in 18:0 and 18:1 fatty acids.

Comparison of Profiles of Fatty Acids of *C. quinquefasciatus* and *C. tritaeniorhynchus* Cells in the Logarithmic and Stationary Phases

a. The *C. quinquefasciatus* cells contained a larger amount of 20:0 fatty acid and a tendency toward a smaller amount of 16:0 fatty acid and a larger amount of 22:0 fatty acid than the *C. tritaeniorhynchus* cells in the lysophosphatidylcholine fraction in the logarithmic phase of growth (Table II). In the stationary growth phase, the *C. quinquefasciatus* cells contained more saturated acids and less monoenes than *C. tritaeniorhynchus* cells. In the same phase of growth, more 20:0 and 22:0 fatty acids and less 16:1 and 18:1 fatty acids were observed in *C. quinquefasciatus* cells than the *C. tritaeniorhynchus* cells.

b. The amounts of saturated acids, monoenes, and polyenes in the phosphatidylcholine fraction were approximately equal in both species in the logarithmic phase (Table III). However, the *C. quinquefasciatus* cells had a smaller percentage of 18:0 in the fatty acid profile. In the stationary phase of growth, the *C. quinquefasciatus* cells had a tendency toward a larger amount of saturated acids, a larger amount of 16:0 fatty acid, and a smaller amount of 16:1 fatty acid than was observed in the *C. tritaeniorhynchus* cells.

c. The *C. quinquefasciatus* cells in comparison to the *C. tritaeniorhynchus* cells in the logarithmic phase generally had a larger percentage of 18:1 fatty acid in the phosphatidylserine fraction. In the stationary phase, *C. quinquefasciatus* cells contained less 16:1 fatty acid than *C. tritaeniorhynchus* cells.

d. The *C. quinquefasciatus* cells, in the logarithmic phase, contained a larger percentage of 16:1 fatty acid and smaller percentages of 16:0 and 18:0 fatty acids than *C. tritaeniorhynchus* cells in the phosphatidylinositol frac-

tion (Table V). The fatty acid profiles of both species of Culex cells were approximately the same in the stationary phase of growth.

e. In the phosphatidylethanolamine fraction, a smaller amount of polyenes was observed in the *C. quinquefasciatus* cells than was found in the *C. tritaeniorhynchus* cells in both growth phases (Table VI). The *C. quinquefasciatus* cells had larger amounts of 16:0 fatty acid in both growth phases and a smaller amount of 18:0 fatty acid in the stationary phase than the *C. tritaeniorhynchus* cells.

f. The *C. quinquefasciatus* cells had a larger percentage of saturated acids and a smaller percentage of polyenic and 18:2 fatty acids than the *C. tritaeniorhynchus* cells in the logarithmic phase in the cardiolipin fraction (Table VII). A suggestion of a decrease in the amount of saturated acids was observed in the *C. tritaeniorhynchus* cells in comparison to the *C. quinquefasciatus* cells in the stationary phase. The fatty acid profiles of the two species of cells were similar in the stationary phase.

DISCUSSION

The phospholipid composition and the fatty acid profiles of the phospholipids analyzed from the Culex cells did not resemble the lipids from calf serum in the growth medium.

Among insects in the Dipteran order, several have phosphatidylethanolamine as the principal phospholipid (1,2). Culex cells grown in a suspension culture contained about equal amounts of phosphatidylcholine and phosphatidylethanolamine (82-84% of the total phospholipids) at both phases of development. The phospholipids, from the Aedes genera of mosquito cells (19) grown under similar conditions, contained 42-54% of phosphatidylethanolamine and 28-37% of phosphatidylcholine.

Variations in the fatty acid profiles of the phospholipid classes between the Culex cells and the medium were observed. These differences suggest that the cells could chain elongate saturated and monoenoic acids. Fast (20) reported that polyunsaturated fatty acids were not synthesized by insects. In the Culex cells, the fatty acid profiles of the phosphatidylcholine and phosphatidylethanolamine fractions were similar. It was suggested by Fernandez-Sousa et al. (21) that the similarity of phosphatidylcholine and phosphatidylethanolamine fatty acid profiles in the egg of *C. capitata* was due to the absence of transacylating reactions.

From the series of studies of lipids of Aedes and Culex species (18,19, 22-25), several distinguishing features of these cells emerged when these cells were grown under the same conditions. Some variations in the fatty acid profiles from lipids of Aedes and Culex mosquito cells occurred between genera and growth phases. In the total lipid, total neutral lipids and total phospholipid fractions, the Aedes species showed an increase in the relative percentage of 18:2 fatty acid from the logarithmic to stationary phases (22), whereas the amount of 18:2 fatty acid in the Culex cells remained approximately the same with aging of the cells (23). The Aedes and Culex cells could be distinguished from each other by comparison of the fatty acid patterns from the ceramide phosphorylcholine fraction at both phases of growth (18). The Aedes species could be separated from the Culex species by the fatty acid patterns of the ceramide phosphorylethanolamines. The lysophosphatidylcholine fraction from the Culex cells contained a larger percentage of 22:0 fatty acid than the Aedes cells (19).

From these data, it would be intriguing to determine if these differences could be associated with variations of virus susceptibility of each of the different mosquito species. Also, the changes observed in aging of mosquito cells might be another factor associated with variation in host susceptibility to virus infection. From these studies, it would appear that it is now possible to selectively study lipid metabolism of mosquito cells using specifically labeled lipid precursors.

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Effects of Protected Cyclopropene Fatty Acids on the Composition of Ruminant Milk Fat

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ABSTRACT

Unsaturated fatty acids can be protected from ruminal hydrogenation, and, when fed to lactating ruminants, the constituent acids are incorporated into milk triacylglycerols. By this means, it has been possible to reduce the melting point of milk triglycerides and to make softer butter fat. This report shows that, by feeding small amounts of protected cyclopropene fatty acids, one is also able to make harder butter fat. *Sterculia foetida* seed oil, a rich source of cyclopropene fatty acids, was emulsified with casein and spray dried to yield a free flowing dry powder. When this material was treated with formaldehyde and fed to lactating goats (ca. 1 g cyclopropene fatty acids per day), there were substantial increases in the proportions of stearic acid and decreases in the proportions of oleic acid in milk fat. Similar results were obtained when the formaldehyde-treated supplements were fed to lactating cows (ca. 3 g cyclopropene fatty acids per day). The effect was considerably less apparent when the *S. foetida* seed oil-casein supplement was not treated with formaldehyde, suggesting that cyclopropene fatty acids are hydrogenated in the rumen as are other unsaturated fatty acids. The effect of feeding protected cyclopropene fatty acids on the stearic: oleic ratio in milk fat is probably due to cyclopropene-mediated inhibition of the mammary desaturase enzymes.

INTRODUCTION

Lipids can be protected against microbial degradation in the rumen by embedding droplets of the lipid within an insoluble formaldehyde-treated protein matrix (1,2). When fed to ruminants, such protected lipids pass through the rumen into the abomasum, where the acid conditions allow the lipid to be released for subsequent digestion. Protected lipid supplements have been used to prepare linoleic acid-enriched beef and sheep meats and milk and dairy products (3,4). The linoleic acid of seeds or seed oils is normally hydrogenated in the rumen, but, by feeding these oils in a protected form, the linoleic acid escapes hydrogenation and is subsequently absorbed from the small intestine. Also, by using the protected lipid process, one is able to feed larger amounts of dietary triacylglycerols without deleterious effects on ruminant digestion and metabolism (5,6). This increase in the caloric density of the diet, irrespective of whether saturated or polyunsaturated fats are protected, will produce increases in milk fat content (7) and may be useful in alleviating the problem of the "low milk-fat syndrome" (8).

The incorporation of protected dietary linoleate into milk fat causes a reduction in the melting point of the fat and thus a softer butter. In this paper, we show how the protected lipid process can also be used to produce harder butters. Furthermore, the studies reported here demonstrate that, by feeding small amounts of a protected biologically active lipid, one can markedly modify lipid metabolism in the ruminant mammary gland.

MATERIALS AND METHODS

Animals and Diets

Lactating cows and goats were fed basal diets of chopped alfalfa hay and crushed oats (1:1, w/w). The spray-dried casein-*Sterculia foetida* seed oil supplements were fed in amounts sufficient to supply cyclopropene fatty acids at the approximate rates of 1 g/day (goats) or 3 g/day (cows).

To ensure consumption, the *S. foetida* seed oil supplements were offered to the animals mixed with ca. 10-fold weight of crushed oat grain, and this mixture was eaten by each animal before the main bulk of feed was given.

Spray-dried *S. foetida* Seed Oil Supplements

Oil was obtained from *S. foetida* seeds by extraction with hexane. The hexane was removed and the oil was emulsified with an equal weight of sodium caseinate in water (24% solids, pH 7.0), and the emulsion was spray dried using a rotary atomizer to yield the "untreated supplement" (2).

The formaldehyde-treated spray-dried material ("treated supplement") was prepared by spraying the untreated dry powder with formalin (37% formaldehyde) (3.7 g formaldehyde/100 g casein). The treated material was then kept in an enclosed container for at least 4 days prior to feeding. This holding time was necessary to ensure sufficient reaction between the protein and the formaldehyde.

The fatty acid composition of the original oil was estimated using procedures described elsewhere (9,10). The oils from the treated and untreated supplements were also recovered by extraction with chloroform:methanol (2:1, v/v) and their fatty acid compositions determined.

Abomasal Infusion of Cyclopropene Fatty Acids

Untreated spray-dried casein-*S. foetida* seed oil (1:1, w/w) supplement was dispersed in water (10% solids) and infused daily into the abomasum of a lactating goat fitted with a permanent cannula. The dispersion was infused over a period of 30 min following the daily milking.

Extraction of Milk Fat and Fatty Acid Analyses

Milk fat was extracted using the Roese-Gottlieb method (11). The extracted fat was saponified and total fatty acids were obtained following acidification of the saponified mixture. Methyl esters were prepared using diazomethane, and the esters were separated by gas liquid chromatography (GLC) (12).

Analysis of *trans* Octadecenoic Fatty Acid in Milk Fat

Fatty acid methyl esters were separated according to their degree of unsaturation by argentation thin layer chromatography (TLC) [Adsorbosil-1 (Applied Science Laboratories, State College, PA); 8% $AgNO_3$; solvent systems; benzene:diethyl ether (95:5, v/v) or petroleum spirit:diethyl ether (99:1, v/v)]. The two solvent systems used for argentation TLC gave similar results.

The separated fatty acid bands were visualized under UV light following treatment with dichlorofluorescein.

The principal component bands from each milk sample were (a) saturates, (b) monoenoics (*trans*), (c) monoenoics (*cis*), and (d) dienoics and trienoics. The component bands from each sample were divided into two approximately equal portions by drawing a line from origin to solvent front which uniformly dissected the bands.

One half of each *trans* monoenoic fatty acid band was then removed by scraping, and the remaining "half bands" from this side were then also scraped off and pooled. The complete set of "half bands" on the other side of the line were also pooled. Following elution of the respective fatty acid methyl esters, the esters were analyzed by GLC as previously described. Each sample of mixed milk fat methyl esters thus yielded three GLC tracings, namely, (a) complete mixture, (b) complete mixture minus *trans* fatty acids, and (c) *trans* fatty acids (this was ca. 90% 18:1).

The percentage *trans* fatty acid in each sample was calculated by difference using an internal reference peak such as palmitate or stearate.

Analysis of Radioactive Fatty Acids in Milk and Abomasal Digesta

The pathways for the biosynthesis of *cis* 18:1 and *trans* 18:1 were demonstrated by feeding [1-^{14}C] trilinolenyl glycerol to a lactating goat and measuring the proportions of radioactive saturated (18:0), *cis* monoenoic (18:1 *cis*), *trans* monoenoic (18:1 *trans*), and polyenoic (18:2, 18:3) fatty acids in the abomasal digesta and in the milk.

The total fatty acids of milk fat were obtained as previously described. The total fatty acids in abomasal digesta were obtained by saponifying the digesta samples, acidifying, and extracting with petroleum ether. The recovered fatty acids from both milk and abomasal digesta were methylated with diazomethane, and the methyl esters were initially separated by argentation TLC using benzene:diethyl ether (9:1, v/v) as the developing solvent and Silica Gel G containing 10% $AgNO_3$ as the support. The bands corresponding to (a) saturates plus *cis* and *trans* monoenoics and (b) dienoics plus trienoics were removed and the methyl esters eluted with diethyl ether. The saturates, *trans* monoenoics, and *cis* monoenoics were separated by further argentation TLC using benzene:petroleum spirit (1:1, v/v) as the developing solvent. The radioactivity of the methyl esters corresponding to (a) saturates, (b) *trans* monoenoics, (c) *cis* monoenoics, and (d) dienoics plus trienoics was measured by liquid scintillation spectrometry (13).

GLC was used to verify the mass composition of the separated methyl esters, but the specific radioactivity was too low to permit GLC verification of the radioactivity distribution. Since the carbon skeleton of dietary long chain fatty acids resists degradation in the rumen and is incorporated intact into milk triacylglycerols (14,15), it has been assumed in this study that all of the radioactive fatty acids in abomasal digesta and milk are 18-carbon acids derived from the [1-^{14}C] linolenic acid.

TABLE I

Fatty Acid Analysis of *Sterculia foetida* Seed Oil Supplements[a]

Fatty acid	Original oil	Oil recovered from: Untreated supplement	Treated supplement
14:0	0.2	Trace	Trace
16:0	17.5	25.0	25.5
18:0	2.1	3.8	3.8
18:1	5.1	9.4	9.5
18:2	6.7	12.3	12.4
18:3	1.4	2.6	2.6
Malvalic acid	12.3	7.3	7.3
Sterculic acid	54.7	39.6	38.9

[a]Values are % by wt of total fatty acids.

RESULTS

Fatty Acid Composition of *S. foetida* Supplements

Although some loss of the cyclopropene fatty acids occurred during the preparation of the supplements, there were no differences in fatty acid composition between the treated and untreated supplements (Table I); both supplements contained ca. 47% cyclopropene fatty acids in the oil phase.

Effects of *S. foetida* Supplements on Milk Fat Composition

The effects of feeding lactating goats with untreated and with a formaldehyde-treated supplement containing *S. foetida* seed oil are shown in Figure 1. Feeding the formaldehyde-treated supplement resulted in an increased proportion of octadecanoic (18:0) relative to octadecenoic (18:1) acid in the milk fat. On the other hand, the untreated supplement had only a slight effect on the 18:0/18:1 ratio in milk fat. The formaldehyde-treated supplements were also effective in increasing the amount of 18:0 relative to 18:1 in milk fat from a cow (Table II). This table also shows the other major component fatty acids in the milk of a cow and goat fed either a control or a supplemented diet. The supplement did not markedly alter the proportions of fatty acids other than 18:0 and 18:1. Furthermore, when the 18:1 fatty acids of the milk fat were fractionated into *cis* and *trans* isomers, it was apparent (Table III) that *cis*-18:1 was the only isomer of octadecenoic acid to be affected by the cyclopropene supplementation; the level of *trans* acid in the milk fat was not altered by feeding the supplement (Table III).

Abomasal Infusion of *S. foetida* Oil Supplement

Daily abomasal infusion of untreated *S. foetida* seed oil supplements also caused an increase in the 18:0/18:1 ratio in goat milk fat,

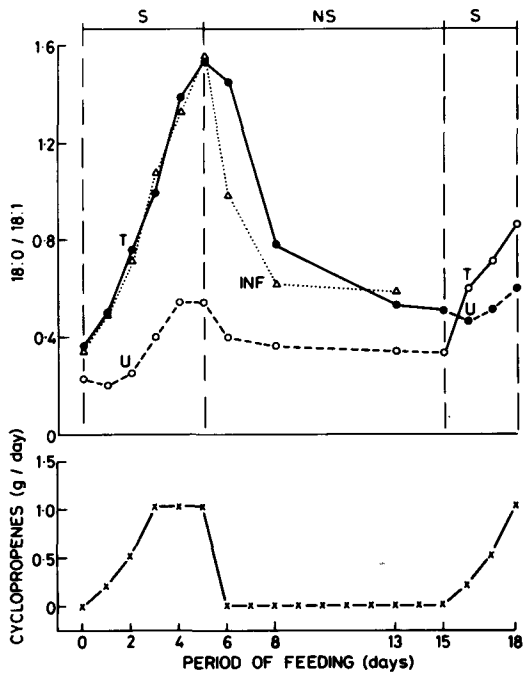

FIG. 1. The effects of feeding supplements containing *Sterculia foetida* seed oil on the ratio of 18:0/18:1 in milk fat from goats. The supplements, either untreated (U) or treated (T) with formaldehyde, were concurrently fed to two lactating goats. At the end of the initial supplementation period and following the return to normal in the fatty acid profile of the milk, the supplementation was reversed. Another goat was given equivalent amounts of *S. foetida* seed oil per abomasum for the first 5 days of the experimental regimen (INF). S = supplemented period, NS = non-supplemented period.

and the magnitude of this effect was analogous to that observed when equivalent amounts of formaldehyde treated *S. foetida* oil supplement were fed (Fig. 1).

Origin of *cis* and *trans* 18:1 in Milk Fat

Analysis of samples from the goat fed

TABLE II

Effect of Feeding Formaldehyde-treated Casein-*Sterculia foetida* Seed Oil Supplement on the Fatty Acid Composition of Ruminant Milk Fat[a]

Fatty acid	Cow		Goat	
	Basal diet	Supplemented[b] diet	Basal diet	Supplemented diet
14:0	11.7	11.2	9.4	10.9
16:0	27.7	28.9	34.5	30.4
18:0	15.1	23.8	9.8	21.6
18:1	27.6	22.0	27.2	15.5
18:2	2.5	1.9	2.6	1.4

[a]Values are % by wt of long chain, i.e., C_{12} and higher, fatty acids.

[b]Each animal was fed the supplement for 3 days at the rate of 5 g/day and 15 g/day (cow).

TABLE III

Relative Proportions of Stearic Acid (18:0), *cis* Octadecenoic Acid (*cis* 18:1), and *trans* Octadecenoic Acid (*trans* 18:1) in Cow Milk Fat[a]

	Basal diet	Supplemented diet[b]
18:0	17.0	24.1
18:1 *(cis)*	22.4	12.9
18:1 *(trans)*	7.2	8.5

[a]Values are % by wt of long chain, i.e., C_{12} and higher, fatty acids.

[b]Formaldehyde-treated casein-*sterculia foetida* seed oil supplement was fed to a cow for 3 days at the rate of 15 g/day.

[1-^{14}C] trilinolenyl glycerol showed that most of the radioactivity in the abomasal digesta was present as stearic (43%) or *trans* octadecenoic (34%) acids; there was relatively little radioactivity in the residual polyenoic acids (18%) and even less in the *cis* octadecenoic fraction (4%) (Fig. 2). The distribution of radioactivity in milk fatty acids was, however, different from that in abomasal digesta fatty acids. In milk fat, most of the radioactivity was present as *cis* octadecenoic acid (44%), and there was a much reduced proportion of radioactive stearic acid (16%). The proportion of radioactivity in *trans* octadecenoic acid was similar for both milk and abomasal digesta samples.

DISCUSSION

Cyclopropene fatty acids are powerful inhibitors of fatty acid desaturating enzyme systems (16,17). This effect has been extensively studied in nonruminant animals. When fed to chickens, small amounts (5-10 mg/kg body weight) of cyclopropene fatty acids such as malvalic acid and sterculic acid can effectively inhibit the conversion of stearic acid to oleic acid in the liver (18). This inhibition results in a markedly increased proportion of stearate and a decreased proportion of oleate in the tissue lipids.

Although the feeding to ruminants of cyclopropene fatty acids (as in cottonseed oil) has a slight effect on the fatty acid composition of milk fat (19), there is no effect on adipose tissue (F.S. Shenstone and A.R. Johnson, personal communication), and it is probable that cyclopropene acids are hydrogenated in the rumen as are other unsaturated fatty acids (20). Ruminal hydrogenation of cyclopropene fatty acids would most likely produce cyclopropane acids, and these do not inhibit desaturase enzyme systems (18).

Bickerstaffe and Johnson (21) have demonstrated that infusion of sterculic acid into the jugular vein of a lactating goat inhibits the desaturase activity of the mammary gland and causes a rise in the 18:0/18:1 ratio in milk fat. The present paper shows that a similar effect can be produced by feeding the cyclopropene fatty acids in a form whereby they are protected against ruminal hydrogenation.

The feeding of formaldehyde-treated spray dried casein-*S. foetida* oil supplements to lactating goats (Fig. 1, Table II) and cows (Table II) caused a considerable increase in the 18:0/18:1 ratio in milk fat. The magnitude of this effect was similar to that observed when equivalent amounts of untreated supplement were infused into the abomasum of a goat (Fig. 1). On the other hand, when untreated *S. foetida* supplements were fed to lactating goats, there were only small increases in the 18:0/18:1 ratio of milk fat (Fig. 1). These results and those of a previous study (21), therefore, show that, when cyclopropene acids are administered to ruminants in such a way as to "bypass" rumen metabolism, they exert the same effect as is evident when these acids are fed to nonruminant species. In short term feeding experiments

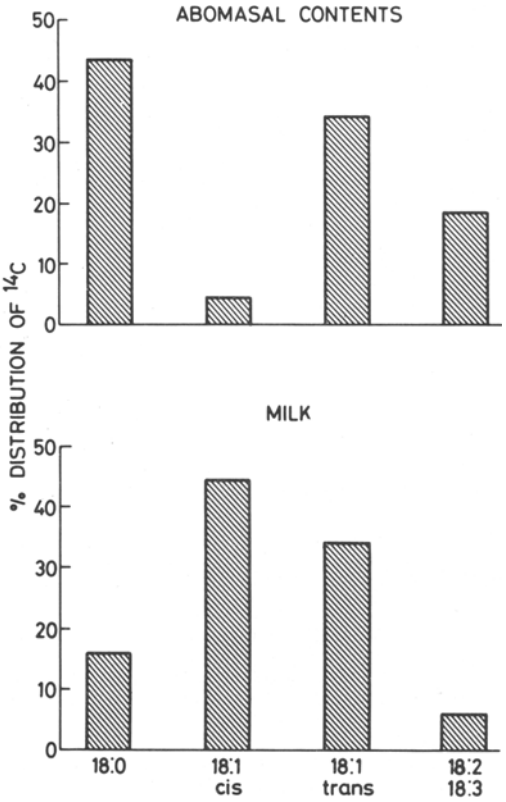

FIG. 2. Carbon-14 radioactivity of C18 fatty acids in the abomasal digesta and milk of goats after feeding [^{14}C] trilinolenyl glycerol. Trilinolenyl glycerol containing [1-^{14}C]linolenic acid was incorporated into a spray-dried linseed oil-casein (1:1, w/w) complex (ca. 0.01 μCi/g), and this (500 g/day) was fed to a goat for 2 days, together with alfalfa hay and oats (1:1, w/w, 1,550 g/day). The abomasal digesta samples were taken on the 2nd day, whereas the milk samples were taken on the 3rd day following the initial feeding of the radioactive components.

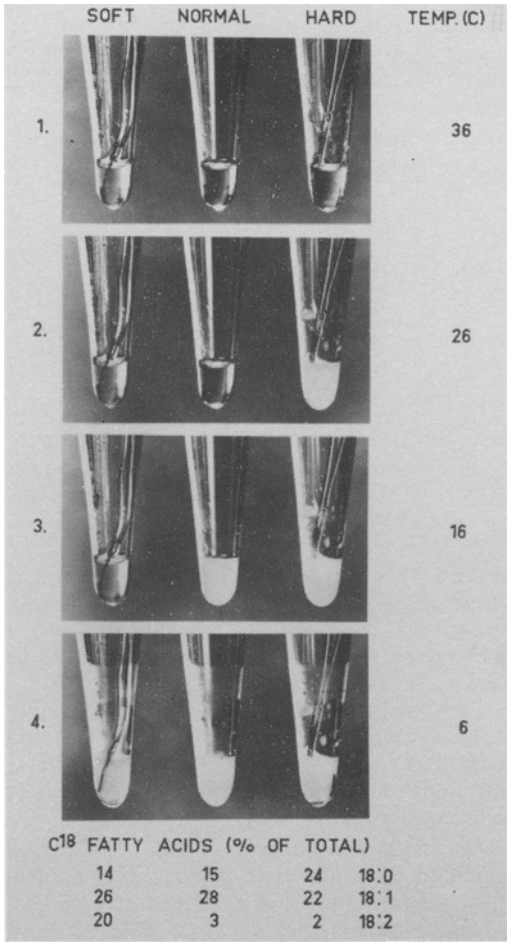

FIG. 3. Liquid-solid transition temperatures of milk fats. The soft milk fat (18:2-enriched) was produced by a cow which had been fed a supplement of formaldehyde-treated spray-dried sunflower oil:casein (1:1, 1 kg/day). The hard (18:0-enriched) milk fat was produced by a cow which received a supplement of formaldehyde-treated spray-dried *Sterculia foetida* seed oil: casein (1:1, 15 g/day). The temperature of all fats was raised to 36 C and progressively lowered to demonstrate the liquid-solid transition temperatures.

of this nature, there were no marked changes in the yield of milk or the milk fat content during the period of protected cyclopropene supplementation (Table IV).

The increased 18:0/18:1 ratio in milk fat from ruminants fed the protected cyclopropene acids is probably a consequence of cyclopropene-mediated inhibition of mammary desaturase enzymes. This conclusion is supported by the observation that the cyclopropene supplement caused a reduction in *cis* 18:1 but not in *trans* 18:1 of milk fat (Table III).

Oleic acid (*cis* 18:1) of milk can be derived by desaturation of stearic acid in the mammary gland (22), but *trans* 18:1 of milk derives exclusively from ruminal hydrogenation/isomerization of dietary unsaturated C_{18} fatty acids (23). Support for these different origins of *cis* and *trans* 18:1 was obtained by feeding [1-^{14}C] trilinolenyl glycerol to lactating goats and comparing the distribution of radioactive fatty acids in milk vis-a-vis abomasal digesta (Fig. 2).

Milk fats from ruminants fed conventional diets have a relatively constant fatty acid composition, and thus the physical properties of fat-enriched dairy products (particularly butter) tend also to be rather constant. Previous work has shown that protected polyunsaturated lipid supplements can be fed to lactating ruminants as a means of markedly increasing the proportion of polyenoic fatty acids in milk

TABLE IV

Effect of Feeding Formaldehyde-treated Casein-*Sterculia foetida* Seed Oil Supplement to Two Lactating Goats[a]

Days of experiment	Dietary treatment	Milk production (l/day)		Milk fat content (%)	
		No. 226	No. 187	No. 226	No. 187
1	No supplement	1.32	1.54	3.7	4.3
3		1.14	1.72	3.6	4.6
4		1.14	1.74	5.2	4.4
5		1.42	1.72	3.6	4.5
7	Supplement	1.12	1.58	4.7	3.1
8		1.02	1.36	4.1	4.5
9		1.20	1.74	5.2	4.8
10		1.25	1.57	3.8	5.0
11	No supplement	1.17	1.14	4.2	4.9
12		1.30	1.83	4.1	4.3

[a]Goats were fed a diet of chopped alfalfa and crushed oats (1:1) at the rate of 2 kg/day (goat 187) and 1.6 kg/day (goat 226). The casein-*S. foetida* seed oil supplement was fed at the rate of 3 g/day.

fat and thus markedly altering the physical properties of milk fat (24). The butters produced by these means are considerably softer due to the lower melting point of the polyenoic fatty acids (24).

The present study clearly shows that protected cyclopropene supplements can be used to markedly increase the stearic:oleic acid ratio in milk fat and thus enable the production of harder butter fats (Fig. 3).

The milk fat samples in Fig. 3 were all heated to 36 C and then progressively cooled. The hard fat (from a cow fed protected cyclopropene supplements) solidified first at 26 C, the normal fat (from a cow on conventional rations) solidified next at 16 C, and the soft fat (from a cow fed protected sunflower oil supplement) solidified last at 6 C. These differences in the liquid to solid transition temperatures were primarily a reflection of the C_{18} fatty acid distribution profile (Fig. 3).

The ability of protected cyclopropene supplements to increase the "hardness" of milk fat may be useful in the production of butters, chocolate, etc., for tropical regions. Furthermore, these supplements may also be useful for enhancing the "hardness" of adipose tissue triacylglycerols from sheep fattened on grain (25). Before these supplements are used in practice, however, their effects on human and animal health should be carefully considered.

ACKNOWLEDGMENTS

S. foetida seeds were donated by the Department of Forestry, Republic of the Philippines. The authors thank P.J. Bready for preparation of the supplements, S. Margan for technical assistance, and M. Horan for care and management of the animals.

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Effect of Bovine Serum Albumin on Monoacyl- and Diacylglycerol 3-Phosphate Formation in Mitochondrial and Microsomal Fractions of Rabbit Hearts[1]

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ABSTRACT

The formation of monoacyl- and diacylglycerol 3-phosphate (P) by rabbit heart mitochondrial and microsomal fractions was studied by varying the concentration of acyl-CoA and that of bovine serum albumin in the assay system. The two subcellular fractions were prepared by the conventional differential centrifugation technique. The optimal concentration of acyl-CoA for both mitochondrial and microsomal acylation of glycerol 3-P was shifted to a higher range of acyl-CoA concentrations by greater amounts of albumin. A similar shift in the acyl-CoA concentration-enzyme activity relationship was observed in the acylation reaction of 1-palmitoylglycerol 3-P by the heart microsomes. The addition of albumin increased slightly the rate of diacylglycerol 3-P accumulation but increased greatly the rate of monoacylglycerol 3-P accumulation at any concentration of acyl-CoA; the effect was observed with mitochondrial or microsomal fraction as the crude enzyme source. Moreover, palmitoyl-CoA and linoleoyl-CoA served equally well as the acyl donor for the acylation reaction. However, relatively more monoacyl- than diacylglycerol 3-P was accumulated in the assays with rabbit heart mitochondrial fraction in the presence of albumin, whereas more diacyl- than monoacylglycerol 3-P was formed by the microsomal fraction. As a result, the microsomal diacyl:monoacyl-glycerol 3-P ratio was invariably greater than the mitochondrial ratio at a given concentration of acyl-CoA and albumin.

INTRODUCTION

Acyl-CoA:glycerol 3-phosphate acyltransferase (EC 2.3.1.15) and acyl-CoA:monoacylglycerol 3-phosphate acyltransferase (EC 2.3.1.52) are the first two enzymes of de novo synthesis of phospho- and neutral glycerides in tissue (1,2). A number of studies have appeared dealing with the properties of these enzymes. It has been found that both *Escherichia coli* (3,4) and hepatic mitochondrial and microsomal fractions (5-9) contain these two enzymes and that monoacylglycerol 3-phosphate (P) is the product of the first and the substrate of the second reaction. We have investigated the properties of these enzymes of the subcellular fractions of rabbit hearts (10-13). The kinetics, substrate preference, and products of these enzymes reactions were studied in previous reports. This paper deals with the effect of bovine serum albumin on the acylation reaction.

EXPERIMENTAL PROCEDURES

Materials

Suppliers of the chemicals are listed as follows: palmitic, stearic, and linoleic acids (99% pure), coenzyme A lithium salt (> 85% pure), and fatty acid free-bovine serum albumin, fraction V (fatty acid < 0.005%) were all purchased from Sigma Chemical Co., St. Louis, MO, and sn-[U-^{14}C]glycerol 3-P (120 μCi/μmol; 98% pure) was obtained from New England Nuclear Corp., Boston, MA. 1-Palmitoylglycerol 3-P was supplied from Serdary Research Laboratories, Inc., London, Ontario. CoA esters of palmitic, stearic, and linoleic acids were synthesized according to the method of Al-Arif and Blecher (14), and their purity was determined by ultraviolet spectrophotometry and thin layer chromatography (TLC) (solvent system, n-butanol: acetic acid:water, 5:2:3, v/v/v). Silica Gel G precoated TLC plates were purchased from Brinkmann Instruments, Rexdale, Ontario.

Methods

The hearts were removed from anesthetized rabbits (4-5 lb.) and homogenized in 0.25 M sucrose - 0.02 M Tris-HCl buffer, pH 7.4, in a glass homogenizer. The mitochondrial fraction was isolated by precipitating the 800 x g homogenate at 10,000 x g for 15 min, as described previously (10). The microsomal fraction was obtained by centrifuging the post-

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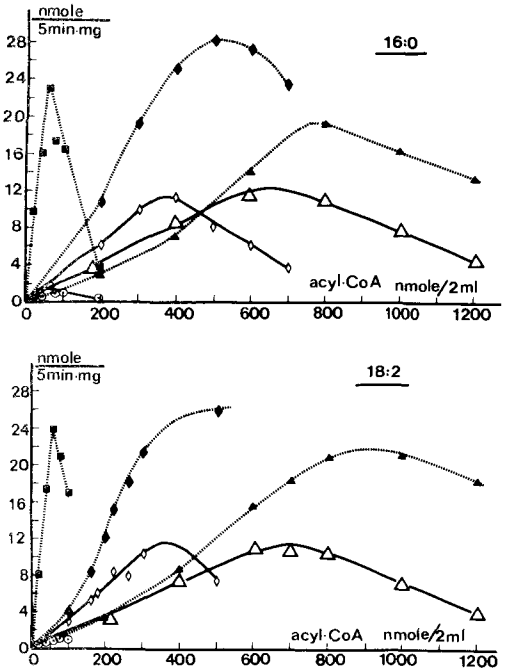

FIG. 1. Effect of bovine serum albumin on the acyl-CoA concentration-enzyme activity relationship of rabbit heart microsomes. The rates of formation of monoacylglycerol 3-P (solid lines) and diacylglycerol 3-P (broken lines) by the microsomal fraction of rabbit hearts are plotted on the ordinate (nmol glycerol 3-P acylated per mg of protein per 5 min incubation time), and the concentrations of palmitoyl-CoA (16:0) and linoleoyl-CoA (18:2) are shown on the abscissa. Results obtained from the assays carried out in the absence of albumin (squares and circles), as well as those from the assays carried out in the presence of two different concentrations of albumin (10 mg = diamonds, and 20 mg = triangles), are illustrated. Each point represents an average of duplicate determinations. See Experimental Procedures for further details of the enzyme assay.

mitochondrial supernatant at 18,000 x g for 15 min and 159,000 x g for 40 min (15). Mitochondrial and microsomal pellets were once washed and then were resuspended in the homogenizing medium at a final protein concentration of ca. 13 mg/ml.

The accumulation of monoacyl- and diacylglycerol 3-P was determined as follows: the assay mixture contained 100 μmol of Tris-HCl, pH 7.4, 6.0 μmol of [^{14}C]glycerol 3-P (731,000 dpm/μmol), 20-1200 nmol acyl-CoA esters, 0, 5, 10, or 20 mg fatty acid free bovine serum albumin (pH 6.9), and 1.0 mg of the microsomal fraction or 2.0 mg of the mitochondrial fraction in a total volume of 2.0 ml (13). An addition of 2.0 mM $MgCl_2$ influenced the rate of reaction only slightly (20-40% increase in diacylglycerol 3-P formation). After a 5 min preincubation at 37 C, the reaction was started by the addition of the subcellular fraction. The reaction was stopped after 5 min (microsomal fraction) or 10 min (mitochondrial fraction) by the addition of 3.0 ml boric acid-saturated butanol, followed by 4.0 ml butanol-saturated 0.1 M boric acid. After mixing, the phases were separated by centrifugation at 10,000 x g for 10 min.

The butanol phase was washed with 6.0 ml of butanol-saturated boric acid. The washed butanol phase was dried under nitrogen gas at ca. 35 C and the residue dissolved in chloroform:methanol mixture (2:1, v/v). The lipid extract was applied to a TLC plate; the plate was developed with a solvent system, chloroform:methanol:3.5 N NH_4OH (65:35:8, v/v/v). The radioactive products were localized by exposure to X-ray films for 3 days. The lipids were scraped into counting vials and their radioactivities determined after the addition of Econofluor (New England Nuclear Corp.). The counting efficiency was > 90%.

Acyl-CoA: *monoacylglycerol* 3-phosphate acyltransferase assays were carried out by measuring the rate of increase of CoA-SH caused by the acyl transfer reaction as described by Lands and Hart (16). The reaction mixture contained in a final volume of 1.0 ml, 80 μmol of Tris-HCl, pH 7.4, 1.0 μmol of 5,5'-dithiobis-(2-nitrobenzoic acid) (pH 7.0), 50 nmol of 1-palmitoylglycerol 3-P (suspended by sonication in 50 mM of sodium borate, pH 6.5), and ca. 0.2 mg of microsomal enzyme protein. After equilibration of 30 C for 2 min, the reaction was started by adding 10 nmol of acyl-CoA. The increase in absorbance, measured at 413 nm in 1 ml cuvettes, was continuously recorded on a 10 mV recorder. Full-scale deflections of 0.04 absorbance unit were used, and the chart speed was adjusted to 2 in./min, giving slopes of ca. 45° The acyl-CoA hydrolase activity, measured in the absence of acyl acceptor, was subtracted optically by a Unicam double-beam spectrophotometer. The values measured by a spectrophotometric and radioactive technique gave good agreement (13). The molar absorption coefficient of 13,600 $M^{-1}cm^{-1}$ was used to calculate the amount of mercaptan released during the reaction (16).

RESULTS

At the optimal concentration of glycerol 3-P (3 mM), the rate of accumulation of the two products of the acylation reaction, monoacyl- and diacylglycerol 3-P, by the microsomal fraction of rabbit hearts was dependent on the con-

centration of both acyl-CoA and albumin in the assay system (Fig. 1). As shown previously (8,13,17), there is an optimal range of concentration of acyl-CoA for the enzyme reaction, and higher concentrations of acyl-CoA are inhibitory. However, the optimal concentration and the range of inhibitory concentration were shifted to higher levels in the presence of bovine serum albumin, and this phenomenon was dependent upon the concentration of the albumin in the assay medium. In addition, Figure 1 shows that, in the absence of albumin, the microsomal enzyme produced almost exclusively the diacylglycerol 3-P at any concentration of acyl-CoA. However, in the presence of 10 mg of albumin, one-half to one-third of the total acylation products was monoacylglycerol 3-P, whereas, in the presence of 20 mg of albumin, the quantities of monoacyl- and diacylglycerol 3-P were nearly equal at almost all acyl-CoA concentrations below the optimum. Therefore, the relative amounts of the acylation reaction products are greatly influenced by the amount of albumin in the assay medium. The two acyl-CoA esters, palmitoyl- and linoleoyl-CoA, were utilized in a similar fashion by the glycerol 3-P acylation reaction of the heart microsomes (Fig. 1).

The kinetics of the mitochondrial acylation reaction were studied in our previous work, in which fatty acid was used as the substrate of the reaction (10). Similar results were obtained using acyl-CoA as substrate; the velocity of the acyltransferase reaction was proportional to the amount of mitochondrial protein up to 2.0 mg, and the reaction was linear up to 10 min for monoacylglycerol 3-P formation and up to 30 min for diacylglycerol 3-P and neutral lipid formation. Ca. 18% of the activity of the total acylation by heart homogenates can be attributed to that by the mitochondrial acylation, although the mitochondrial fraction participated as much as 33% in the total monoacylglycerol 3-P accumulation of the homogenates (unpublished results). However, no attempt was made in this study to characterize independent mitochondrial enzymes of the rabbit heart. Therefore, acyltransferase activities of the mitochondrial fraction described below may not solely be due to those of the mitochondrial enzymes.

The rate of accumulation of the two products of acylation reaction by the mitochondrial fraction of rabbit hearts was also dependent upon the concentrations of both acyl-CoA and albumin in the assay system (Fig. 2). As in the microsomal acylation, excess amounts of acyl-CoA inhibited the acylation reaction; the inhibitory concentration of acyl-

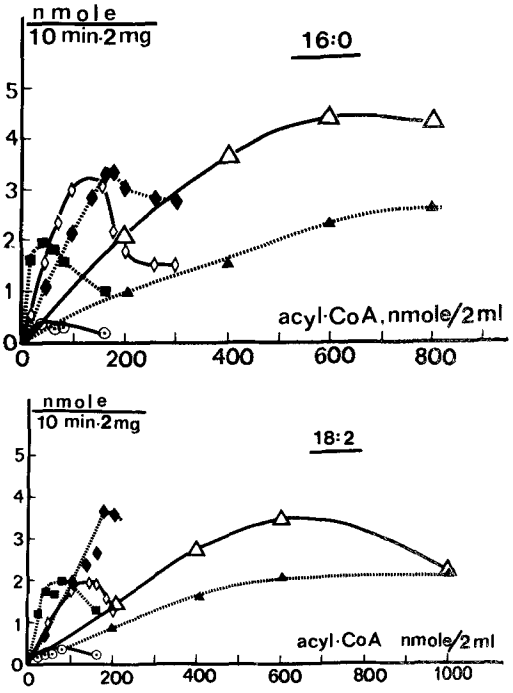

FIG. 2. Effect of bovine serum albumin on the acyl-CoA concentration-enzyme activity relationship of rabbit heart mitochondria. The rates of formation of monoacylglycerol 3-P (solid lines) and diacylglycerol 3-P (broken lines) are indicated on the ordinate in nmol glycerol 3-P acylated per 2 mg protein per 10 min incubation, and the concentrations of palmitoyl CoA (16:0) and linoleoyl-CoA (18:2) are plotted on the abscissa. Results obtained from the assays carried out in the absence of albumin (squares and circles) and in the presence of two different amounts of albumin (5 mg = diamonds, and 20 mg = triangles) are illustrated. Each point represents an average of duplicate assays. See Experimental Procedures for further details of the enzyme assays.

CoA was greater at larger amounts of albumin in the assay. However, the mitochondrial acylation reaction was characterized by a relatively large quantity of monoacylglycerol 3-P formation in the assay containing albumin. Thus, in the presence of 20 mg albumin, monoacylglycerol 3-P accumulation represented approximately two-thirds of the total acylation products at any concentration of palmitoyl- or linoleoyl-CoA (Fig. 2). In the presence of 5.0 mg of albumin in the assay, monoacylglycerol 3-P formation was approximately equal to diacylglycerol 3-P formation at low acyl-CoA concentrations. Only in the absence of albumin was the major products of mitochondrial acylation diacylglycerol 3-P. The substrate concentration-activity relationship obtained with palmitoyl-CoA was similar to that with lino-

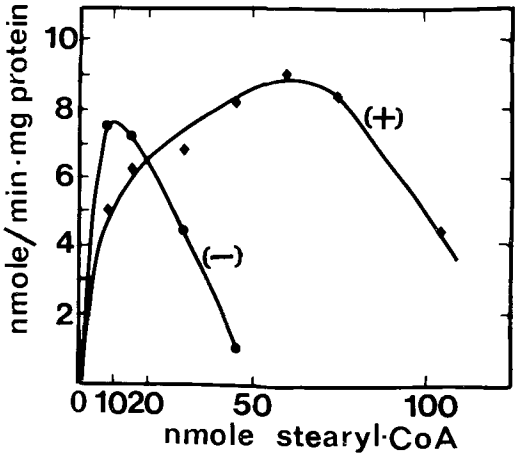

FIG. 3. The effect of albumin on the substrate concentration-activity relationship of microsomal acyl-CoA:monoacyl-glycerol 3-phosphate acyltransferase. Assays were carried out spectrophotometrically as described in Experimental Procedures. The ordinate indicates the rate of CoA-SH release (nmol/min per mg protein), and the abscissa, the amount of stearoyl-CoA (nmol/ml). The amount of microsomal protein in the assays was 0.23 mg and that of 1-palmitoylglycerol 3-phosphate was 50 nmol. (+) indicates the assays performed in the presence of 1 mg bovine serum albumin/ml and (-) indicates those performed in its absence. Each point represents an average of duplicate determinations.

leoyl-CoA under these experimental conditions (Fig. 2).

The effect of bovine serum albumin on the in vitro acylation of 1-palmitoylglycerol 3-P by the cardiac microsomal fraction was examined separately (Fig. 3). The acyl-CoA concentration-enzyme activity relationship showed a pattern similar to that obtained with glycerol 3-P acylation reaction. The optimal acyl-CoA concentration was ca. 10 nmol in the absence of albumin, and it shifted to a higher range of acyl-CoA concentrations by the addition of 1 mg albumin per ml (Fig. 3). The effect of albumin was similar with linoleoyl- or oleoyl-CoA as an acyl donor (not shown) to that observed with stearoyl-CoA (Fig. 3).

DISCUSSION

The sole product of glycerol 3-P acylation in mammalian system had been believed to be diacylglycerol 3-P until Fallon and Lamb (5), Daae and Bremer (6), and Monroy et al. (7) demonstrated accumulation of monoacylglycerol 3-P in the hepatic subcellular fraction. Monoacylglycerol 3-P was detected because these workers used an improved lipid extraction technique and albumin in their assay medium. The present study demonstrated that the rate of accumulation of monoacylglycerol 3-P was dependent upon the amount of albumin in the assay system. In the absence of albumin, a relatively small amount of monoacylglycerol 3-P was accumulated in the assay with mitochondrial or microsomal fraction of rabbit hearts. Addition of albumin up to 20 mg to the assay medium increased substantially the amounts of monoacylglycerol 3-P accumulation but increased only slightly the amounts of diacylglycerol 3-P formation at the optimal acyl-CoA concentration. This resulted in a decreased ratio of diacyl:monoacyl-glycerol 3-P and in an increased rate of total glycerol 3-P acylation both in the assay containing the mitochondrial fraction and that containing the microsomal fraction. Similarly, the maximal rate of the acyl-CoA:1-palmitoylglycerol 3-phosphate acyltransferase reaction was slightly accelerated by the albumin addition. Previously, the same enzyme of guinea pig liver microsomes was shown to be activated by the heat denatured microsomal protein or serum albumin (16). This activation was attributed to the binding of inhibitory acyl-CoA by the protein (16). Fallon and Lamb (5) similarly reported albumin stimulation of glycerol 3-P acylation reaction.

The effect of albumin may, in part, be explained by assuming that albumin binds with the intermediate of acylation, monoacylglycerol 3-P; consequently, the latter accumulates. Previously, Monroy et al. (18) also found that albumin stimulated monoacylglycerol 3-P formation by rat liver mitochondria without influencing diacylglycerol 3-P formation. Alternatively, albumin may activate phospholipase activity of the subcellular particles, thereby modifying the rate of transesterification, i.e., the rate of labeling of monoacylglycerol 3-P; the importance of the latter pathway in the rat liver was recently stressed by Tzur and Shapiro (19).

The fact that high concentrations of acyl-CoA are inhibitory to some enzyme reactions and that this inhibitory effect can be curtailed by the addition of albumin has been described (8,20,21). The effective substrate concentration for the enzyme reaction is thus decreased by the binding to the albumin. Our finding that the optimal acyl-CoA concentrations shifted to a higher concentration range by the addition of albumin and that substrate specificity of both mitochondrial and microsomal acyltransferases was unchanged may be attributed to such unspecific binding by exogenous albumin. In contrast to the rabbit heart mitochondria and microsomes (10,13), rat liver mitochondria and microsomes both possess distinct fatty acid

specificity (7,9,17,18,22).

The ratio of diacyl:monoacyl-glycerol 3-P formed by the microsomal fraction was invariably greater than the ratio calculated for the mitochondrial acylation in the presence of an equal albumin concentration. This difference suggests that the mitochondrial enzymes may be different from the microsomal enzymes, as was postulated previously (6,7,10). However, it is not known whether the mitochondrial enzymes themselves are different from the microsomal enzymes in the rabbit heart, as was the case in rat liver (18), or whether mitochondrial protein, by acting in a manner similar to albumin, modifies the reaction catalyzed by the microsomal enzymes. With respect to the latter point, our work currently in progress provides another piece of evidence to support a suggestion that the cardiac mitochondrial fraction contains acyltransferase activities separate from the microsomal enzymes; in this work, the rate of glycerol 3-P acylation by rat heart mitochondria was found to be as high as that of the microsomes. Furthermore, the activities of the two subcellular fractions changed independently under various experimental conditions (manuscript in preparation).

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Inhibition of Acid Esterase in Rat Liver by 4,4′-Diethylamino-ethoxyhexestrol

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ABSTRACT

The effect of 4,4′-diethylamino-ethoxyhexestrol (DH) on acid esterase in rat liver was studied in vivo and in vitro. The acid esterase activity in the livers of rats treated with 0.125% DH for 1 week was found to decrease more than 60% as compared with that in untreated rats. The addition of DH to the incubation medium caused considerable inhibition of the acid esterase activity in lysosome from untreated rat liver, and the inhibition type appears to be noncompetitive. The acid lipase activity in rat liver lysosome was also inhibited by DH. Some antihistamic agents and chloroquine also inhibited the acid esterase activity in rat liver lysosome.

INTRODUCTION

Lipidosis induced by 4,4′-diethylamino-ethoxyhexestrol (DH) is known to be accompanied by the accumulation of various classes of lipids, including unusual phospholipids such as bis-(monoacylglycerol) phosphate or acyl phosphatidylglycerol (1-3). This disease has also been known to form the intracellular particles with myelin-like structure in liver cells (4,5). However, neither mechanism, the induction of lipidosis nor the formation of myelin-like bodies, has yet been clarified. Saito et al. (6) recently reported that a decrease in phospholipase A activity was caused in all subcellular fractions of the livers of DH-treated rats. The present paper is a study on the inhibitions of acid esterase and acid lipase by DH in rat liver.

METHODS

Preparation of Liver Homogenates of DH-treated Rats

Twelve male rats of Wistar strain, weighing 150-200 g, were divided into two groups of control and DH-treated rats. DH was fed ad libitum mixed with ground Oriental Company chow at 0.125% for 1 week to the rats of treated group. The rats of control group were given the same diet but without DH for 1 week. At the end of experimental feeding, the animals were fasted overnight and sacrificed by decapitation. The livers were homogenized in ice-cold 0.25 M sucrose (9 ml per g of fresh tissue) in a Potter-Elevehjm homogenizer.

Preparation of Rat Liver Lysosomal Fraction

Male rats of Wistar strain, weighing 200-250 g, were sacrificed as described above. The livers were submitted to subcellular fractionation according to the procedure reported by Murota et al. (7). The livers (ca. 30 g) were minced with scissors and then homogenized in the same volume of 0.25 M sucrose-0.2 M KCl (1:1, v/v) in a Waring Blendor at top speed for 30 sec. The slurry was diluted twice with 0.25 M sucrose-0.2 M KCl and was centrifuged at 1000 x g for 10 min at 4 C. The sediment was discarded, and the supernatant was diluted three times with 0.25 M sucrose-0.2 M KCl and centrifuged at 1700 x g for 5 min. This sediment was then discarded, and the supernatant was centrifuged at 5900 x g for 20 min. The supernatant was discarded, and the sediment was suspended in 80 ml of 0.7 M sucrose-0.2 M KCl (1:1, v/v), and this suspension was centrifuged at 1700 x g for 5 min. The sediment was discarded, and the supernatant was centrifuged at 9500 x g for 20 min. The resulting sediment, representing a lysosomal fraction, was suspended in 0.25 M sucrose and stored at -20 C until use. In the lysosomal fraction, the specific activity of acid phosphatase was increased eightfold as compared with the original homogenate.

Assays of Enzymes and Chemical Components

Acid phosphatase was determined by the method of Gianetto and De Duve (8), and the liberated phosphate was estimated by the Fiske-Subbarow method (9). Acid esterase was determined by the method of Mahadevan and Tappel (10) using p-nitrophenyl myristate as substrate. Acid lipase was measured by the method of Hayase and Tappel (11) using tricaprin as substrate, and the liberated fatty acids were estimated according to Duncombe's method (12). The enzyme activity was measured after disruption of lysosomal particles by adding Triton X-100 at a final concentration of 0.1% or by homogenizing in a Waring Blendor for 3 min.

TABLE I

Effect of 4,4'-Diethylaminoethoxyhexestrol (DH) on Lipid and Protein Contents and Acid Esterase and Phosphatase Activities in Rat Livers In Vivo[a]

	Control	DH treated
Total lipids (g/100 g wet wt of tissue)	5.70 ± 0.29	7.16 ± 0.30
Protein (mg/0.1 g wet wt of tissue)	19.8 ± 0.52	18.4 ± 0.40
Acid esterase[b] (mμmol p-nitrophenol liberated/min/mg of protein)	49.4 ± 1.67	17.9 ± 1.41
Acid phosphatase (mμmol phosphate liberated/min/mg of protein)	30.7 ± 1.07	33.5 ± 0.76
DH and its metabolite (mg/g wet wt of tissue)		0.73 ± 0.06

[a]Values are represented as the mean ± SE of six experiments.

[b]The reaction mixture for acid esterase contained 0.17-0.22 mg of homogenate protein; 100 μmol of glycine-HCl buffer, pH 3.6; 7.5 μmol of p-nitrophenyl myristate; and 25 mg of Triton X-100 in a total volume of 1.5 ml. Incubations were for 20 min at 37 C.

TABLE II

Effect of 4,4'-Diethylaminoethoxyhexestrol (DH) on Acid Esterase In Vitro[a]

Concentration of DH (mM)	Specific activity (mμmol/min/mg protein)	Inhibition (%)
0	217.5	-
0.05	215.9	0.7
0.1	211.3	2.9
0.25	183.9	15.4
0.5	158.1	27.3
1.0	132.3	39.2
1.5	111.2	48.9
2.0	100.9	53.6

[a]Values are represented as the mean of three experiments. The reaction mixture was the same as described in Table I, except for 58 μg of rat liver lysosomal protein, and DH was added in the amount described above.

Protein concentration was determined by the method of Lowry et al. (13). Lipids were extracted by the method of Folch et al. (14) and were determined as total lipids by weight. 4,4'-Diethylaminoethoxyhexestrol and its main derivative were isolated from phospholipid fraction as described in a previous report (3) and were determined by measuring the absorbance at 278 nm in chloroform:methanol (1,1:v/v).

Chemicals and Drugs

p-Nitrophenyl myristate was prepared according to the method of Huggins and Lapides (15) and recrystallized from diethyl ether. Tricaprin was prepared from capric acid chloride and glycerol by the usual method (16) and recrystallized from ethyl alcohol. Other reagents were analytical reagent grade obtained from commercial suppliers. Drugs were also purchased from commercial suppliers and used after recrystallization.

RESULTS AND DISCUSSION

As shown in Table I, the acid esterase activity in DH-treated rat livers was decreased 64% as compared with that in untreated rat livers. On the other hand, the acid phosphatase activity in DH-treated rats was slightly higher than that in untreated rats. DH and its metabolite accumulated in the livers in a considerable concentration of 0.73 mg per g of fresh tissue. Calculating from this amount, the sum of the concentration of DH and its metabolite was 9 μM in our incubation medium with the treated rat liver. These substances were not supposed to affect acid esterase activity at a concentration of this level because our preliminary experiment showed that the addition of DH to control medium in a concentration of 10 or 20 μM did not affect this activity. The total lipid content of DH-treated rat livers was higher than that of the control rats, as already reported by Adachi et al. (2). No difference was

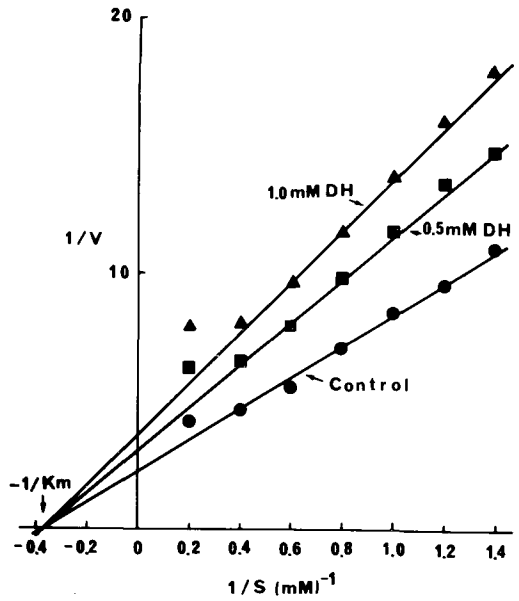

FIG. 1. Effect of 4,4'-diethylaminoethoxyhexestrol (DH) on acid esterase with p-nitrophenyl myristate as varying concentration of substrate. Experimental conditions were as described in the text, Table I, and II. The data are plotted by the method of Lineweaver and Burk (19). The ordinate gives the reciprocal of the velocity (mμmol p-nitrophenol liberated/20 min/mg of protein).

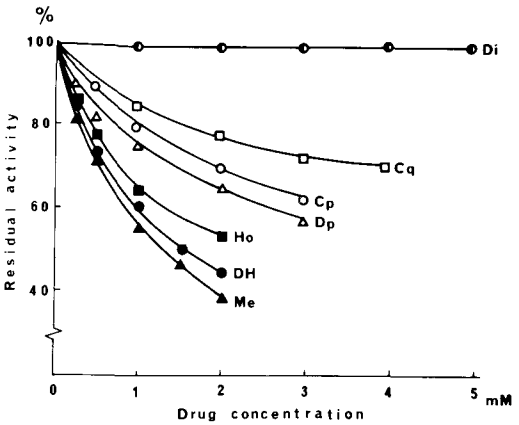

FIG. 2. Effect of various drugs on acid esterase. Experimental conditions were as described in the text, Table I, and II. DH = 4,4'-Diethylaminoethoxyhexestrol, Me = meclizine, Ho = homochlorcyclizine, Dp = diphenylhydramine, Cp = chlorpheniramine, Cq = chloroquine, Di = 2-diethylaminoethanol.

found in the protein content between control and treated rat (Table I).

In Table II, the effect of DH on acid esterase activity in vitro is presented. The inhibition occurred at 0.25 mM of DH, and its inhibitory constant was 1.75 mM as estimated by Dixon's method (17). The above result in vivo is seemingly contradictory to the result in vitro presented here in view of the concentration of accumulated DH and its metabolite, for the result in vivo showed that the concentration of DH and its metabolite in the assay system was 9 μM, which by itself was insufficient to cause inhibition of acid esterase, but a substantial inhibition was observed. The in vitro inhibition did not become substantial until the concentration of 250 μM. Therefore, these data indicate that, although the in vivo concentration of DH and its metabolite in fresh tissue is 1.56 mM, which is sufficient to cause inhibition, additional long-lasting effect on enzyme also occurs in the livers. The amount of DH in clinical case has been reported to be 0.2-0.7% by wet weight of the tissue (18). This amount is equivalent to 2-7 mg per g of the fresh tissue and as high as 3-10 times the amount described above in treated rat livers. These findings in our experiments suggest the possibility that the inhibition of acid esterase is also caused in human tissues. The effect of pH on the inhibition of acid esterase activity by DH was also observed. The inhibition by DH became weak, with elevating pH in a ragne of 3.0-6.0, and disappeared at pH 6.0.

Kinetics of inhibition of acid esterase by DH are shown in Figure 1. This type of inhibition was found to be noncompetitive, as the data were plotted by the method of Lineweaver and Burk (19). The Michaelis constant was 2.6 mM, and this value does not agree with the result of Mahadevan and Tappel (10). This disagreement may be due to the difference of assay system between us and them.

Some antihistamic agents and chloroquine were also found to inhibit acid esterase (Fig. 2). The inhibitory effect was strong in the following order: meclizine, DH, homochlorcyclizine, diphenylhydramine, chlopheiramine, and chloroquine. 2-Diethylaminoethanol scarcely affected acid esterase activity, although diethylaminoethoxy group constitutes DH as a part of its structure. These drugs have diphenylethyl, diphenylmethyl, piperazinyl, diethylaminoethoxy, dimethylaminoethoxy, dimethylaminoethyl, and diethylaminobutyl as a comparatively common group in their molecular structures. Among these drugs, chloroquine has been previously reported to inhibit lysosomal degradative processes in culture cells (20). Hruban et al. (21) have indicated that the formation of myeloid bodies in livers was induced to rat by feeding chlorcyclizine, norchlorcyclizine, homochlorcyclizine, triparanol, and chloroquine. In formation of the myelin-like material,

TABLE III

Effect of 4,4'-Diethylaminoethoxyhexestrol (DH) on Acid Lipase In Vitro[a]

Concentration of DH (mM)	Specific activity (mμmol/min/mg protein)	Inhibition (%)
0	323.5	-
0.25	237.4	26.6
0.5	217.8	32.7
1.0	196.9	39.1
2.0	156.4	51.7

[a]Values are represented as the mean of two experiments. The reaction mixture contained 0.38 mg of rat liver lysosomal protein; 100 μmol of citrate-phosphate buffer, pH 5.2; 7.5 μmol of tricaprin; and 25 mg of gum arabic in a total volume of 1.5 ml. Incubations were for 20 min at 37 C.

the drugs may selectively inhibit lysosomal lipolytic enzymes concerned with membrane digestion. SKF 525-A and Lilly 18947, well-known potent inhibitors of hepatic microsomal drug-metabolizing enzymes (22), also have a molecular structure similar to DH.

Hayase and Tappel (11) suggest that the enzymes described as lysosomal esterase (10) and lysosomal lipase (23) may be identical in substrate specificity, pH optimum, and a behavior on hydroxylapatite column chromatography. As shown in Table III, the acid lipase was inhibited by DH as was expected, and its inhibition was of a degree relatively similar to acid esterase.

Deficiencies of acid lipase and acid cholesteryl ester hydrolase in Wolman's disease and cholesteryl ester storage disease have recently been reported (24-28). Our findings described above suggest that DH-induced lipidosis may be attributed to overloading of lysosomes, which results from the occurrence of DH and its metabolites in lysosomal system as inhibitors of lipolytic hydrolases (20).

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Fatty Acid Variability of Plasma Lipids and Cholesteryl Esters in Adult Male Twins and Their Brothers

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ABSTRACT

Variation in the fatty acid composition of fasting plasma lipids and of cholesteryl esters was studied in 69 sets of adult male twins and 25 of their brothers. Genetic variances were estimated using the twin model. In general, monozygotic (MZ) twins were characterized by the smallest within-pair variance, and brothers of twins by the largest. Variation within dizygotic pairs fell intermediate to that of MZ twins and brothers. The present study did not reveal consistent significant ($P<0.05$) genetic variation in plasma fatty acids from total plasma lipids or cholesteryl esters.

INTRODUCTION

The implication of blood lipids, particularly of cholesterol and triglycerides, as risk factors in the development of coronary heart disease has stimulated increased interest in the nature of the factors determining their variation. The fact that genotype and environment play roles in determining the quality and quantity of blood lipids has been fairly well established. Studies of plasma cholesterol level have indicated that genotype plays a major part in the determination of familial hyperlipoproteinemia. Significant genetic variation in plasma lipids has been reported by several investigators (1-3).

To date, studies of variation between and within monozygotic (MZ) and dizygotic (DZ) twin pairs have provided one of the most useful methods for the determination of the relative roles of genotype and of environment in explaining observed variation in quantitative traits in man (4). The purpose of the present study was to measure the variation in concentrations of fatty acids of plasma cholesteryl esters and of total plasma lipids in twins and their brothers in order to determine the proportions of the variation due to genetic and environmental influences.

MATERIALS AND METHODS

Subjects

The subjects were part of the National Research Council Twin Panel, which listed all male Caucasian World War II and Korean Veteran twins who were born between the years 1917 and 1927 (5). Zygosity determination was made by 14 blood group and electrophoretic markers. Based on genotyping results, 41 sets of twins were discordant for one or more systems and were considered dizygotic (DZ). The remaining 28 sets were MZ twins. This study included a total of 25 brothers of these twin pairs, each of whom had at least two brothers. The ages of these brothers ranged from 37 to 62 years.

Extraction and Analysis of Lipids

For the fatty acid analysis, venous blood was collected in tubes containing EDTA following a 12-14 hr overnight fast. Plasma was separated

TABLE I

Fatty Acid Composition (percentage ± standard deviation) of the Plasma Cholesteryl Esters for Monozygotic (MZ) and Dizygotic (DZ) Twins and Their Brothers

Fatty acid	MZ (24 pairs)	DZ (32 pairs)	Brothers[a]
14:0	2.5 ± 0.64	2.2 ± 0.58	2.8 ± 0.82
16:0	14.2 ± 6.93	15.6 ± 6.66	14.8 ± 8.04
16:1	3.2 ± 2.02	2.7 ± 3.22	2.5 ± 2.01
18:0	1.1 ± 2.46	1.4 ± 2.48	2.7 ± 5.06
18:1	23.9 ± 8.27	22.5 ± 8.64	22.4 ± 10.50
18:2	52.5 ± 8.81	53.1 ± 8.28	51.6 ± 10.67
18:3	2.0 ± 0.56	1.6 ± 0.83	2.3 ± 1.59
20:4[b]	0.6[b] -	0.9 -	0.9 -

[a]Eleven brother sets with 25 individuals.

[b]Standard deviations were not determined because 20:4 was a sporadic and often very minor component.

TABLE II

Fatty Acid Composition (percentage ± standard deviation) of the Total Plasma Lipids for Monozygotic (MZ) and Dizygotic (DZ) Twins and Their Brothers

Fatty acid	MZ (28 pairs)	DZ (41 pairs)	Brothers[a]
14:0	3.1 ± 0.74	2.9 ± 0.89	2.2 ± 1.08
16:0	32.1 ± 9.86	28.4 ± 8.06	27.9 ± 10.36
16:1	3.4 ± 1.69	4.1 ± 2.26	3.2 ± 2.55
18:0	3.5 ± 2.54	4.9 ± 3.46	4.0 ± 3.27
18:1	36.5 ± 7.58	44.3 ± 6.90	41.5 ± 8.38
18:2	18.8 ± 9.52	16.5 ± 8.70	18.4 ± 7.99
18:3	1.3 ± 2.0	0.9 ± 1.61	2.1 ± 1.59
20:4	0.9 ± 1.68	0.7 ± 1.17	1.0 ± 1.07

[a]Eleven sets with 25 individuals.

TABLE III

Among and Within Twin-Set Mean Squares (percentage^2) for Fatty Acid Components of Plasma Cholesteryl Esters

	Monozygotic		Dizygotic		Brothers	
Fatty acids	Among	Within	Among	Within	Among	Within
14:0	0.580	0.237	0.423	0.259	0.814	0.738
16:0	51.400	44.925	65.240	24.090	124.057	28.271
16:1	5.098	3.114	13.967	7.002	6.450	2 992
18:0	9.886	2.281	11.048	1.385	37.400	21.873
18:1	102.597	35.942	80.178	69.397	187.443	70.366
18:2	126.031	30.953	79.382	58.081	244.164	26.540
18:3	0.403	.227	1.019	0.363	4.112	1.824

from the erythrocytes, and the lipids were extracted with chloroform-methanol using an antioxidant, 2,6-di-tert-butyl-4-methyl-phenol, in the chloroform. The cholesteryl esters were separated by thin layer chromatography (TLC) on Silica Gel G using a solvent of equal parts chloroform and cyclohexane. Known quantities of a standard mixture of lipid compounds and the sample were applied to lanes on each plate. The position of the cholesteryl esters was determined by the exposure of the reference lane to iodine vapor. Lines were drawn above and below the spot across the plate. Cholesteryl ester spots were then scraped into tubes and saponified.

The cholesteryl ester fatty acids and the fatty acids in an aliquot of plasma extract were converted to methyl esters by heating for 5 min at 100 C with 15% boron trifluoride in methanol (6). The methyl esters of fatty acids were separated on a 6 ft x 4 mm column of 10% diethylene glycol succinate on 80/100 mesh Chromosorb W (Supelcoport, Supelco, Inc., Bellefonte, PA) in a Packard Model 419 gas chromatograph equipped with a flame ionization detector. Column temperature was 185 C, nitrogen flow rate 40 cc/min, inlet temperature 250 C, and detector temperature 240 C. Qualitative determinations were made by comparing relative retention times of known fatty acids methyl esters (AOCS Oil reference mixtures RM-3 and RM-5). The relative proportion of each fatty acid methyl ester was determined by dividing the area under each peak by the total peak area, and was expressed as a percentage. All lipid analyses were done on duplicate determinations from a single fasting blood sample from each individual. Several gas chromatographic injections were made from each sample.

Estimates of genetic variances and test procedures followed the method developed for twin analysis in this laboratory (7).

RESULTS AND DISCUSSION

Means and standard deviations for plasma total and cholesteryl ester fatty acids are presented in Tables I and II. The mean values obtained are comparable to previously reported results in all cases except arachidonic acid (8,9). The mean values for arachidonic acids are lower than previously reported, and the gas-liquid chromatographic system used does not appear to be optimal from quantitative calibration data. However, the values were reproducible and have been reported without corrections. The mean percentages of various fatty acids were not significantly different ($P>0.05$)

TABLE IV

Among and Within Twin-Set Mean Squares for Fatty Acid Components of Plasma Total Lipids

	Monozygotic		Dizygotic		Brothers	
Fatty acids	Among	Within	Among	Within	Among	Within
14:0	0.631	0.483	0.947	0.668	2.018	0.713
16:0	139.879	49.171	83.384	47.268	214.781	38.899
16:1	4.014	1.734	6.643	3.630	12.778	2.614
18:0	8.955	4.112	16.841	7.218	17.605	7.363
18:1	64.958	50.218	57.840	37.817	97.524	64.359
18:2	103.282	78.483	87.713	64.043	81.268	65.275
18:3	5.986	2.126	3.066	2.078	3.225	3.004
20:4	3.002	2.708	1.857	0.910	0.819	1.502

TABLE V

Sums of Mean Squares and Genetic Variance Estimates for Cholesteryl Fatty Acids from Monozygotic (MZ) and Dizygotic (DZ) Twins

	Sum of mean squares			Genetic variance estimates[a]		
Fatty acid	MZ	DZ	F	Among	Within	Among component
14:0	0.818	0.687	1.191	0.153	0.022	0.087
16:0	96.325	89.330	1.078	-13.840	-20.834	-17.337
16:1	8.212	20.970	2.553[b]	-8.869	3.888[c]	-2.490
18:0	12.167	12.434	1.021	-1.162	-0.895	-1.028
18:1	138.539	149.575	1.079	22.418	33.455[c]	27.937
18:2	156.984	137.463	1.142	46.649	27.128[c]	36.888
18:3	0.632	1.383	2.189[b]	-0.615	0.136	-0.239

[a]Genetic variance estimates: Among = among MZ mean square - among DZ mean square; Within = within DZ mean square - within MZ mean square; Among component = 1/2 (genetic variance estimate among + genetic variance estimate within).

[b]$P<0.2$ (an increased significance level was used in order to determine if environmental variance common to both types of twins is present).

[c]$P<0.01$.

among MZ twins, DZ twins, or their brothers. While the predominant fatty acids of both lipid fractions are palmitic (16:0), oleic (18:1), and linoleic (18:2) acids, the proportions of these fatty acids in cholesteryl ester and total plasma lipids are quite different. This confirms previous findings that cholesteryl esters transport proportionally more unsaturated fatty acids than does the plasma total lipid fraction (10).

Tables III and IV show analyses of variance for various fatty acids from plasma total and cholesteryl esters in the three groups (MZ, DZ, brothers). Considerable variation in fatty acid concentration is found between members of a twin pair (reflected in within pair mean squares), as well as among twin pairs and brother sets (among sets mean squares). Separate analyses of variance of multiple determinations, not listed in the tables, indicated that laboratory error accounted for only a small portion of the total variation in major fatty acid components. Laboratory error did, however, seem to account for a greater part of the total variation (13-26%) for the minor components (16:1, 18:3, 20:4). In all cases, with the exception of arachidonic acid in total plasma lipids, the within twin pair mean squares for fatty acid components were significantly greater ($P<0.05$) than the laboratory error mean squares from duplicate sample analysis for each individual.

It was expected that the variation within brother sets would be the greatest in magnitude and that within MZ twins the smallest. Average genetic differences between two brothers should be the same as between members of a DZ twin pair; however, differences between members of a DZ twin pair due to environmental factors, particularly age, should be smaller than those of brothers.

In all cases, with one exception (Table IV, 20:4 for brothers), the variation among sets was greater than that within sets. Variation within brother sets was greater than that within MZ or DZ pairs for three fatty acids (14:0, 18:0, and 18:1), while variation in palmitic acid concen-

TABLE VI

Sums of Mean Squares and Genetic Variance Estimates for Plasma Total Lipid Fatty Acids from Monozygotic (MZ) and Dizygotic (DZ) Twins

Fatty acid	Sum of mean squares		F	Genetic variance estimates[a]		
	MZ	DZ		Among	Within	Among component
14:0	1.115	1.615	1.448[b]	-0.315	.184	-0.065
16:0	189.05	130.652	1.447[b]	56.495	-1.903	27.296
16:1	5.749	10.274	1.787[b]	-2.628	1.896[c]	-0.366
18:0	13.068	24.060	1.841[b]	-7.885	3.106	-2.389
18:1	115.117	95.657	1.204	7.118	-12.401	-2.641
18:2	181.765	151.756	1.197	15.569	-14.440	0.504
18:3	8.134	5.145	1.570[b]	2.920[c]	-0.048	1.436

[a]Genetic variance estimates: Among = among MZ mean square - among DZ mean square; Within = within DZ mean square - within MZ mean square; Among component = 1/2 (genetic variance estimate among + genetic variance estimate within).

[b]$P<0.2$.

[c]$P<0.05$.

tration within MZ pairs was greater than that within either DZ twins or brothers.

The sums of mean squares and various estimates of genetic variance are shown in Tables V and VI. The total variances of MZ and DZ twins differed in five of the seven fatty acids measured from plasma total lipid fatty acids as opposed to differing in only two of those measured from cholesteryl ester fatty acids. Three genetic variance estimates obtained from the within mean squares for cholesteryl esters were significant, in contrast to only one for total lipids.

There was no significant correlation between any fatty acid proportion and age of the individual studied. This finding could possibly be explained by the relatively narrow age range of most of the subjects examined in this studv.

While there have been many studies on the genetic variance of plasma lipids, particularly cholesterol (1,5,11), the genetic variances of cholesteryl ester and total plasma lipid fatty acids have not been examined to any large degree. One report concerning the genetic constitution of the fatty acids of sphingomyelin in twins (12) indicated that several fatty acids of sphingomyelin (18:1, 18:2, 20:0, and 23:0) showed significantly larger ($P<0.05$) intrapair variation for DZ twins as compared to MZ twins. Results of this study were difficult to interpret because of small sample size (15 pairs) and because of the method of statistical analysis of data. Each member of the twin pair was assigned arbitrarily in the analysis, and a "residual" was partitioned from the intrapair variance.

Several investigators have shown that diet or environment may influence fatty acid patterns in plasma (13,14). This is in agreement with the findings presented herein since no significant genetic variation was found in any of the plasma fatty acids examined from among component estimates. The distributions of fatty acids within cholesteryl esters and total plasma lipids are quite different; however, the level of these fatty acids does not seem to be determined by the individual's genotype.

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Phospholipid Synthesis in Mammary Tissue. Choline and Ethanolamine Kinases: Kinetic Evidence for Two Discrete Active Sites

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ABSTRACT

Choline and ethanolamine kinases are located in the high speed supernatant of lactating bovine mammary gland. Maximum activities of choline and ethanolamine kinases were observed at pH 9.2 and 8.0, respectively, with the rate of ethanolamine phosphorylation being 1/15 that of choline phosphorylation. Activation energies of 29 joules (Q_{10} - 1.5) and 31 joules (Q_{10} = 1.5) were calculated between 3.4 and 31.3 C for choline kinase and ethanolamine kinase, respectively. Above 31.3 C, the Arrhenius plot deviated from linearity for both enzymes, suggesting that denaturation was occurring. An apparent Km of 0.25 mM for choline was obtained for choline kinase activity. The apparent Km of ethanolamine kinase for ethanolamine was unusually high (17 mM), and activity was not linear with increasing protein concentration. Activity was tripled and the Km decreased to 2.5 mM when the enzyme preparation was washed with butanol: benzene mixture, suggesting the presence of an endogenous competitive inhibitor(s), with respect to ethanolamine. Choline kinase was not affected by the solvent wash. Substrate competition studies revealed that choline kinase was slightly inhibited competitively by ethanolamine (apparent Ki = 19-21 mM), whereas choline was a potent competitive inhibitor of ethanolamine kinase (apparent Ki = 0.33-0.50 mM). The results indicated that these two kinase activities were mediated by two distinct active sites, possibly on a single protein. The significance of choline in the regulation of phosphatidylethanolamine synthesis is discussed.

INTRODUCTION

ATP:choline phosphoryltransferase (choline kinase, EC.2.7.1.32) and ATP:ethanolamine phosphotransferase (ethanolamine kinase) are the initiating enzymes in the cytidine or Kennedy pathway for the de novo synthesis of 3-sn-phosphatidylcholine (PC) and 3-sn-phosphatidylethanolamine (PE), respectively. Because these enzymes may catalyze the controlling steps in the synthesis of PC and PE and since mammary cells display an apparent preferential synthesis of PC compared to PE (1), we studied the relative activities of these enzymes in lactating mammary tissue.

Ethanolamine kinase activity has been demonstrated in crude and partially purified choline kinase preparations (2-4). A highly purified choline kinase from rabbit brain has also been shown to have ethanolamine kinase activity (5). Weinhold and Rethy (6) resolved the ethanolamine kinase from rat liver into two fractions by DEAE-cellulose chromatography. One of the fractions (ethanolamine kinase II) copurified with choline kinase. The ratio of these two activities remained constant during DEAE-cellulose and Sephadex G-200 chromatography, and both activities showed similar stabilities (6). The copurification of ethanolamine and choline kinase activities from rat liver has recently been confirmed by Brophy and Vance (7) by affinity chromatography. These studies suggest that both kinase activities are associated with the same protein in rabbit brain and rat liver. However, Sung and Johnstone (3) reported indirect evidence that two distinct proteins are responsible for these two kinase activities in Ehrlich ascites cells. Broad and Dawson (8) partially separated these two kinases from the rumen protozoan *Entodinium caudatum* by gel filtration.

If, indeed, choline and ethanolamine kinases are associated with the same protein, a question that must be answered is whether the same active site is responsible for these two kinase activities, or if two or more specific sites are involved. We studied the properties of these enzymes from bovine mammary tissue, compared their activities under different assay conditions, and used the kinetic technique of substract competition to answer this question. Results indicate that the choline and ethanolamine kinase activities are mediated by at least two distinct active sites.

MATERIALS AND METHODS

Materials

(Me-^{14}C) choline chloride (61 μCi/μmol,

diluted tenfold for enzyme assays) was purchased from Amersham/Searle Corp. (Arlington Heights, IL). (1,2-^{14}C) Ethanolamine-HCl (30 μCi/μmol, diluted fourfold for enzyme assays) was purchased from New England Nuclear (Boston, MA). Choline bromide, monoethanolamine-HCl, and phosphatidylethanolamine (dipalmityl, synthetic) were purchased from Calbiochem (San Diego, CA). Lecithin (bovine) was purchased from P.L. Biochemical Inc. (Milwaukee, WI). Egg lysolecithin was purchased from Pierce Chemicals (Rockford, IL). Tween-20 and adenosine-5′-triphosphate (disodium crystalline, 99-100%, low calcium content) were purchased from Sigma Chemical Co. (St. Louis, MO).

All common chemicals were of reagent grade or of the highest purity of commercially available materials. Glass redistilled and deionized water was used in all solutions and buffers.

Methods

Enzyme preparation: Mammary tissue was excised from lactating Holstein cows immediately after slaughter and stored in an ice chest (4 C). Connective tissue was removed, and the secretory tissue was minced, in a meat grinder. This material was suspended in Tris-HCl buffer (0.14M, pH 8) in a 1:1 (v/v) ratio and homogenized in a Waring blendor for 30 sec. This homogenate was further rehomogenized in a small mill (Polyscience Corp., IL) for 30 sec. All manipulations were carried out at 4 C.

The final homogenate was centrifuged in a refrigerated Sorvall centrifuge using a GSA rotor (r = 5.75") at 15,000 x g for 20 min at 4 C. The supernatant was decented and strained through two layers of cheese cloth to separate the fat layer and centrifuged in a Beckman model L2-65 preparative ultracentrifuge using a type-21 fixed angle rotor at 44,000 x g for 75 min. The final supernatant was quickly frozen in a round bottom flask using a dry ice-acetone bath and lyophilized in a Virtis freeze-drier (at 0.03 mm Hg pressure at 24 C for 24 hr). The resulting powder was ground to a fine consistency and stored in plastic-capped vials at −25 C. This powder was used as the enzyme source.

Butanol:benzene wash: The lyophilized supernatant was suspended in n-butanol in a 1:10 (w/v) ratio and immediately centrifuged at 3,000 x g in a refrigerated Sorvall centrifuge for 10 min. The resulting supernatant was decanted, and the wash operation was repeated three more times. The final pellet was further washed four times with a 2:1 (v/v) butanol: benzene mixture following the same procedure used for the butanol wash. All preparations were carried at 4 C. The final pellet was dried under a stream of dry nitrogen. This powder was stored at −25 C and called the "delipidated enzyme preparation."

Preparation of phospholipid dispersions: With the exception of lyso-PC, all phospholipids (20 μmol) were first dissolved in 0.5 ml benzene followed by the addition of 5.0 ml of 0.01%(v/v) Tween 20 in water. The suspension was sonicated for 10 min in a water bath sonicator (model 8845-3, Cole-Parmer Instrument Co., Chicago, IL), and the organic solvent was then evaporated under a stream of dry nitrogen. The phospholipid suspension, free of organic solvent, was sonicated (ca. 20 min) until the opalescence disappeared.

Assay conditions: Unless otherwise stated, all assays were performed in a total volume of 375μl containing 50 mM pH 8.0 Tris-HCl and ca. 1.0 mg of protein. Stock solutions of ethanolamine were adjusted to pH 8.0 with Tris-HCl buffer. Initial velocities were obtained with 15-30 min incubations. Routine incubations were performed at 36 C in a shaking water bath, stopped with 50 μl of 1.0 M trichloroacetic acid, and subsequently centrifuged in a refrigerated Sorvall centrifuge at 5,000 x g for 5 min at 4 C. Aliquots of the 5,000 x g protein free supernatant were subsequently analyzed for radioactive substrates and products.

The rationale of the substrate competition technique used in this study to determine whether two alternate substrates are utilized by the same active site has been discussed in detail (9,10).

All kinetic constants obtained in these experiments must be considered as apparent ones, since true kinetic constants and their composition in terms of invididual rate constants can only be obtained after the kinetic mechanism has been elucidated.

Analytical: The protein was determined by the method of Lowry et al. (11) using crystalline bovine serum albumin as a standard. Choline, P-choline, and CDP-choline were separated by paper chromatography (10 x 10", Whatman 3 MM) using a modification of the solvent system described by Plageman (12). The developing solvent contained 2.7 M ammonium acetate buffer pH 5.0 and 95% ethanol in a 3:7 (v/v) ratio. The Rf's were 0.82, 0.48, and 0.27 for choline, P-choline, and CDP-choline, respectively. The same solvent system was found to resolve ethanolamine, P-ethanolamine, and CDP-ethanolamine, with Rf's of 0.75, 0.32, and 0.15, respectively. Authentic standards of choline, ethanolamine, and their respective phosphate and CDP- esters were always chromatographed along with the assay aliquots. Choline

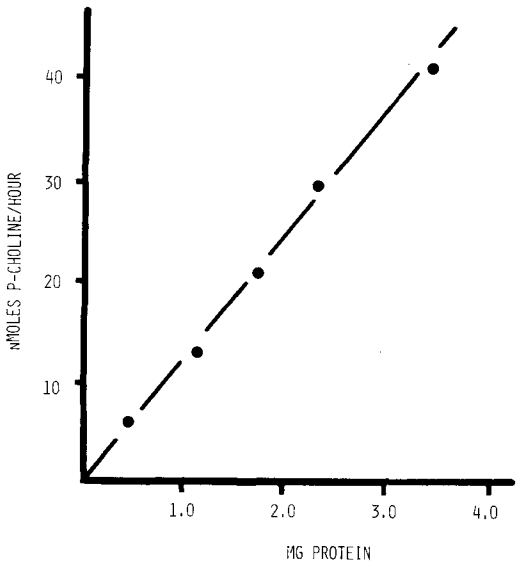

FIG. 1. Bovine mammary choline kinase activity as a function of protein concentration. The specified amounts of high speed supernatant from mammary tissue were incubated in medium containing Mg-ATP^{-2}, 3.3 mM, free Mg^{2+}, 0.4 mM, and (Me-^{14}C) choline, 0.15 mM. Other experimental procedures as in "Methods."

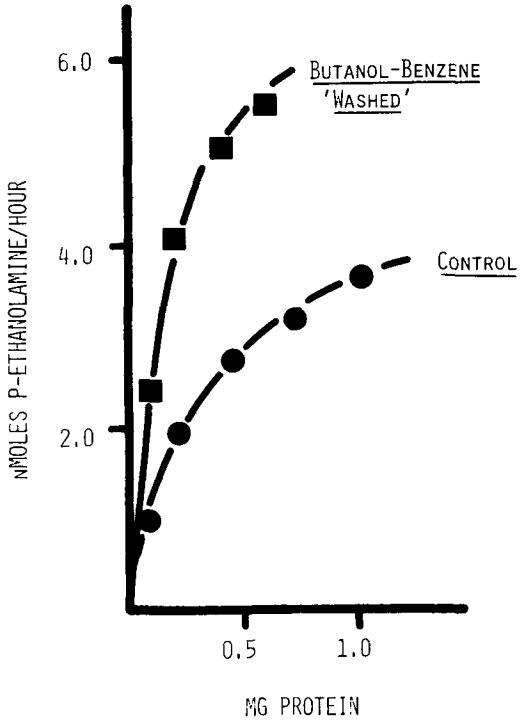

FIG. 2 Activity of bovine mammary ethanolamine kinase as a function of protein concentration. The specified amounts of protein from high speed supernatant (normal and butanol:benzene "washed") were incubated in a medium containing Mg-ATP^{-2}, 3.6 mM, free Mg^{2+}, 0.43 mM, and (1,2-^{14}C)-ethanolamine, 0.37 mM. Other experimental procedures as in "Methods."

spots were detected by iodine vapors. The phosphate ester was detected by the phosphorus spray of Hanes and Isherwood (13) as modified by Dawson (14). Ethanolamine and P-ethanolamine were detected by a ninhydrin spray. CDP-choline and CDP-ethanolamine were visualized under ultraviolet light. Following identification of the standards, the appropriate spots were cut from the paper chromatograms and counted in a Packard scintiallation spectrometer (15).

Radiopurity of ^{14}C-labeled compounds and recovery of radioactivity in the assays were routinely checked, and both were 98%. CDP-(^{14}C)-choline or CDP-(^{14}C) ethanolamine were not detected in the assays.

Calculations of Mg-ATP^{-2} and free Mg^{2+} concentrations were made using the Mg-ATP^{-2} stability constant of 20 mM^{-1} (16).

RESULTS

Choline kinase activity assayed with a limiting concentration of choline (0.15 mM) was directly proportional to protein concentration over a tenfold range, i.e., 0.36-3.6 mg of protein (Fig. 1). This indicated that no detectable concentrations of endogenous substrates, inhibitors, or activators were present in the enzyme preparation at the protein levels used.

Initial velocity experiments with choline as the variable reactant produced nonlinear double reciprocal plots at choline concentrations below 0.07 mM. Therefore, the substrate competition experiments in this study were performed above this level of choline. Detailed studies on the kinetic mechanism of mammary choline kinase will be published separately.

The activity of ethanolamine kinase was not linear with protein concentration, suggesting that an inhibitor(s) was present in the preparation (Fig. 2). When the lyophilized preparation was washed with a butanol:benzene mixture, ethanolamine kinase activity increased threefold and deviation from linearity decreased (Fig. 2), indicating that part of the inhibition was being removed by the solvent treatment. In contrast, choline kinase showed only a slight increase (10%) in activity after the same solvent wash, suggesting that no inhibitors nor activators of this enzyme were present in the preparation at the concentration of protein used (Table I). This agrees with the observed linearity of choline kinase activity as a function of protein

TABLE I

Effects of Solvent Extraction of Crude Enzyme on Choline and Ethanolamine Kinase Activities[a]

Enzyme treatment	Specific activity	
	Choline kinase	Ethanolamine kinase
Normal preparation	22	4.2
Benzene extracted	17	4.1
Butanol extracted	21	12
Benzene:butanol extracted	25	13

[a]Incubation medium contained (Me-^{14}C) choline, 0.3 mM for the choline kinase assay; (1,2-^{14}C) ethanolamine, 2.5 mM for the ethanolamine kinase assay. Mg-ATP^{-2} and free Mg^{2+} concentrations were 3.6 and 0.43 mM, respectively, in both enzyme assays. Other experimental procedures as in "Methods." Specific activity is indicated in nmol phosphorylcholine or phosphorylethanolamine formed per mg protein per hour.

concentration.

Choline kinase activity was remarkably stable in freeze-dried preparations, showing only 5% loss in activity after 15 months storage at −25 C. Its stability in buffer solution at 4.0 C was limited to ca. 5 hr, after which the activity decreased according to a first order reaction ($k = 5.1$ min^{-1}) over a 32 hr period, with the half life ($t_{½}$) of 8.2 hr (Fig. 3). Perhaps the high protein concentration of the holding stock solution contributed to the stability of the enzyme. The initial 5 hr stability period was of sufficient duration for performance of experiments. The stability of choline kinase at 24 C was more limited. Activity diminished over a 32 hr period following first order rate kinetics ($k = 10$ min^{-1}), with $t_{½}$ equal to 4.0 hr. The stability of

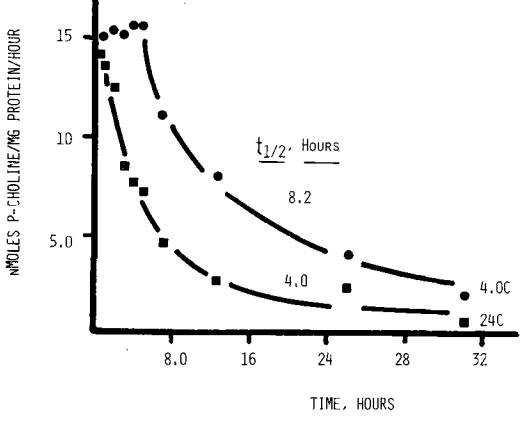

FIG. 3. Stability of choline kinase activity from bovine mammary tissue at 4 and 24 C. Enzyme solutions containing 75 mg protein per ml of Tris-HCl buffer (0.3 M pH 8.0) were held at the indicated temperatures for the specified periods. The activity of aliquots containing 1 mg protein was assayed in a medium containing Mg-ATP^{-2}, 3.3 mM, free Mg^{2+}, 0.41 mM, and (Me-^{14}C)-choline, 0.15 mM. Other procedures as in "Methods."

ethanolamine kinase in solution followed the same pattern as choline kinase, and freeze-dried preparations retained ethanolamine kinase activity over a period of 15 months.

Optimum pH of choline and ethanolamine kinase activities: The pH profiles of both choline and ethanolamine kinase activities had characteristic bell shapes, but each showed a distinctly different pH optimum. Choline kinase showed its maximum activity at pH 9.0, with a steep decrease in activity on the acid side of the curve; a 17-fold difference was observed between pH 6 and 9 (Fig. 4A). Ethanolamine kinase reached its maximum activity at pH 8.0, showing only a 60% lower rate at pH 6.0 (Fig. 4B). The buffer N-(2-acetamido) iminodiacetic acid (ADA) was strongly inhibitory to both enzymes. This inhibition is consistent with its Mg^{2+} binding properties, i.e., log $K_{assoc} = 2.5$ (17). The other buffers used have negligible metal binding constants (18), thus their competition for Mg^{2+} can be ruled out.

Effect of temperature on activity of choline and ethanolamine kinases: The effect of temperature on the activity of both enzymes was studied between 3.5 C and 40.7 C. From rate data obtained between incubation temperatures of 3.4 C and 31.3 C, Arrhenius activation energies (Ea) of 29 joules ($Q_{10} = 1.5$) and 31 joules ($Q_{10} = 1.5$) were calculated for choline kinase and ethanolamine kinase, respectively (Fig. 5). Above 31.3 C, the Arrhenius plot deviated from linearity for both enzymes. Suggesting that denaturation was occurring.

Initial velocity patterns with ethanolamine and choline was variable reactants: An apparent Km of 17 mM for ethanolamine, as the variable reactant, was obtained from a double reciprocal plot of initial velocities (Fig. 6A). Because this Km was abnormally high, the presence of an endogenous competitive inhibitor in the enzyme preparation was suspected. Consistent

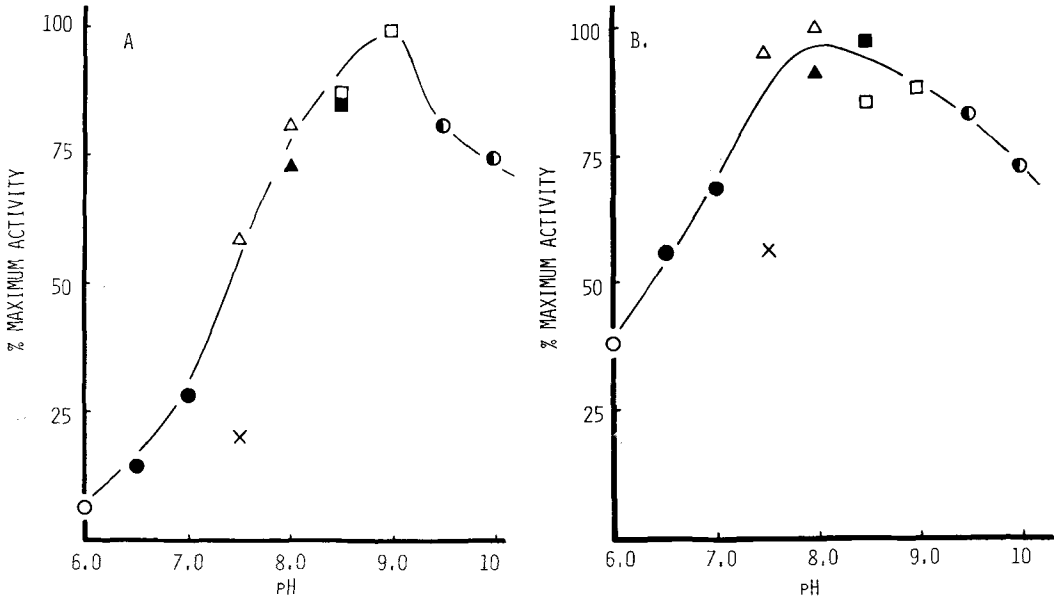

FIG. 4. Phosphoryl transfer activity as a function of pH. Assay medium for choline kinase (A) contained Mg-ATP^{-2}, 3.6 mM, free Mg^{2+}, 0.43 mM, and (Me-^{14}C) choline, 2.37 mM. Assay medium for ethanolamine kinase (B) contained Mg-ATP^{-2}, 3.6 mM; free Mg^{2+}, 0.43 mM, and (1,2-^{14}C) ethanolamine, 1.9 mM. Buffer concentration was 80 mM; —○—, 2-(N-morpholino) ethane sulfonate (MES); —●— N,N-bis (2-hydroxymethyl)-2-aminoethane sulfonate (BES); —△— N-2-hydroxyethyl piperazine-N-2-ethane sulfonate (HEPES); —X— N-(2-acetamido) iminodiacetate (ADA); —▲—, 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris); —■—, N,N-bis (–2-hydroxyethyl) glycine (BICINE); —□—, glycyl glycine (GLY-GLY); —◐— glycine. Other experimental procedures as in "Methods."

with this was the fact that activity was not linear with increasing protein concentration (Fig. 2). Ethanolamine kinase activity was tripled when the lyophilized enzyme preparation was washed with butanol or butanol: benzene while the choline kinase activity remained relatively constant. Kinetic studies revealed that the butanol:benzene extraction of the enzyme preparation caused a large decrease in the apparent Km for ethanolamine of ethanolamine kinase, i.e., from 17 mM to 2.5 mM (Fig. 6B), while the Vmax remained unaffected. These data indicated that a kinetically competitive inhibitor was being removed from the enzyme preparation by the solvent wash.

The phospholipids 3-sn-phosphatidylcholine, monoacyl-sn-glycero-3-phosphoryl-choline, and 3-sn-phosphatidylethanolamine, in Tween 20 (final concentrations, 1.3 mM and 0.03%, respectively), were not inhibitors of ethanolamine kinase because, when added to the delipidated enzyme preparation, they caused no inhibition with respect to the control containing Tween 20 (Table II). The surfactant Tween 20 (0.03%) produced 37% inhibition of ethanolamine kinase. On the other hand, choline kinase was unaffected by the butanol:benzene wash, phospholipids, or Tween 20.

Substrate competition experiments showed that choline was a strong competitive inhibitor (apparent Ki = 0.50 mM) of ethanolamine kinase activity (Fig. 6B), while choline kinase activity (apparent Km = 0.25 mM) was only slightly inhibited by ethanolamine (apparent Ki = 21 mM). This inhibition was also competitive (Fig. 7A).

To minimize the effect of possible activators and/or inhibitors, the same substrate competition studies were done using the delipidated enzyme preparation. The activity of choline kinase was not changed, its apparent Km for choline being 0.28 mM, and it was slightly inhibited by ethanolamine, i.e., apparent Ki = 19 mM (Fig. 7B). In the case of ethanolamine kinase, its ethanolamine apparent Km decreased to 2.5 mM (Fig. 6B). The inhibition by choline was slightly increased compared to the original preparation, i.e., apparent Ki = 0.33 mM compared to an apparent Ki of 0.50 mM, respectively (Fig. 6A).

DISCUSSION

Previous studies indicated that the lactating mammary gland synthesized phospholipids de novo and that the classical Kennedy pathway

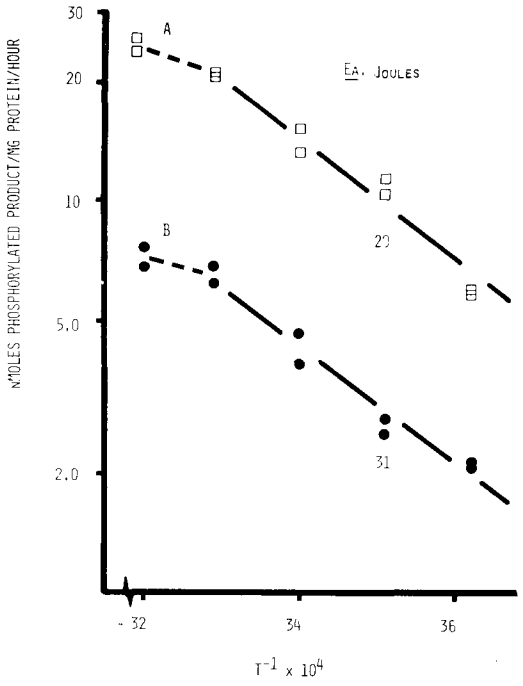

FIG. 5. Arrhenius plot of bovine mammary choline kinase and ethanolamine kinase activities. Assay medium for choline kinase (A) contained Mg-ATP^{-2}, 3.6 mM, free Mg^{2+}, 0.43 mM, and (Me-^{14}C) choline, 0.30 mM. Assay medium for ethanolamine kinase (B) contained Mg-ATP^{-2}, 3.6 mM; free Mg^{2+}, 0.43 mM, and (1.2-^{14}C) ethanolamine, 2.3 mM. Other experimental procedures as in "Methods."

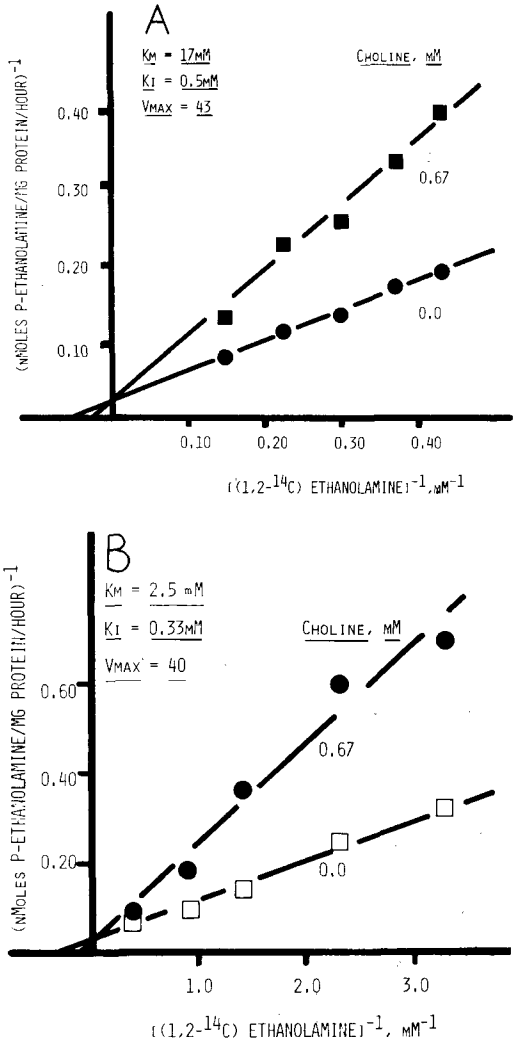

FIG. 6. Double reciprocal plots of initial velocities of bovine mammary ethanolamine kinase activity in the presence of choline. Assay medium contained Mg-ATP^{-2}, 3.6 mM, free Mg^{2+}, 0.43 mM, and varying levels of (1,2-^{14}C) ethanolamine. Other experimental procedures as in "Methods." A. Unwashed enzyme. B. Butanol:benzene washed enzyme.

was the major route (1,19,20). The present data corroborate this.

The broad alkaline pH optima for choline and ethanolamine kinases from bovine mammary tissue are in agreement with reports for these enzymes from other tissues (2,3,21-26). However, there are no comparative studies on the pH optima for these two enzymes under the same conditions. The fact that, when identical assay conditions were used in this study, ethanolamine kinase activity showed a lower pH curve than choline kinase activity suggests that these bases are not phosphorylated by the same active site. These data are also consistent with the possibility that a cationic nitrogen is needed for the binding of ethanolamine and choline to the enzyme active site. In the case of choline, the quaternary ammonium cation ($-N^+-(CH_3)_3$) holds a permanent charge independent of pH. Ethanolamine, however, has a dissociable proton on the primary amine nitrogen (pK = 9.2) at 36 C (27). At the optimum pH (pH 8.0), 94% of the ethanolamine would be in the protonated form ($-H_3N^+$), conceivably providing maximum binding to the enzyme active site. At pH 9.0, however, only 62% of the ethanolamine species would be in the cationic form. The possibility that a single active site could have different pH optima for these two substrates cannot be excluded if a cationic nitrogen on the base is needed for binding reaction to occur. These data suggest that an anionic group on both enzymes is involved in the binding of both choline and ethanolamine.

The Arrhenius activation energies (EA's) obtained for choline and ethanolamine, i.e., 29 and 31 joules, respectively, correspond to a

TABLE II

Effects of Tween-20 and Phospholipids on Choline and Ethanolamine Kinase Activities from Bovine Mammary Tissue[a]

Addition		Enzyme activity (nmol product formed mg^{-1} hr^{-1})	
Tween-20 (% v/v)	Phospholipid (mM)	Choline kinase	Ethanolamine kinase
0.0	0.0	25	13
0.03	0.0	26	8.2
0.03	3-sn-PC, 1.3	25	10
0.03	Lyso-PC, 2.7	26	10
0.03	3-sn-PE, 1.3	22	9.1

[a]Incubation medium contained (Me-^{14}C) choline, 0.3 mM for choline kinase assay; (1,2-^{14}C) ethanolamine, 2.5 mM for the ethanolamine kinase assay. Mg-ATP^{-2} and free Mg^{2+} concentration were 3.6 and 0.43 mM, respectively, in both assays. Solvent extracted enzyme preparation was used. Other experimental procedures as in "Methods." PC and PE denote phosphatidylcholine and phosphatidylethanolamine, respectively.

Q_{10} of 1.5. Choline kinase from brain tissue showed an EA of 13 joules (3.0 Cal) (25). The almost identical activation energies for the two mammary kinases reported here are consistent with the hypothesis that these two activities are associated with the same protein. However, these data can also be rationalized on the grounds that, since EA is a function of the bond energies involved in the reaction from the same phosphoryl donor (Mg-ATP^{-2}) to similar acceptors (choline or ethanolamine) they should have similar EA's. This assumes that the effect of temperature on the active conformation of the enzyme is negligible or of equal magnitude in both instances, if the enzymes are different proteins. The parallel loss of linearity of the Arrhenius plot observed above 31 C for the activity of the two enzymes is also consistent with the above suggestions.

The weak inhibition of choline kinase by ethanolamine (apparent Ki = 21 mM) is in line with the results obtained by Sung and Johnstone (3) for the enzyme from Ehrlich ascites cells and rat brain tissue. These authors also reported that ethanolamine did not inhibit choline phosphorylation in mouse liver, spleen, and kidney tissue.

The apparent Km (2.5 mM) obtained for ethanolamine kinase is probably higher than the real Km since the washed preparation may have still contained residual amounts of a competitive inhibitor. This strengthens the argument for the presence of two active sites, as the actual differences between the ethanolamine Km and Ki may be even larger than the experimentally observed ones. Ethanolamine kinase preparations may contain inhibitors; e.g., Weinhold and Rethy (23) suggested that choline was the endogenous inhibitor in their crude supernatant from rat. This does not seem to be the case for the mammary enzyme since ethanolamine kinase decreased its apparent Km for ethanolamine sevenfold (from 17 to 2.5 mM)

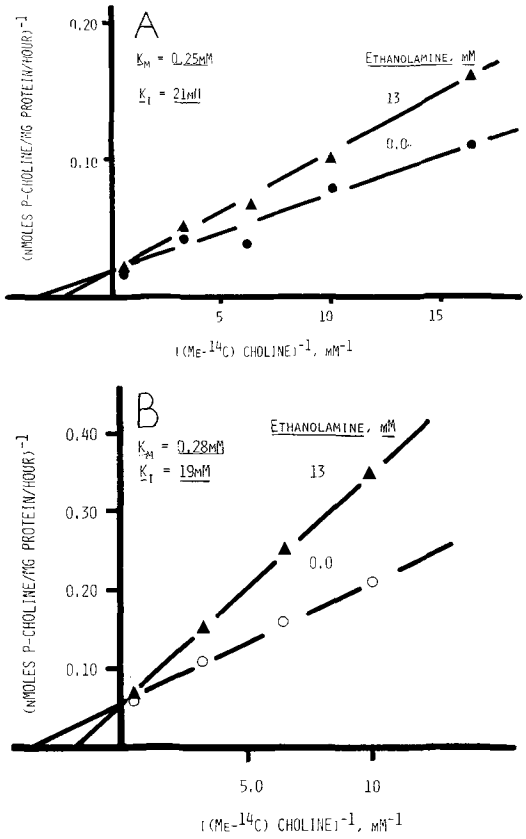

FIG. 7. Double reciprocal plots of initial velocities of bovine mammary choline kinase in the presence of ethanolamine. Assay medium contained Mg-ATP^{-2}, 3.6 mM, free Mg^{2+}, 0.43 mM, and varying levels of (Me-^{14}C) choline. Other experimental procedures as in "Methods." A. Unwashed enzyme. B. Butanol: benzene washed enzyme.

after the solvent wash, while its choline apparent Ki showed only a slight decrease (from 0.50 50 0.33 mM); thus, the small amount of choline that might have been extracted by the solvent cannot account for the large decrease in the apparent Km for ethanolamine. Furthermore, the observed linearity of choline kinase activity with increasing levels (10-fold increase) of protein, when assayed with limiting concentrations of choline (0.15 mM), suggests that little or no endogenous choline was present in the high speed supernatant in the protein range used. The possibility that endogenous ethanolamine was the inhibitor extracted by the solvent wash is very unlikely because of its insolubility in the benzene:butanol mixture.

The strong inhibition of ethanolamine kinase by choline is consistent with the results of Sung and Johnstone (3) and Weinhold and Rethy (23). The latter authors, however, reported a noncompetitive inhibition with respect to ethanolamine but competitive with respect to ATP.

From the substrate competition studies using the delipidated preparation, it is clear that choline kinase and ethanolamine kinase activity do not have a common active site. If this were not the case, the Ki of choline kinase inhibition by ethanolamine would be identical to the ethanolamine Km of ethanolamine kinase activity. This was not observed; therefore, on kinetic grounds these two activities were mediated by at least two distinct active sites. The fact that the apparent Ki of ethanolamine kinase inhibition by choline was similar to the choline Km of choline kinase suggests that the binding site of ethanolamine kinase may recognize choline with the same or higher affinity than ethanolamine. The converse, however, is not true; the binding site of choline kinase can discriminate between the two nitrogenous bases. An obvious difference between these bases is the trimethyl quaternary ammonium moiety of choline. Since the dealkylated substrate (ethanolamine) is such a weak competitive inhibitor (apparent Ki = 19 mM) of choline kinase, it is clear that this enzyme recognizes these three methyl groups which apparently are essential for the substrate binding to this enzyme. Wittenberg and Kornberg (2) showed that the Km's were directly influenced by the number of N-methyl groups of the substrate; i.e., the Km's for the N-methyl-, N,N-dimethyl-, and N,N,N-trimethyl-ethanolamine (choline) were 0.6, 0.1, and 0.02 mM, respectively.

An alternative explanation for the similarities between the choline apparent Km of choline kinase and its apparent Ki for ethanolamine phosphorylation is that the site of inhibition of the latter activity by choline is the choline kinase site. This would be possible if these two activities are associated with the same protein and a "one way negative interaction" operates between these two sites. By this mechanism, occupation of the choline kinase site would prevent the activity of the ethanolamine kinase site; however, the converse would not occur.

As the cytidine pathway is the predominant mechanism whereby 3-sn-phosphatidylcholine and 2-sn-phosphatidylethanolamine are synthesized de novo in most animal tissues (28-36), and since choline is a potent inhibitor of ethanolamine kinase, it is conceivable that the intracellular concentration of choline and of the endogenous inhibitor may control the activity of existing ethanolamine kinase. If this phosphorylation step is rate limiting, the choline:ethanolamine ratio would have the potential to regulate the synthesis of PE in vivo. This control mechanism may play a role in vivo in determining that PC is the major phospholipid in mammalian tissues, and it may explain the increased level of PE found in livers from choline deficient rats (37,38) by release of the inhibition of ethanolamine kinase activity.

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Effect of Dietary Fats on Pentobarbitone-induced Sleeping Times and Hepatic Microsomal Cytochrome P-450 in Rats

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ABSTRACT

Female Wistar-derived rats were fed diets containing either sunflowerseed oil or tallow for 4 weeks. Rats fed the sunflowerseed oil diet had longer pentobarbitone-induced sleeping times and lower concentrations of hepatic microsomal cytochrome P-450 than rats fed the tallow diet. The addition of pentobarbitone to the drinking water of rats caused approximately 2-fold increases in the concentrations of hepatic microsomal cytochrome P-450 but did not alter the relative effect of the diets.

INTRODUCTION

The microsomal mixed function oxygenase enzyme system, which contains the hemoprotein cytochrome P-450, is responsible for the metabolism of a variety of compounds, including foreign materials such as various drugs, pesticides, and carcinogens. As these enzymes are involved in the metabolism of many carcinogens, they may be important factors in carcinogenesis (1-4).

The activity of this enzyme system fluctuates markedly in response to various environmental stimuli (3). It is possible that dietary fats could be important in determining the activity of the mixed function oxygenases, as these enzymes have at least one specific lipid requirement. Phosphatidylcholine has been shown to be essential for microsomal metabolism by allowing enzymatic reduction of cytochrome P-450 (5). Caster et al. (6) have examined the effect of dietary fat on drug metabolism by hepatic microsomes from rats and reported that as the dietary intake of corn oil was increased to 3% of the diet, there was an increased ability of the liver, in an in vitro system, to metabolize drugs. Measurements of drug-induced sleeping times confirmed these findings. Increases in microsomal drug metabolism and the content of cytochrome P-450 in hepatic microsomes from rats fed high levels of corn oil in the diet were also reported by Norred and Wade (7). Rats and mice fed diets containing polyunsaturated fats have been shown to be more susceptible to the action of phenobarbital in stimulating drug detoxification (8,9). Greatest stimulation of drug metabolism was observed in phenobarbital-pretreated animals fed linseed or menhaden oil, and least induction found in animals fed beef fat or low levels of corn oil. These results are supported by those of Marshall and McLean (10), who reported that the addition of herring oil (a highly polyunsaturated oil) or linoleic acid to the diet allowed large increases in the concentration of cytochrome P-450 in response to phenobarbitone feeding. Addition of coconut oil (a highly saturated oil) to the diet did not permit this induction of cytochrome P-450.

In this paper, we have examined the influence of the type of dietary fat on the sleeping times of pentobarbitone-dosed rats. The concentrations of cytochrome P-450 have been determined in the livers of rats fed the diets with or without pentobarbitone pretreatment.

MATERIALS AND METHODS

Weanling female Wistar rats bred in the Animal Breeding Establishment of the Australian National University were maintained on a normal low fat laboratory rat pellet diet manufactured by Bunge Australia Pty. Ltd., Murrumburrah, NSW 2595. At 32 days of age, the rats were transferred to high fat diets containing either sunflowerseed oil or tallow. These diets were manufactured by Rural Chemical Industries Pty. Ltd., Glenorie, NSW 2157. The proportions of ingredients, proximate analyses, and fatty acid compositions of the high diets are given in Tables I-III, respectively. The principal fatty acids in the sunflowerseed oil diet were linoleic (59%) and oleic (27%) acids, and in the tallow diet were palmitic (28%), stearic (19%), and oleic (37%) acids. The high fat diets were stored at -15 C until used. There were no differences in growth rate, as assessed by body weights, between rats fed either of the high fat diets. All rats were allowed free access to food and water.

The fatty acid composition of the diets was determined on lipids extracted from the diets by the method of Folch et al. (11). Butylated hydroxytoluene was added to the extraction mixture to prevent lipid autoxidation (12). Fatty acid methyl esters, transesterified using 2,2-dimethoxypropane (13), were analyzed

TABLE I

Compositions of the High Fat Diets Containing Either Sunflowerseed Oil or Tallow[a]

Ingredient	Sunflowerseed oil diet	Tallow diet
Wheat	41.4	41.4
Dehydrated lucerne meal	5.5	5.5
Soybean meal	32.0	32.0
Dicalcium phosphate	0.5	0.5
Lime	2.5	2.5
Vitamin and mineral premix[b]	0.5	0.5
Methionine	0.1	0.1
Tallow	-	17.5
Sunflowerseed oil	17.5	-

[a]Values listed are percentage composition by weight.

[b]The vitamin and mineral premix supplemented the diets to the following amounts per kg total diet: vitamin A, 13,200 IU; vitamin D_3, 1320 IU; vitamin E, 53 IU; vitamin K_3, 4.9 mg; vitamin B_1, 12 mg; vitamin B_2, 27 mg; vitamin B_6, 12 mg; vitamin B_{12}, 12μg; vitamin C, 233 mg; biotin, 66μg; folic acid, 7.3 mg; nicotinic acid, 146 mg; pantothenic acid, 43 mg; p-aminobenzoic acid, 49 mg; inositol, 244 mg; choline, 480 mg; iron, 70 mg; copper, 20 mg; cobalt, 50μg; iodine, 1.6 mg; manganese, 60 mg; zinc, 60 mg.

using a gas chromatograph (model 1840, Varian Aerograph, Walnut Creek, CA) equipped with two 3 m x 1/8 in. stainless steel columns and dual hydrogen flame ionization detectors. One column was packed with 10% ethylene glycol succinate methyl silicone polymer (EGSS-X) on Gas-Chrom Q, 80-100 mesh, and the other with 3% dimethylpolysiloxane (JXR) on Gas-Chrom Q, 100-120 mesh. All column packing materials were obtained from Applied Science Laboratories Inc., State College, PA. Samples were analyzed using both columns and the individual fatty acid methyl esters identified by comparison with standards obtained from Nu-Chek-Prep Inc., Elysian, MN. The relative proportions of fatty acids were calculated from the product of the peak height and retention time.

Pentobarbitone-induced sleeping times and concentrations of hepatic microsomal cytochrome P-450 were determined in rats which had been fed either of the high fat diets for 4 weeks. Some of the rats received sodium pentobarbitone (0.1% w/v) ("Nembutal," Abbott Labs, Kurnell, NSW 2231) in the drinking water for 2 weeks before they were killed, and the amount of pentobarbitone consumed was estimated from the loss of water from the drinking bottles. There were no statistically significant differences between the amounts of pentobarbitone consumed per kg body weight by the two groups of rats. Each group of rats received ca. 52 mg pentobarbitone/kg body weight/day. Pentobarbitone-induced sleeping times were determined after each rat had received an intraperitoneal injection of sodium pentobarbitone (35 mg/kg body weight) in saline. The ability of rats to elicit two righting

TABLE II

Proximate Analyses of the High Fat Diets Containing Either Sunflowerseed Oil or Tallow[a]

Component	High fat diets
Protein	20.5
Fat	18.6
Fiber	4.1
Calcium	1.16
Phosphorus (available)	0.52
Phosphorus (total)	0.76
Methionine	0.40
Methionine and cysteine	0.80
Lysine (available)	1.11
Choline	0.18

[a]Values listed are percentage composition by weight.

TABLE III

Fatty Acid Composition of High Fat Diets[a]

Fatty acids	Sunflowerseed oil diet	Tallow diet[d]
14:0[b]	Trace[c]	3.2 ± 0.05
16:0	9.1 ± 0.03	28.0 ± 0.05
16:1	Trace	2.3 ± 0.05
18:0	3.5 ± 0.10	19.3 ± 0.05
18:1	27.2 ± 0.20	37.1 ± 0.15
18:2	59.0 ± 0.05	10.1 ± 0.05
18:3	1.2 ± 0.05	-

[a]Values listed are the mean ± standard error of two determinations on each of two samples of feed. Units are mol % of total fatty acids.

[b]Number of C atoms:number of double bonds.

[c]<0.5%.

[d]It was not possible to distinguish between the cis and trans isomers of the unsaturated fatty acids which are known to be present in tallow.

TABLE IV

Concentrations of Hepatic Microsomal Cytochrome P-450 in Rats Fed Diets Containing Either Sunflowerseed Oil or Tallow[a]

	Experiment 1		Experiment 2	
	Sunflowerseed oil diet	Tallow diet	Sunflowerseed oil diet	Tallow diet
Concentration of cytochrome P-450 (μmol/kg body weight)	0.59 ± 0.040	0.66 ± 0.043	0.44 ± 0.041	0.50 ± 0.041
Concentration of cytochrome P-450 (μmol/g microsomal protein)	0.74 ± 0.44	0.77 ± 0.042	0.61 ± 0.035	0.63 ± 0.041
Liver weight (g/kg body weight)	40.3 ± 0.62	41.1 ± 0.97	35.1 ± 1.07	36.5 ± 1.15
Microsomal protein (mg/g liver)	19.8 ± 1.06	21.0 ± 1.59	20.2 ± 1.13	21.7 ± 0.87

[a]Each value is the mean ± standard error of determinations on 15 animals.

TABLE V

Pentobarbitone-induced Sleeping Times of Rats Fed Diets Containing Either Sunflowerseed Oil or Tallow[a]

	Sunflowerseed oil diet	Tallow diet
Sleeping time (min)	63.0 ± 4.04	48.4 ± 2.71[b]

[a]Each value is the mean ± standard error of determinations on 23 animals.

[b]Comparisons by Student's *t*-test: $P<0.05$.

reflexes within a 30 sec period was used to indicate the loss or reappearance of consciousness (6).

Rats were killed by exsanguination and livers perfused in situ with ice cold KCl (1.15% w/v). A representative sample (2 g) of liver was homogenized in 8 ml of the KCl solution using a Potter-Elvehjem teflon-glass homogenizer. The homogenate was centrifuged (centrifuge Model RC2, Ivan Sorvall Inc., Norwalk, CT) at 9000 x g for 20 min to remove the cell debris and mitochondria. After the floating fat layer had been aspirated, the supernatant was decanted and centrifuged at 105,000 x g for 60 min in an ultracentrifuge (Model L3-50, Beckman Instruments Inc., Spinco Division, Palo Alto, CA). The resulting microsomal pellet was resuspended in 8 ml of the KCl solution and centrifuged again at 105,000 x g for 60 min. The microsomal pellet was resuspended in 8 ml of 0.1 *M* phosphate buffer at pH 7 and the suspension diluted with 0.1 *M* phosphate buffer to give a protein concentration of ca. 4 mg/ml. The concentrations of cytochrome P-450 were determined by the method of Omura and Sato (14) using a recording spectrophotometer (Model SP 8000, Pye Unicam Ltd., Cambridge, England). The concentrations of protein in microsomal preparations were determined using the method of Schacterle and Pollack (15), which was recommended by Albro (16) for use with microsomal preparations.

RESULTS

In Table IV are the results of Experiment 1, including the concentrations of cytochrome P-450 and microsomal protein and the liver

TABLE VI

Concentrations of Hepatic Microsomal Cytochrome P-450 in Rats Receiving Pentobarbitone in the Drinking Water and Fed Diets Containing Either Sunflowerseed Oil or Tallow[a]

	Sunflowerseed oil diet	Tallow diet
Concentration of cytochrome P-450 (μmol/kg body weight)	1.27 ± 0.039	1.63 ± 0.086[b]
Concentration of cytochrome P-450 (μmol/g microsomal protein)	1.43 ± 0.040	1.82 ± 0.069[b]
Liver weight (g/kg body weight)	42.9 ± 0.70	45.4 ± 0.89[c]
Microsomal protein (mg/g liver)	20.9 ± 0.65	19.6 ± 0.69

[a]Each value is the mean ± standard error of determinations on 15 animals.

[b,c]Comparisons by Student's *t*-test: [b]$P<0.01$, [c]$P<0.05$.

weights of rats not receiving pentobarbitone and fed either of the diets. The mean concentration of cytochrome P-450/kg body weight was greater in rats fed the tallow diet than in rats fed the sunflowerseed oil diet. This difference resulted from increases in the concentration of cytochrome P-450/g microsomal protein, the concentration of microsomal protein/g liver and the liver weights/kg body weight. However, none of these increases were statistically significant. This experiment was therefore repeated (Experiment 2) using a second group of rats, and the results are also presented in Table IV. The results of Experiment 2 confirmed those of Experiment 1. Although some loss of microsomes would be expected during the centrifugation of microsomal preparations, the comparisons of concentrations of microsomal protein/g liver and cytochrome P-450/kg body weight are valid as relatively large numbers of animals were used and similar losses would be expected in each sample. Similar comparisons of concentrations of cytochrome P-450 have also been made by other workers (7,10).

Pentobarbitone-induced sleeping times of rats which did not receive pentobarbitone in the drinking water and were fed either the sunflowerseed oil or the tallow diet are shown in Table V. These measurements were made on animals which were not used for the determination of concentrations of cytochrome P-450. Rats fed the sunflowerseed oil diet slept for significantly longer times than rats fed the tallow diet.

The concentrations of cytochrome P-450 and the liver weights of rats which received pentobarbitone and were fed either of the diets are shown in Table VI. The addition of pentobarbitone to the drinking water of these rats caused approximately a 2-fold increase in the concentration of cytochrome P-450/kg body weight. This increase was slightly greater in rats fed the tallow diet than in rats fed the sunflowerseed oil diet. The increase in the concentration of cytochrome P-450/kg body weight in rats fed the tallow diet resulted from increases in the concentration of cytochrome P-450/g microsomal protein and in the liver weights/kg body weight.

DISCUSSION

These results show that rats fed a diet containing tallow had greater concentrations of hepatic microsomal cytochrome P-450 than rats fed a diet containing sunflowerseed oil. As expected, rats with greater concentrations of cytochrome P-450 slept for shorter times after injection with pentobarbitone.

Other workers (8-10) have shown that the rate of microsomal metabolism and the concentration of hepatic microsomal cytochrome P-450 are increased when rats are fed diets containing polyunsaturated rather than more saturated fats. It is possible that either the degree of saturation of the dietary lipids or the propensity to form lipid peroxides was responsible for the results of these experiments (10). As we have taken particular care to reduce lipid peroxidation in our diets to a minimum, it is possible that greater amounts of lipid peroxides in the diets used by other workers may be responsible for the discrepancy between their results and the results presented here.

Attempts to rationalize the interaction of a wide range of substrates with a common cytochrome P-450 have led to the discovery of a multiplicity of cytochrome P-450 and the existence of substrate specificity (17). As dietary factors may preferentially influence one or more forms of the cytochrome, this topic requires further investigation. The effect of diet on several monooxygenase activities, such as the conversion of aniline to p-aminophenol and laurate to ω-hydroxylaurate, and on NADPH-cytochrome P-450 reductase would be of interest.

The activity of the microsomal enzymes may be an important factor in human carcinogenesis, as it is thought that as many as 90% of cancers seen in man are caused by environmental factors, mostly chemical (18). Many chemical carcinogens are converted into active carcinogenic derivatives by these enzymes (19). In addition to producing more active derivatives, these enzymes also produce less active derivatives (19). The balance between the activating and deactivating reactions is important in determining the carcinogenic effects of many compounds. This topic has been discussed more extensively elsewhere (20). A considerable number of in vivo experiments have shown that chemical carcinogenesis is inhibited by the induction of microsomal enzymes (21). The decreased microsomal enzyme activity of rats fed the sunflowerseed oil diet compared to rats fed the tallow diet may, therefore, have enhanced their susceptibility to the action of chemical carcinogens. Other work from this laboratory (22) has shown that the incidence of 7,12-dimethylbenz(a)anthracene-induced tumors is greater in rats fed the sunflowerseed oil diet than in rats fed the tallow diet. However, the activity of the microsomal enzymes may not have been important in determining the tumor incidence in these rats, as only the diet fed after DMBA administration influenced

the tumor incidence.

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Lipids of Some Thermophilic Fungi

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ABSTRACT

Total lipid content in the thermophilic fungi–*Thermoascus aurantiacus, Humicola lanuginosa, Malbranchea pulchella* var. *sulfurea,* and *Absidia ramosa*–varied from 5.3 to 19.1% of mycelial dry weight. The neutral and polar lipid fractions accounted for 56.4 to 80.2% and 19.8 to 43.6%, respectively. All the fungi contained monoglycerides, diglycerides, triglycerides, free fatty acids, and sterols in variable amounts. Sterol ester was detected only in *A. ramosa.* Phosphatide composition was: phosphatidyl choline (15.9-47%), phosphatidyl ethanolamine (23.4-67%), phosphatidyl serine (9.3-17.6%), and phosphatidyl inositol (1.9-11.9%). Diphosphatidyl glycerol occurred in considerable quantity only in *H. lanuginosa* and *M. pulchella* var. *sulfurea.* Phosphatidic acid, detected as a minor component only in *M. pulchella* var. *sulfurea* and *A. ramosa,* does not appear to be a characteristic phosphatide of thermophilic fungi as suggested earlier. The 16:0, 16:1, 18:0, 18:1, and 18:2 acids were the main fatty acid components. In addition, *A. ramosa* contained 18:3 acid. Total lipids contained an average of 0.93 double bonds per mole of fatty acids, and neutral lipids tend to be more unsaturated than phospholipids.

INTRODUCTION

The remarkable ability of thermophilic organisms to survive and grow at high temperatures is attributed either to their exceptional capacity to rapidly synthesize and replace cellular constituents that are destroyed or to the intrinsic stability of their biomaterials at elevated temperatures (1). Since many cellular functions are membrane linked, it is conceivable that lipid components of these organisms play a role in thermophily. However, lipids of thermophilic organisms have not been studied adequately. Mumma and coworkers (2-4) compared the fatty acid compositions of a number of thermophilic and mesophilic fungi and concluded that the fatty acids of thermophilic fungi are more saturated than those of corresponding mesophilic species. The neutral and phospholipid composition of only one thermophilic fungus, *Humicola grisea* var. *thermoidea,* was reported (5). A high concentration of phosphatidic acid was noted in this organism. It was suggested that the relatively high concentration of phosphatidic acid may be characteristic of thermophilic fungi. We have investigated the phospholipid compositions of four thermophilic fungi and report that the above generalization is untenable.

MATERIALS AND METHODS

Thermoascus aurantiacus and *Humicola lanuginosa* were isolated from soil and horse dung, respectively, using the temperature enrichment culture technique (1). *Malbranchea pulchella* var. *sulfurea* and *Absidia ramosa* were isolated from compost. *Thermoascus aurantiacus, Humicola lanuginosa,* and *Absidia ramosa* were cultured in 250 ml Erlenmeyer flasks containing 50 ml of medium of the following composition: 20 g glucose, 4 g L-asparagine, 1 g K_2HPO_4, and 0.5 g $MgSO_4 \cdot 7\ H_2O$ and 0.1 ml Vogel's trace element mixture (6) per liter at pH 7.3. *M. pulchella* var. *sulfurea* was cultured in 2% corn steep liquor supplemented with 2% glucose at pH 6.0. All organisms were grown in stationary culture. *T. aurantiacus, H. lanuginosa,* and *M. pulchella* var. *sulfurea* were harvested after 6 days of growth at 45 C. *A. ramosa* was grown both at 45 C and 29 C and harvested after 3 days of growth. Except *H. lanuginosa,* all fungi had begun sporulating. No attempt was made to harvest the fungi at a comparable stage of growth, but the culture period was sufficient to obtain maximum growth in all cases.

Mycelial mats were transferred to Buchner funnels, washed with distilled water, and blotted dry with filter paper. Dry weight was determined by keeping wet mycelia in a vacuum desiccator over KOH pellets until constant weight was attained. Lipids were extracted from the wet mycelia as described by Mumma et al. (2). The chloroform extract was evaporated to dryness under reduced pressure, and the residue was stored in chloroform: methanol (2:1 v/v) at 0 C. Neutral and polar lipid fractions were separated by chromatography on silicic acid. Samples (50 mg of total lipids) were applied on a 5 g column of silicic acid (Mallinckrodt, St. Louis, Mo). Neutral lipids were eluted with 50 ml of chloroform, and polar lipids were eluted with 50 ml of

TABLE I

Lipid Composition of Thermophilic Fungi

Organism	Mycelium extracted (dry wt [g])	Total lipids (% dry weight)	Neutral lipids[a] (% total lipids)	Polar lipids[a] (% total lipids)	Phospholipids[b] (% total lipids)
Humicola lanuginosa	0.81	19.1	80.2	19.8	8.9
Thermoascus aurantiacus	1.07	7.7	65.1	34.9	30.3
Malbranchea pulchella var. *sulfurea*	1.81	5.3	56.5	43.6	25.6
Absidia ramosa (45 C)	2.28	18.5	56.4	40.6	36.9
Absidia ramosa (29 C)	0.55	14.5	51.4	48.7	39.2

[a]Determined by weight after separation by silicic acid column chromatography.
[b]Obtained by estimating phosphorus in total lipid extract and multiplying by 25.

methanol. Gravimetric analysis indicated that essentially all the lipid applied on the column was recovered in the two fractions. Thin layer chromatography (TLC) was performed on 0.5 mm layers of Silica Gel C (ACME synthetic chemicals, Bombay, India). Neutral lipids were separated in a one dimensional double development system with hexane:ethyl ether:acetic acid (90:10:1 by vol) (7). Polar lipids were separated by two dimensional chromatography with chloroform:methanol:water:28% ammonia (130:70:8:0.5 by vol) as the solvent in the first direction and chloroform:aceton:acetic acid: methanol:water (100:70:20:20:10 by vol) as solvent in the second direction (5). Spots were visualized by exposure to iodine and identified by spraying with ninhydrin (7), Dragendorff (7), and phosphate reagents (8) and by comparison with authentic standards. For further confirmation, the polar lipid fractions were also resolved on silicic acid impregnated paper according to the procedure of Marinetti (9).

Dry weights were determined by keeping the samples in a vacuum desiccator over KOH pellets until constant weights were reached. Phosphorus in lipid samples as well as in the spots on TLC plates was estimated by Bartlett's procedure (10). Fatty acid methyl esters from neutral and polar fractions were prepared by refluxing the dried samples (5 mg) with 4.5 ml of 0.7 N methanolic HCl for 1 hr (11). Gas chromatography was carried out on a 6 ft column of 15% polyethylene glycol adipate at 200 C with nitrogen as carrier gas and flame ionization detector.

RESULTS

The total lipid content in the four species varied from 5.3 to 19.1% of dry weight (Table I). It may be noted that *A. ramosa* grown at 45 C contained a considerably higher amount of lipid than when it was grown at 29 C. Of the total lipids, neutral lipid content varied from 56.4 to 80.2% and polar lipid varied from 19.8 to 43.6% among the different thermophilic fungi (Table I). *A. ramosa* grown at 29 C contained less neutral lipid and more polar lipid compared to its composition when grown at 45 C. In all fungi, the quantity of neutral lipid was higher than that of polar lipid. These data suggested that both neutral and polar lipid composition is highly variable in thermophilic fungi. When the amount of phospholipid was estimated by determining lipid phosphorus in the polar lipid fractions, only a part of the polar lipids could be accounted as phospholipids (Table I). The major portion of

polar lipid fraction appears to be phospholipid in the case of *T. aurantiacus* and *A. ramosa,* but only 45.6 and 58.8% of polar lipid fraction is phospholipid in *H. lanuginosa* and *M. pulchella* var. *sulfurea,* respectively. Probably, the pigments present in these organisms contribute considerably to the polar lipid fractions of these organisms.

All the thermophilic fungi examined contained monoglycerides, diglycerides, and sterols (Fig. 1). *M. pulchella* var. *sulfurea* showed a higher concentration of monoglycerides and diglycerides than the other thermophilic fungi. Free fatty acids were present in notable amounts only in *H. lanuginosa* and in *A. ramosa* grown at 45 C and could not be detected in *T. aurantiacus* and *M. pulchella* var. *sulfurea.* Triglycerides were present in all organisms with some variation in concentration. Trace amounts of sterol esters were detected in *A. ramosa.* Small amounts of unidentified, fast-moving nonpolar components were present in *T. aurantiacus* and *H. lanuginosa.*

Figure 2 shows the separation of polar lipid components of *M. pulchella* var. *sulfurea* by two dimensional TLC. The principal components were identified as phosphatidic acid, phosphatidyl serine, phosphatidyl inositol, phosphatidyl choline, phosphatidyl ethanolamine, and diphosphatidyl glycerol by their reaction with various reagents. Besides the above components, a minor unidentified lipid spot was observed in *M. pulchella* var. *sulfurea.* Chromatography of polar lipid fractions on silicic acid-impregnated papers showed essentially the same pattern. In all the organisms studied, phosphatidyl choline and phosphatidyl ethanolamine are the most abundant phosphatides (Table II). Phosphatidyl serine and phosphatidyl inositol are the next most abundant phosphatides. Diphosphatidyl glycerol was found in notable quantities only in *H. lanuginosa* and *M. pulchella* var. *sulfurea* and was not detected in the other fungi. Phosphatidic acid was not detected in both *H. lanuginosa* and *T. aurantiacus,* and was only a minor component in *M. pulchella* var. *sulfurea* and *A. ramosa.* In *A. ramosa,* an increase in the concentration of phosphatidyl inositol and phosphatidyl choline with a concomitant decrease in phosphatidyl ethanolamine was observed at 29 C when compared to 45 C.

In all the fungi studied, 16:0, 18:1, and 18:2 acids are the most predominant fatty acid components with considerable amounts of 16:1 and 18:0 acids (Table III); 18:3 was found only in *A. ramosa.* No unusual fatty acid was observed in any of these organisms. The degree of unsaturation of fatty acids, calculated as the number of double bonds per mol of fatty acid (12), is comparable in the four thermophilic fungi studied. However, a distinct increase in unsaturation in total lipids was observed in *A. ramosa* when grown at 29 C. At this tempera-

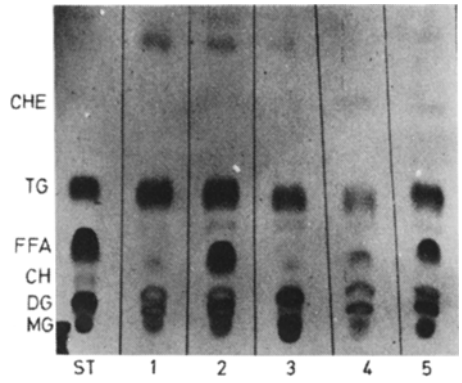

FIG. 1. TLC of neutral lipid fractions of thermophilic fungi. The plate was developed twice with the solvent system hexane:ethyl ether:acetic acid (90:10:1 by vol) and the spots were visualized by exposure to iodine. MG = monoglyceride; DE = diglyceride, CH = cholesterol, FFA = free fatty acid; TG = triglyceride, CHE = cholesterol ester, ST = standard, 1 = *Thermoascus aurantiacus*, 2 = *Humicola lanuginosa*, 3 = *Malbranchea pulchella* var. *sulfurea*, 4 = *Absidia ramosa* (29 C), 5 = *Absidia ramosa* (45 C).

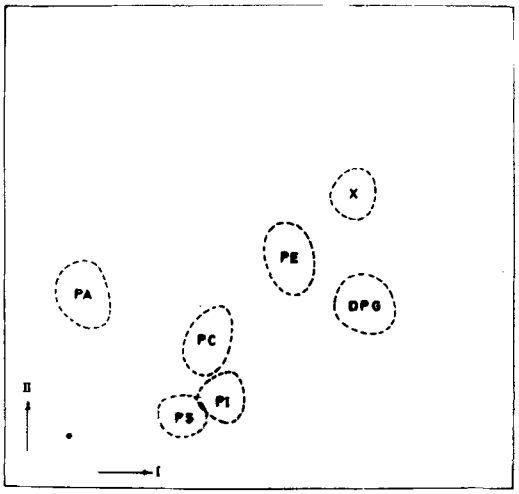

FIG. 2. Two dimensional TLC of polar lipids of *Malbranchea pulchella* var. *sulfurea.* The plate was developed in the I direction with chloroform:methanol:water:28% ammonia (130:70:8:0.5 by vol) and in the II direction with chloroform:acetone:acetic acid:methanol:water (100:70:20:20:10 by vol). Spots were visualized by exposure to iodine. PA = phosphatidic acid, PS = phosphatidyl serine, PI = phosphatidyl inositol, PC = phosphatidyl choline, PE = phosphatidyl ethanolamine, DPG = diphosphatidyl glycerol, X = unknown.

TABLE II

Phospholipid Composition of Thermophilic Fungi

Organism	Percent of total phospholipids						
	Phosphatidic acid	Phosphatidyl inositol	Phosphatidyl serine	Phosphatidyl choline	Phosphatidyl ethanolamine	Diphosphatidyl-glycerol	Unknown
Humicola lanuginosa	ND[a]	11.3	10.3	34.3	23.4	20.8	
Thermoascus aurantiacus	ND	7.9	11.0	47.0	33.8	ND	
Malbranchea pulchella var. *sulfurea*	1.0	6.7	17.6	36.3	30.4	7.0	0.7
Absidia ramosa (45 C)	5.8	1.9	9.3	15.9	67.0	ND	
Absidia ramosa (29 C)	6.2	11.9	11.3	29.8	40.2	ND	

[a]ND = not detected.

TABLE III

Fatty Acid Composition (%) of Total Lipids of Thermophilic Fungi

Organsim	14:0	16:0	16:1	18:0	18:1	18:2	18:3	Unsaturation (Δ/mol)
Humicola lanuginosa	0.4	19.5	1.8	6.0	52.3	20.5	-	0.950
Thermoascus aurantiacus	-	23.5	1.9	6.3	52.5	15.8	-	0.860
Malbranchea pulchella var. *sulfurea*	-	17.2	-	11.7	43.2	27.9	-	0.990
Absidia ramosa (45 C)	1.3	14.4	3.5	13.5	48.8	12.1	5.2	0.921
Absidia ramosa (29 C)	1.5	12.6	7.6	13.9	29.5	21.6	13.4	1.205

TABLE IV

Fatty Acid Composition (%) of Neutral and Polar Lipids of Thermophilic Fungi

Organism		12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	Unsaturation (Δ/mol)
Humicola lanuginosa	N[a]	-	0.4	10.2	-	5.2	59.5	24.7	-	1.089
	P[a]	-	-	25.1	2.0	7.0	47.8	18.2	-	0.862
Thermoascus aurantiacus	N	0.9	1.9	16.6	-	8.5	58.3	13.8	-	0.859
	P	-	-	29.4	1.2	1.6	53.2	14.6	-	0.836
Malbranchea pulchella var. *sulfurea*	N	-	-	14.7	1.6	9.3	46.2	28.0	-	1.038
	P	-	-	21.1	2.9	2.7	53.6	19.6	-	0.957
Absidia ramosa (45 C)	N	-	0.9	15.7	2.3	16.2	51.0	10.0	4.0	0.853
	P	-	1.0	19.9	3.1	3.0	47.2	14.9	10.9	1.128
Absidia ramosa (29 C)	N	1.5	2.6	21.4	8.8	15.9	20.5	18.4	11.0	0.991
	P	-	1.4	20.0	6.3	8.7	32.6	21.5	9.5	1.104

[a]N = neutral, P = polar.

ture, the polyunsaturates increased with a concomitant decrease in 18:1 acid. The fatty acid composition of neutral and polar fractions (Table IV) is similar to the composition observed with total lipids. The degree of unsaturation in the two fractions varied only marginally, and, in general, there was a tendency of neutral lipids to be more unsaturated than polar lipids. Only in *A. ramosa* did the polar lipids show higher unsaturation. Even in this organism, the increased unsaturation in fatty acids was in the neutral lipid fraction only when the culture temperature was decreased from 45 to 29 C.

DISCUSSION

The ability of fungi to adapt to a variety of environmental conditions, especially elevated temperatures, makes them versatile tools to understand the role of lipids in biomembranes. However, except for yeasts, relatively few studies are available on neutral and phospholipid composition of fungi in general (see review [13]) and thermophilic fungi in particular. Neutral and phospholipid composition of only one thermophilic organism, *H. grisea* var. *thermoidea,* has so far been reported (5). Such data are now presented on *T. aurantiacus, H. lanuginosa, M. pulchella* var. *sulfurea,* and *A. ramosa.* The effect of elevated temperature on lipid composition was also examined by growing the same strain of *A. ramosa* at two temperatures (29 C and 45 C).

The total lipid content varied from 5.3 to 19.1% of dry weight in the four organisms, which agrees with earlier reports (2,4). The total lipid content of *M. pulchella* var. *sulfurea* was found to be 5.3%, which is much less than the value (24.8%) reported by Mumma et al. (2). Thus it appears that the lipid content in different species of thermophilic fungi is highly variable and probably also depends on the culture conditions. The proportions of neutral and polar lipids also varied considerably in the four organisms studied, which agrees with the results of Mumma et al. (3,4). In all species, neutral lipids accounted for the bulk of the total lipid and contained monoglycerides, diglycerides, triglycerides, free fatty acids, and sterols in different quantities. Sterol esters could be detected only in *A. ramosa.* This pattern of neutral lipid distribution is similar to that found in *H. grisea* var. *thermoidea,* where, however, the sterol composition was more complex (5).

Phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, and phosphatidyl inosital are the major phospholipid components observed in the four species examined. Diphosphatidyl glycerol occurred in notable amounts only in *H. lanuginosa* and *M. pulchella* var. *sulfurea.* Phosphatidic acid could be detected only as a minor component in *M. pulchella* var. *sulfurea* and *A. ramosa.* Thus, the phospholipid composition found in this study is not in agreement with the report of Mumma et al. (5) on *H. grisea* var. *thermoidea.* In all four organisms studied, considerably higher amounts of phosphatidyl ethanolamine and phosphatidyl serine were observed. More importantly, phosphatidic acid was found only as a minor component in contrast to the nearly 30% reported in *H. grisea* var. *thermoidea.* Phosphatidic acid is usually found only in trace quantities in biological materials, and it is not surprising that it is a minor component in thermophillic fungi also. It must be pointed out that in this study the phosphatide composition was determined chemically while Mumma et al. (5) estimated radiochemically. It is possible that fungal species may vary in phospholipid composition, but it appears more likely that all phosphatides may not have achieved isotopic equilibrium with precursor P^{32} in the experiments of Mumma et al. (5), which would vitiate their quantitative estimation. It is known that incorporation of P^{32} phosphate into acidic phosphatides, particularly phosphatidic acid, is much faster than for other phosphatides, as has been shown with *Mycobacterium phlei* (14). Based on the higher quantitative distribution of phosphatidic acid in *H. grisea* var. *thermoidea* and an increase of phosphatidic acid in *Pythium ultimum* when the temperature was elevated from 20 C to 30 C (15), it was suggested that high concentrations of phosphatidic acid may contribute to membrane stabilization at elevated temperatures (5). Our data do not support this contention.

The fatty acid composition of total lipids as well as neutral and polar fractions was reported in a number of thermophilic and mesophilic fungi (2-4). Such data in the present study are in complete agreement with earlier findings and support the suggestion that saturated fatty acids predominate at elevated temperatures. In a given organism, neutral lipids seem to be more unsaturated than polar lipids, though there are exceptions such as *A. ramosa* for this generalization. Even in *A. ramosa,* increased unsaturation caused when the growth temperature was lowered was mainly in neutral lipids. Thus, it would appear that unsaturated fatty acids are preferentially incorporated into neutral lipids in thermophilic fungi.

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Lipid Content and Fatty Acid Composition of Heart and Muscle of the BIO 82.62 Cardiomyopathic Hamster

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ABSTRACT

Microscopic and analytical studies of the lipids in the heart and muscle of the BIO 82.62 myopathic hamsters and age-matched normal animals at the average ages of 33, 67, and 108 days were performed. Microscopic examinations did not show increased lipid depositions in the hearts of the diseased animals as was found in the BIO 14.6 strain. No consistent differences in the lipid content of the cardiomyopathic hamsters (BIO 82.62) and age-matched controls were observed in the three age groups except in the cholesterol content of muscle. Cholesterol increased significantly ($P<0.01$) in the 67 and 108 day old animals. This increase elevated the cholesterol/phospholipid ratio. Analysis of the fatty acid composition of triglycerides showed that the cardiomyopathic hamsters store more saturated fatty acids in both heart and muscle than do their normal counterparts. The abundance of more saturated fatty acids may imply that either the desaturation mechanism is altered in the diseased animals or that unsaturated fatty acids are preferentially utilized in other processes.

INTRODUCTION

The BIO 14.6 and other cardiomyopathic lines of the Syrian golden hamster provide a good model for investigative cardiology because the clinical course of the disease in these animals resembles certain nonvascular, myocardial diseases of man (1). Histological, morphological, and clinical investigations have yielded valuable information detailing and characterizing the progression of the disease in this animal model (2). Biochemical investigations have shown alterations in several vital structures and functions such as membrane structure (3), sarcoplasmic reticulum (4,5), oxidative phosphorylation (6,7), and lipid metabolism. There have been reports of an abnormally enhanced accumulation of intracellular lipid droplets in the hearts of these animals (8), depressed fatty acid oxidation (9), and changes in the activities of some of the enzymes that contribute to the synthesis of lipids (10). Since many of these studies were performed on animals in the advanced stages of the disease, it is difficult to differentiate between primary and secondary changes. Thus, we initiated a systematic study of lipid metabolism covering three stages in the life of the animals: age 33 days, 67 days, and 108 days. These ages approximate the three stages in the development of the myopathy (2).

Our initial observations showed a depression in the activities of several of the enzymes of de novo fatty acid synthesis in the liver and adipose tissue of the BIO 82.62 hamster throughout the above three stages (11). The present study was undertaken in order to determine quantitatively if there are any differences in the total lipid content of the heart and muscle of the cardiomyopathic hamsters (BIO 82.62) during the same stages. Such a quantitative study was seen as necessary because the reports of enhanced lipid accumulations were based mainly on histological observations of animals in the advanced stages of the disease.

METHODS

Animals

Both BIO 82.62 and randomly bred female hamsters were purchased from TELACO (Trenton Experimental Laboratory Animal Company, Bar Harbor, ME) when they were ca. 25 days old and were reared in our facilities under identical conditions. At an average age of 33, 67, and 108 days, 10 animals from the BIO 82.62 strain and 10 age-matched normal animals were sacrificed by decapitation, and the heart and the gastrocnemius muscle group were excised and suspended in ice cold 1.15% KCl. The tissues were then weighed and the apex of the heart was cut and preserved for histological work.

Histological Examinations

For the visualization of lipid droplets, the apex of the heart, from either the cardiomyopathic or randomly bred hamsters, was cut and immersed in 10% neutral buffered formalin. After formalin fixation, the tissue was frozen

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TABLE I

Relationship Between Age and Number of Lipid Droplets and Relative Estimate of *Lipid* Content in the Randomly Bred (RB) and the BIO 82.62 Strain (BIO)[a]

		30-35 Days		65-70 Days		105-110 Days	
		RB	BIO	RB	BIO	RB	BIO
Average number of drops per square[b]	1st Observer	9.1	7.8	8.6	8.1	10.5	9.0
	2nd Observer	7.3	4.0	7.6	7.3	7.1	5.4
	Average	8.2	5.9	8.1	7.7	8.8	7.2
Estimate quantity[c]	1st Observer	2.1	1.7	2.3	2.6	2.9	2.4
	2nd Observer	2.3	1.7	2.4	2.5	2.3	1.8
	Average	2.2	1.7	2.4	2.6	2.6	2.1

[a]Sections from the apex of the heart were cut and stained with oil red 0 stain.

[b]Random areas from each slide were examined, and the number of lipid droplets per square area, disregarding size, were counted.

[c]The quantity of lipids was rated on an arbitrary scale of 1-3, with 1 being a low, 2 a medium, and 3 a high amount of lipids.

TABLE II

Lipid Content of Hearts of Randomly Bred (RB) and Cardiomyopathic BIO 82.62 (BIO) Hamsters at Three Ages[a]

	30-35 Days		65-70 Days		105-110 Days	
Lipid class	RB	BIO	RB	BIO	RB	BIO
Total lipid	17.7 ± 1.0	19.1 ± 1.0	22.1 ± 0.8	21.7 ± 0.6	32.7 ± 2.3[b]	25.7 ± 1.8[b]
TG	4.2 ± 0.4	5.4 ± 1.2	3.1 ± 0.3	3.5 ± 0.3	5.9 ± 0.9[c]	3.1 ± 0.5[c]
Chol.	0.86 ± 0.08	0.85 ± 0.1	0.80 ± 0.03	0.77 ± 0.07	1.42 ± 0.07[d]	1.25 ± 0.05[d]
PL	14.95 ± 0.65[e]	18.20 ± 0.65[e]	15.60 ± 1.3	15.60 ± 0.65	22.10 ± 0.65	20.80 ± 0.65
Cardiolipin	1.20 ± 0.12[f]	1.68 ± 0.18[f]	2.04 ± 0.06	1.92 ± 0.12	2.76 ± 0.12	2.40 ± 0.18
PE	3.36 ± 0.18[g]	4.62 ± 0.18[g]	4.68 ± 0.30	4.32 ± 0.18	5.98 ± 0.24	5.58 ± 0.18
PC	5.67 ± 0.35	6.44 ± 0.42	6.30 ± 0.49	6.16 ± 0.28	7.70 ± 0.35	7.70 ± 0.28
Chol./PL PO_4	0.35 ± 0.03	0.30 ± 0.03	0.36 ± 0.03	0.34 ± 0.02	0.41 ± 0.02	0.39 ± 0.03

[a]The values shown are of the mean ± SEM of at least eight observations. Total lipid, triglyceride (TG), and cholesterol (Chol.) content are expressed as mg/g tissue. The total phospholipid (PL) content and that of the various phospholipid classes is expressed as mg phospholipid/g tissue (mg phospholipid/g wt, was calculated by assuming a mol wt of 650 for total PL, 600 for PE, 700 for PC, and 1200 for cardiolipin.

[b-g]Values with similar superscripts are statistically significant ($P<0.01$).

and cryostat sections were collected in distilled water and stained with oil red 0.

For the determination of the number of droplets and the estimation of the amount of lipids, two observers examined the slides when the identification markings were covered in order to minimize bias. The number of droplets in an area of 0.25 mm^2 at 100 x magnification was arrived at by examining five spots on each slide. The amount of lipids was estimated arbitrarily on a scale of 1-3, where 1 was given to areas of low lipid content, 2 for normal, and 3 for above normal.

Lipid Analysis

Lipids from the heart and muscle were extracted and separated as previously described (12). Total lipid and triglyceride content were determined by the acid-dichromate method (13,14), cholesterol by the sulfophosphovanillin reaction (15) as modified by Tapscott and Dohm (16), and phospholipids by the method of Bartlett (17) as modified by Kankare and Souvaniemi (18).

Gas Liquid Chromatography (GLC)

For GLC studies, lipid samples were spotted on thin-layer plates and separated in the nonpolar solvent system referenced above. Spots corresponding to triglycerides were scraped and eluted off the gel twice with diethyl ether. Saponification and methyl ester preparation were performed according to the procedures of Ast (19) and Metcalfe et al. (20), respectively. Fatty acid methyl esters were dissolved in CS_2 and then fractionated by GLC using a Perkin-Elmer 900 dual flame chromatograph equipped with 6 ft x 2 mm ID glass columns packed with 10% EGSS-X on 100/120 mesh Gas-Chrom Q (Applied Science Laboratories, State College,

TABLE III

Lipid Content of Muscles of Randomly Bred (RB) and Cardiomyopathic BIO 82.62 (BIO) Hamsters at Three Ages[a]

Lipid class[b]	30-35 Days		65-70 Days		105-110 Days	
	RB	BIO	RB	BIO	RB	BIO
Total lipid	20.2 ± 1.4	18.6 ± 1.2	22.4 ± 2.2	17.9 ± 0.8	21.2 ± 1.1	21.4 ± 1.6
TG	12.6 ± 1.3	11.5 ± 1.0	9.3 ± 1.6	5.6 ± 1.1	9.3 ± 1.5	9.5 ± 1.3
Chol.	0.61 ± 0.05	0.65 ± 0.05	0.51 ± 0.04[c]	0.79 ± 0.03[c]	0.55 ± 0.07[d]	0.81 ± 0.05[d]
PL	9.10 ± 0.52	7.80 ± 0.26	9.75 ± 0.65	9.75 ± 0.46	9.10 ± 0.52	10.40 ± 0.33
Cardiolipin	0.66 ± 0.12	0.54 ± 0.06	0.66 ± 0.06	0.66 ± 0.12	0.90 ± 0.12	0.90 ± 0.06
PE	1.86 ± 0.12	1.80 ± 0.06	1.80 ± 0.18	1.86 ± 0.18	1.92 ± 0.12[e]	2.34 ± 0.12[e]
PC	3.50 ± 0.28	2.80 ± 0.28	4.27 ± 0.35	4.06 ± 0.18	4.41 ± 0.14[f]	5.04 ± 0.14[f]
Chol./PL PO_4	0.45 ± 0.05	0.53 ± 0.04	0.35 ± 0.01[g]	0.54 ± 0.03[g]	0.43 ± 0.05	0.52 ± 0.02

[a]Conditions were as described in the legend to Table II.

[b]TG = triglyceride, PL = phospholipid, PE = phosphatidylethanolamine, PC = phospahtidylcholine.

[c-g]Values with similar superscripts are statistically significant ($P<0.01$).

TABLE IV

Relative Percent Fatty Acid Composition of Heart Triglycerides from Randomly Bred (RB) and BIO 82.62 (BIO) Hamsters[a]

Fatty acid	30-35 Days		65-70 Days		105-110 Days	
	RB	BIO	RB	BIO	RB	BIO
14:0	1.8 ± 0.4	1.5 ± 0.3	1.2 ± 0.1	1.4 ± 0.4	0.8 ± 0.1	0.6 ± 0.1
16:0	24.3 ± 2.6	26.4 ± 2.5	18.5 ± 1.0	22.0 ± 2.4	19.2 ± 1.9[b]	23.9 ± 1.3[b]
16:1	5.1 ± 1.2	4.5 ± 0.8	2.1 ± 0.3	2.1 ± 0.3	2.8 ± 0.4	1.9 ± 0.4
18:0	7.9 ± 0.5	7.6 ± 0.8	5.8 ± 0.6[c]	7.7 ± 0.6[c]	5.3 ± 1.0	6.4 ± 0.7
18:1	32.4 ± 3.6	29.5 ± 0.5	30.3 ± 1.3[d]	23.0 ± 2.3[d]	33.3 ± 1.2[e]	27.2 ± 0.5[e]
18:2	24.1 ± 4.3	25.0 ± 4.3	36.3 ± 1.5	35.4 ± 1.5	34.8 ± 1.8	36.8 ± 2.3
18:3	1.5 ± 0.6	2.6 ± 0.3	3.1 ± 1.0	1.6 ± 0.5	2.3 ± 0.4	1.7 ± 0.2
Others	2.9 ± 0.4	3.0 ± 0.5	2.6 ± 0.5	2.4 ± 0.4	1.5 ± 0.2	1.5 ± 0.1

[a]Fatty acid methyl esters were prepared and fractionated as described in Methods. The area under all the peaks was taken as 100%, and the area of individual methyl esters was calculated as a percentage of that. Values are expressed as the mean ± SEM of at least five observations. For statistical analysis, percentages were converted to arcsins (23), and Neuman-Keuls (24) analysis were performed.

[b-e]Values with similar superscripts are statistically signficant ($P<0.01$).

PA); detector temperature, 220 C; column temperature, 198 C; injector temperature, 220 C; and the flow rate of carrier gas (N_2), 30 ml/min.

RESULTS

Table I shows the results of the microscopic examinations of heart apex sections that were stained specifically for lipids. Despite the subjectivity of the estimation of the lipid content, the observations of the two observers were surprisingly very close. Both observers found that the average number of droplets per square area was similar in the cardiomyopathic and normal hamsters. Similarly, the estimated quantity of lipids was not different in the two animal strains (Table I).

Tables II and III, respectively, summarize the results of lipid analysis of heart and muscle of the cardiomyopathic and age-matched hamsters at three stages in their development. At an average age of 33 days, the cardiomyopathic hamster hearts (Table II) showed a significant ($P<0.01$) increase in total phospholipids, cardiolipin, and phosphatidylethanolamine. There was no significant difference in the concentration of any of the lipids during the second stage (age 67 days). In the third age group (108 days), there was a significant ($P<0.01$) decrease in total lipids, triglycerides, and cholesterol in the cardiomyopathic hamsters but no change in the amounts of phospholipids. There was no significant difference in the cholesterol/phospholipid ratio between the diseased animals and their normal counterparts.

The results of muscle lipid analysis are summarized in Table III. No difference between the proportions of lipid classes in the two strains was observed. In the second stage, only

TABLE V

Relative Percent Fatty Acid Composition of Muscle Neutral Lipids from Randomly Bred (RB) and BIO 82.62 (BIO) Hamsters[a]

Fatty acid	30-35 Days		65-70 Days		105-110 Days	
	RB	BIO	RB	BIO	RB	BIO
14:0	1.1 ± 0.2[b]	2.2 ± 0.3[b]	1.1 ± 0.1	1.2 ± 0.3	1.7 ± 0.3	1.1 ± 0.8
16:0	22.9 ± 0.7	29.1 ± 3.1	23.9 ± 0.8	26.4 ± 0.8	24.3 ± 2.0	22.1 ± 2.2
16:1	8.4 ± 0.3	7.6 ± 0.5	7.0 ± 0.7[c]	4.4 ± 0.6[c]	7.9 ± 0.5[d]	4.1 ± 0.8[d]
18:0	3.7 ± 0.2	4.0 ± 0.2	3.6 ± 0.3[e]	4.9 ± 0.2[e]	3.4 ± 0.3[f]	4.5 ± 0.2[f]
18:1	38.1 ± 0.4[g]	33.0 ± 2.3[g]	36.4 ± 0.6[h]	30.7 ± 1.3[h]	34.8 ± 0.8	33.9 ± 1.9
18:2	22.5 ± 0.8	21.5 ± 1.2	25.5 ± 0.4[i]	29.4 ± 1.5[i]	25.1 ± 2.0[j]	31.4 ± 2.0[j]
18:3	2.4 ± 0.1	1.8 ± 0.4	2.1 ± 0.3	2.1 ± 0.4	2.1 ± 0.7	1.8 ± 0.3
Others	0.9 ± 0.1	0.8 ± 0.3	0.6 ± 0.1	1.0 ± 0.2	1.1 ± 0.3	1.1 ± 0.2

[a]Conditions were as described in the legend to Table IV.

[b-j]Values with similar superscripts are statistically significant ($P<0.01$).

the cholesterol content was significantly elevated ($P<0.01$). During the third stage, there was a significant ($P<0.01$) increase in the amount of cholesterol, phosphatidylethanolamine, and phosphatidylcholine in the cardiomyopathic hamsters. Such changes elevated the cholesterol/phospholipid phosphorous ratio in the diseased animals.

The relative fatty acid composition of triglycerides from the hearts of the cardiomyopathic and the randomly bred hamsters is shown in Table IV. No significant differences were observed at age 33 days. During the second stage, stearate was significantly higher ($P<0.01$) but oleate significantly lower ($P<0.01$) in the hearts of the diseased animals. At age 108 days, palmitate was elevated but oleate was depressed.

Table V shows the distribution of fatty acids of triglycerides from muscle. During the first stage, myristate was elevated and oleate depressed. Palmitoleate and oleate were depressed, but stearate and linoleate were elevated during the second stage. In the last stage, palmitoleate was depressed but stearate and linoleate were elevated significantly ($P<0.01$) in the cardiomyopathic hamsters.

Analyses of the fatty acid composition of total phospholipids from heart and muscle of the same age groups were performed. No significant changes between the diseased animals and the age-matched controls were found.

DISCUSSION

The reports of the accumulation of lipid droplets observed microscopically during the early (8) and later (10) stages in the development of the myopathy in the BIO 14.6 line of the cardiomyopathic hamster prompted this investigation into the nature and the possible causes of such abnormal accumulations. Furthermore, to investigate the initiation and duration of such accumulations, a systematic study of the lipid content of heart and muscle of this animal model throughout the active phases of the disease was necessary.

Examination of heart preparations stained specifically for lipids (red 0 stain) showed that there were no differences between the diseased animals and normal controls in either the number of lipid droplets or the amount of lipids when measured by the subjective scales used (Table I). Chemical analysis of total lipids from the hearts of the cardiomyopathic hamsters and age-matched normal controls showed essentially the same results as the microscopic examinations. No differences in the total lipid content were found in the first two age groups (age 33 and 68 days), but the total lipid content was significantly ($P<0.01$) depressed in the hearts of 108-day old BIO 82.62 hamsters. The depression in total lipids is the result of a significant ($P<0.01$) drop in the triglyceride and cholesterol content of the same animals since the phospholipid content was unchanged. Such alterations in the concentrations of these lipids may be related to other alterations occurring in other organs. We previously reported (11) a significant ($P<0.05$) decrease in the activities of several of the enzymes of de novo fatty acid synthesis in the liver and adipose tissue of the diseased animals. It is conceivable that the depression in lipogenesis might result in a relative and concomitant depression in triglyceride synthesis in these and other organs. This could be possible if the concentration of fatty acids becomes a limiting factor, thus reducing the rate of glyceride synthesis particularly in the later stages of the myopathy. It is obvious that more work is needed to verify these questions.

Electron microscopic studies of premyolytic areas in the heart of the BIO 14.6 line reported

by Paterson et al. (10) showed hypertrophy and hyperplasia of the mitochondria. In later stages, many mitochondria showed vacuolation which increased with the progression of the lesions (10). Such changes in the mitochondrial structure could possibly be related to some of our findings. The increase in total phospholipid, phosphatidylethanolamine, and especially the cardiolipin content (Table II) of the cardiomyopathic hamsters at age 33 days could be due to the increase in the number and size of mitochondria that was reported to occur at approximately the same time (10). The lack of increase in second and third stages may be a reflection of increased vacuolation and destruction of mitochondrial membranes.

No major changes in the lipids of the muscle of the diseased animals were observed in the first age group (Table III). During the second and third stages, however, there was a significant ($P<0.01$) rise in the cholesterol concentration of muscle from the BIO 82.62 hamsters. The rise in the cholesterol concentration of muscle resulted in a relative increase in the cholesterol/phospholipid ratio in this tissue. Elevation in the cholesterol/phospholipid ratio was reported to occur in both the skeletal and cardiac muscle of the cardiomyopathic hamsters (3,21,22). At variance with these reports are our findings of no significant changes in the cholesterol/phospholipid ratio in the hearts from the three age groups of the BIO 82.62 animals. One possible reason for this discrepancy is age. Owens et al. (3) have reported no change in the cholesterol/phospholipid ratio in 30-day old BIO 14.6 hamsters, but this ratio was elevated in the hearts of 230-day old hamsters. Similar findings were reported by Borowski et al. (22) in UM-X7.1 strain of cardiomyopathic hamsters with an advanced degree of myopathy. Since the oldest animals that we examined were 110 days old, it is possible that alterations in this ratio may occur later.

The fatty acid composition of triglycerides from heart and muscle (Tables IV and V) is noteworthy. There is an apparent trend toward more saturated fatty acids in both the heart and muscle of the BIO 82.62 hamsters. This trend is more prominent in muscle where 16:1 and 18:1 are lower but 16:0 and 18:0 higher in the diseased animals. The increase in the amounts of saturated fatty acids in these two tissues of the cardiomyopathic hamster could be a result of altered (decreased) activity of desaturases or because of preferential utilization of the unsaturated fatty acids in other metabolic processes. In either case, there is a lesser amount of unsaturated fatty acids available for esterification.

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Determination of Background Serum Density for Lipoprotein Ultracentrifugation

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ABSTRACT

It is necessary to know the density of serum exclusive of its macromolecules (background density) prior to density adjustment with solid potassium bromide for ultra-centrifugal separation of lipoprotein fractions. To evaluate this, we compared the densities of the corresponding ultrafiltrates or dialysates of both human and equine sera produced by ultrafiltration and equilibrium dialysis methods for macromolecule removal. Excellent correlation is found between background densities determined following ultrafiltration or equilibrium dialysis. These data validate the use of ultrafiltration as a simple, direct method for determination of background serum densities but reveal equilibrium dialysis to be more time consuming and less precise. Using ultrafiltration, we find the background density for equine serum to be 1.004 g/ml, and initial investigation suggests this value may be altered by freezing, prolonged refrigeration (3 months), or heating to inactivate lecithin:cholesterol acyltransferase.

INTRODUCTION

Ultracentrifugal separation of lipoprotein fractions has become routine, but many methods are presently used to adjust serum density (1). With small serum samples, when dilution must be kept to a minimum, the solid potassium bromide density adjustment is preferable (2). This procedure requires that the density of the serum exclusive of its macromolecules (background density) be known. For human serum, the value of 1.006 g/ml is generally used, but values for other species are not apparent in the literature (1). Furthermore, concise methods to determine background serum densities are not described. Theoretical considerations suggest that both ultrafiltration and equilibrium dialysis should provide satisfactory determinations, because the 10,000-12,000 mol wt cut-off produced by either method virtually eliminates all major serum proteins. In practice, equilibrium dialysis proves time consuming, may require precise volume controls, and necessitates a greater number of density measurements. We find ultrafiltration to be simple and direct, making speed and precision important requirements for evaluation.

MATERIAL AND METHODS

Equine test subjects were kept on a constant dietary regimen for several months prior to study, and human test subjects were clinically normal, with stable body weight, and receiving no medication. All subjects fasted for 16 hr prior to donating blood. Fasted blood samples were allowed to clot, and the resulting serum was drawn off and placed in screw-top vials. Fresh serum samples were kept at 4 C and used within 48 hr. Fresh samples and those frozen or refrigerated samples that were stored for future study did not receive any merthiolate or EDTA that might alter background density. For ultrafiltration, a Diaflo Model 65 Cell and UM10 filters (Amicon Corporation, Lexington, MA) were used. These have a 10,000 mol wt cut-off. Cellulose dialysis tubing (VWR) was used for equilibrium dialysis. This tubing has a 12,000 mol wt cut-off. A MicroMettler Westphal apparatus (Mettler Corporation, Princeton, NJ) was used for specific gravity (density) determinations with a theoretical precision of ± 0.0004 g/ml (± standard error of mean density produced by a maximized weighing error of 0.001 g) using 25 ml samples (3). All measurements were made at 20 C. The method of Robie et al. was used for polyacrylamide disc-gel electrophoresis (4). Ultrafiltration and equilibrium dialysis were evaluated by separate background density determinations for at least three fresh equine serum samples. A single sample was evaluated for the equine background alteration and human serum samples considered.

Ultrafiltration

A 60 ml sample volume was used, and a 25 ml filtrate was collected. New filters were used for each determination, with 2 ml of the initial filtrate being discarded to prevent dilution from prior filter wash. Ultrafiltrations were run at 40 lb/sq. in. and 4 C for 2-5 hr. The clear background material passing through the filter was

collected and its density determined as described above.

Immunodiffusion

The detectable presence of interfering serum proteins in the ultrafiltrate was ruled out by immunodiffusion in Ouchterlony plates (5). One percent agarose in barbital buffer at pH 8.2 served as the diffusion medium. Rabbit antiserum to equine serum proteins was obtained from Grand Island Biological Co. (Grand Island, NY). This antiserum demonstrated sensitivity to equine serum diluted 1:1000, the lowest dilution evaluated since any less protein would have no measurable effect on density. No serum proteins were detected in any ultrafiltrate samples.

Equilibrium Dialysis

Aliquots of the same serum samples used in ultrafiltration were also evaluated by equilibrium dialysis. Ten milliliter serum samples were placed in dialysis tubing and suspended in beakers of distilled water covered with Parafilm (American Can Company, Greenwich, CT). Small stirring magnets were used to ensure adequate mixing of the distilled water. A 6:1 (water: sample) ratio provided a convenient working system. All dialysis experiments were allowed to proceed for 48 hr at 4 C to ensure attainment of equilibrium.

Background density of the serum was calculated from the density change of the distilled H_2O as follows:

Background density of fresh equine samples was also determined by equilibrium dialysis against a NaCl solution of density 1.004 g/ml. This dialysis procedure first described by Lee and Alaupovic (6) served as an additional reference method but presumes that an estimation of the background density value is known. The same procedure for equilibrum dialysis against water was used for the NaCl solution dialysis, except that 25 ml serum samples were dialyzed against 100 ml of NaCl solution.

Background density of the serum was calculated as follows:

$$\text{Background density} = (d_{\text{serum}}) - (d_{\text{dialyzed serum}} - d_{\text{dialysate}})$$

RESULTS AND DISCUSSION

We find excellent correlation between the ultrafiltration and equilibrium dialysis results (Table I). The ultrafiltration method is precise (±0.0002 g/ml), rapid, and direct, requiring a single density determination. Immunodiffusion indicates removal of any interfering serum protein by the ultrafiltration process. Small volume changes are incurred by both equilibrium dialysis techniques, with the NaCl solution dialysis minimizing this effect. Equilibrium dialysis against water requires two density and two volume determinations, while that against a NaCl solution requires four density determinations. However, identical results were found in either case (Table I, footnote f). Both equilibri-

1. $(d_{\text{dialysate at equilibrium}}) - (d_{H_2O \text{ initial}}) = \Delta\, d_{H_2O}$

2. $(\Delta\, d_{H_2O}) \times (V_{\text{dialysate}}) = (\text{weight of background material in dialysate})$

3. $(\text{weight of background material in dialysate}) \,/\, (\text{fraction of total background material that is in dialysate at equilibrium}) = (\text{total weight of background material in original sample})$

4. $(\text{total weight of background material in original sample}) \,/\, (V_{\text{sample initial}}) = (\text{actual density of background material})$

5. $(\text{actual density of background material}) + (d_{H_2O \text{ initial}}) = d_{\text{background}}$

Equations 1 through 5 can be reduced to:

$$\frac{(d_{H_2O}) \times (V_{\text{dialysate}} + V_{\text{sample}})}{(V_{\text{sample}})} + (d_{H_2O \text{ initial}}) = d_{\text{background}}$$

where $V_{\text{dialysate}} \cong$ original H_2O volume; V_{sample} = initial sample volume.

TABLE I

Background Serum Density of Human and Equine Samples Determined by Ultrafiltration and Equilibrium Dialysis

Sample	Ultrafiltration (g/ml at 20 C)[a]	Equilibrium dialysis against water (g/ml at 20 C)[b]
Fresh equine serum	1.004 ± 0.0002	1.005 ± 0.0010[f]
Fresh human serum	1.006 ± 0.0002	1.007 ± 0.0010
Equine serum, heat-inactivated[c] and refrigerated (3 months)[d]	1.006 ± 0.0002	1.009 ± 0.0010
Equine serum, heat-inactivated and frozen (3 months)[e]	1.009 ± 0.0002	–
Equine serum refrigerated (3 months)	1.006 ± 0.0002	–
Equine serum, frozen (1 week)	1.006 ± 0.0002	–
Fresh equine serum, heat-inactivated	1.006 ± 0.0002	–

[a]Mean ± standard error of mean based on separate determinations of three fresh equine samples for ultrafiltration.

[b]Mean ± standard error of mean based on separate determinations of three fresh equine samples for equilibrium dialysis.

[c]All heat inactivation at 60 C for 25 min.

[d]Refrigeration at 4 C.

[e]Frozen storage at −25 C.

[f]Mean ± standard error of mean based on separate determinations of four fresh equine samples for NaCl solution dialysis produced this same value.

um dialysis methods are inherently less precise (±0.0010 g/ml) than ultrafiltration but still provide comparable results. As might be expected, the 2,000 mol wt higher cut-off of the equilibrium dialysis membrane produces slightly higher background density values than the UM10 filters of the ultrafiltration procedure. In equilibrium dialysis, the Donnan Effect is ignored and proves of no apparent significance.

Heat inactivation (60 C for 25 min) may slightly elevate background density of a serum sample. This heat inactivation has routinely been used to inactivate the lecithin:cholesterol acyltransferase enzyme (EC 2.3.1.43) (7). Polyacrylamide disc-gel electrophoresis showed an increase in staining material at the origin with heat-inactivated samples which indicated electrophoretic alteration of serum proteins (4). Freezing also appears to elevate background density, perhaps paralleling the reported modification of lipoproteins by freezing (1,8). In addition, prolonged storage (3 months) of serum samples at 4 C produces a small increase in background density.

These background density alterations produced by various serum treatments introduce errors in the 3-9% range (0.002-0.005 g/ml for a 0.057 g/ml adjustment) in initial KBr density adjustment. This error will be cumulative in all subsequent density adjustments to a specific sample. Appreciable changes in the nature of the serum and lipoproteins may be reflected in these background elevations. Extremes of serum treatment may cause denaturation of liproproteins, resulting in random unfolding of the apoprotein and subsequent modification of native properties. Elevation of background density might be produced by the liberation of ions from denatured protein complexes, which remain complexed and are removed by filtration from unaltered serum.

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Structure-Function Activity of Azasterols and Nitrogen-containing Steroids

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ABSTRACT

Thirty-nine nitrogen-containing steroids were tested against two gram-negative, five gram-positive, and two yeast organisms. Many of these steroids have been previously reported to inhibit various metabolic processes involving sterol metabolism. While low minimal inhibitory concentration (MIC) values were recorded for sterol producing yeast, growth of bacteria which contain no sterols was also inhibited. Structure-function studies provided no relationship between biological activitiy and hypocholesteremic effects of these azasteroids. A hypothesis put forward is that amino and azasteroids are effectors of membrane which, in the case of mitochondria, lead to changes in adenosine triphosphate levels and/or dehydrogenase activity. Their effects on sterol metabolism, therefore, may be of secondary consideration.

INTRODUCTION

In our attempts to elucidate the mechanism of action of hypocholesteremic drugs, simple model systems have been examined. This has resulted in reports on the effects of such compounds on mitochondria (1) and microorganisms (2; J.J. Kabara, unpublished data). Because two hypocholesteremic compounds, 20,25-azacholesterol (Kabara, unpublished data) and 3 β-(β-dimethylaminoethoxy)-androst-5-en-17-me (3), have been reported to suppress growth of microorganisms, it was important to further explore this relationship with other N-steroid derivatives. There is considerable evidence that a large number of steroids have some antimicrobial effects in vitro on bacteria, molds, yeasts, and protozoa (4,5).

Bacteria, except for mycoplasm, neither use nor synthesize sterols (6). Therefore, the mechanism by which some N-steroids, which are also hypocholesteremic agents, could effect sterol levels and also effect bacteria is discussed. It is hoped that such studies will uncover some structure-function relationships which may lead to a better understanding of the mechanism of hypocholesteremic drugs and/or the discovery of new antimicrobial agents.

MATERIALS AND METHODS

Steroids

The nomenclature and structure of each steroid is given in the accompanying tables. The azasteroid samples were generously supplied by the following: R.E. Counsell (University of Michigan), R. Ranney (G.D. Searle Laboratories), G.B. Whitfield (The Upjohn Company), P. Catsoulacos (Nuclear Research Center, Greece), and J.A. Svoboda (ARS, USDA, Beltsville, MD).

One hundred milligrams of steroid were dissolved in 0.5 ml of absolute methanol. To this volume of alcohol, 100 ml of Trypticase soy broth (TSB:BBL) was added aseptically. If the resulting solution was not clear, the suspension was carefully heated (ca. 60 C) to increase drug solubility. Routinely standard solutions (or suspensions) containing 1,000 μg/ml were diluted tenfold with additional sterile broth.

Organisms

The microorganisms used for our screening studies were those frequently encountered in clinical specimens. In general, the clinical isolates (CI) are more resistant to chemical inactivation than those registered with the American Type Culture Collection (ATCC). The clinical isolates were identified and kindly made available to us by Dr. Joseph P. Truant. The following gram-negative organisms were used: *Escherichia coli* (Ec) and *Pseudomonas aeruginosa* (ATCC-10145) (Pa). The gram-positive organisms used were *Streptococcus faecalis* (Group D) (Sf), *Streptococcus pyogenes* (Sp), *Staphylococcus aureus* (Sa), *Corynebacterium sp.* (ATCC-10700) (Csp), and *Nocardia asteroides* (ATCC-3308) (Na). *Candida albicans* (Ca) and *Saccharomyces cerevisiae* (Sc) were examples of yeast isolates.

Screening Technique

A test inoculum consisted of 0.05 ml of an 18-24 hr TSB culture (ca. 10^9 organism/ml). The inoculum was aseptically delivered into all dilutions of the compound, well mixed, and incubated at 36 C in a 5% CO_2 plus 95% air

TABLE I

Minimal Inhibitory Concentration (μg/ml) of Monoaza Sterols

Microorganisms	20-Aza	22-Aza	23-Aza	24-Aza	25-Aza	25-Aza Coprostane	25-Aza Δ^8-Lanostenol
Escherichia coli	NI[a]	NI	NI	NI	NI	NI	NI
Pseudomonas aeruginosa	--	NI	NI	NI	NI	NI	--
Streptococcus faecalis (Group D)	32	25	NI	1,000	1,000	100	32
Streptococcus pyogenes	--	25	NI	10	50	10	--
Staphylococcus aureus	250	25	NI	100	1,000	10	NI
Corynebacterium sp.	--	12.5	5	100	50	100	--
Nocardia asteroides	--	25	25	100	250	100	--
Candida albicans	--	12.5	2.5	100	50	NI	NI
Saccharomyces cerevisiae	NI	5	2.5	10	10	10	--

[a]NI = non-inhibitory at 1,000 μg/ml.

atmosphere. A tube of inoculated broth without drug served as a positive control; also, an uninoculated set of drug solutions was incubated. After 18 hr of incubation, the minimal inhibitory concentration (MIC) of each compound against each organism was determined. In our study, the MIC is defined as the lowest concentration of compound at which no macroscopic evidence was observed when turbidity of the inoculated broth dilutions was compared with that of the control tubes.

In those cases in which the test compound itself caused turbidity so that the MIC could not accurately be determined, a sample (0.015 ml) of the well-agitated broth in question was inoculated onto a Trypticase soy agar plate containing 5% defibrinated sheep blood, incubated at 35 C, and examined after 24 and 48 hr for bactericidal end points.

It was found that turbidity owing to the compounds did not "confuse the readings" since most of the compounds were inhibitory at low concentrations where solubility was almost complete.

The pH of the broth was monitored throughout the study by the use of an Accutint set (Anachemia, Montreal, Quebec, Canada) and was found to be within the range of 7.3 ± 0.2. Also, at the concentration used, methanol was found not to be inhibitory, as demonstrated by controlled test experiments.

RESULTS

Data for seven monoazasterols are presented in Table I. Because of limited quantities of compounds available, it was not always possible to screen against all of the organisms in the test. The first five compounds are monoazacholesterol derivatives, while the last two compounds are 25-azacoprostane and 25-aza-Δ^8-lanostenol. The effect of changes in azasteroid structure to antimicrobial activity was partially dependent upon the type of organism used in the challenge screen. Despite the lack of a general structure-function relationship, a few generalities can be commented upon. The 22-azacholesterol was the most active of the seven monoaza derivatives screened. In general, the yeasts are more affected than are the gram-positive organisms. The gram-negative organisms used in our screen were not affected.

The last two sterol derivatives (25-azacoprostane and 25-aza-Δ^8-lanostenol) indicated that changes in sterol structure could be detected in our microbiological system. The loss of the 3 β-OH group, a change in the stereochemistry of the A/B ring, and the hydrogenation at position 5 and 6, as in the 25-azacoprostane, did

TABLE II

Minimal Inhibitory Concentration (μg/ml) of Azacholesterol derivatives[a]

Microorganisms	20,25-Diaza-cholesterol I	25-Nor II	20-Nor III	20-CHO-25-Nor IV	25-Aza-N-oxide V
Escherichia coli	1,000	NI	1,000	NI	NI
Pseudomonas aeruginosa	NI[b]	NI	NI	NI	NI
Streptococcus faecalis (Group D)	1,000	500	1,000	1,000	NI
Streptoccus pyogenes	250	125	250	100	NI
Staphylococcus aureus	1,000	500	1,000	125	NI
Corynebacterium sp.	250	100	250	25	1,000
Nocardia asteroides	500	250	500	250	NI
Candida albicans	25	25	50	50	1,000
Saccharomyces cerevisiae	10	5	12.5	25	500

[a]I = 17β-[[3-(dimethylamino)propyl]methylamino] androst-5-en-3β-ol, II = 17β-[methyl[3-(methylamino)propyl]amino]androst-5-en-3β-ol, III = 17β-[[3-(dimethylamino)propyl]amino]androst-5-en-3β-ol, IV = N-[3-(methylamino)propyl]-N-(3β-hydroxy-androst-5-en-17β-yl), V = 25-azacholesterol, N-oxide, hydrate.

[b]NI = non-inhibitory.

not lead to a loss of antimicrobial activity. In fact, the coprostane derivative was more active than the cholesterol derivatives. This indicated that unsaturation at Δ^5, the configuration of the A/B ring, and the presence of the 3 β-OH group were not necessary for antimicrobial activity.

In a second series of experiments, a number of simple structural changes were investigated (Table II). The model compound, 20,25-diazacholesterol, showed similar but less activity than 25-azacholesterol. Since the 20-aza derivative was not very active, the aza group in position 25- takes on added importance. This can also be seen when the 25-aza derivative is changed to an N-oxide. In this case, almost all the activity is lost.

The removal of an N-methyl group from either position 20- or 25- has little effect on the parent compound activity.

The mild oxidation of the methyl group at position 20- and the removal of the methyl group at 25- to form a (N-[3-methyl-aminopropyl]-N-[3 β-hydroxy-androst-5-en-17β-yl])-formamide also was without effect on steroid antimicrobial activity.

Data from Table II indicates that while the methyl group at position 20- or 25- was not necessary for compound antimicrobial activity, the aza group at position 25- is very essential.

A group of homo-azasteroidal esters were also screened for antimicrobial activity (Table III). Except for very slight activity shown by a 17-azasteroid (PC 3), none of these compounds exhibited any antimicrobial activity.

In an attempt to study a more simple structure than a sterol, a number of available N-progesterone derivatives were studied. The data are presented in Table IV. In the first instance, it was found that the 3-oxopregnane-4-ene-20β-carboxaldehyde dioxime was less active than the corresponding 20α-isomer. Both isomers had low toxicity. The 11β-hydroxyprogesterone dioxime showed a little more activity, but was still considered to be inactive. A comparison of this compound to a carbamate derivative of an 11α-hydroxyprogesterone dioxime indicated that neither the geometrical position of the hydroxyl group nor its conversion to a carbamate would provide a more active structure. The 3 β-(1-pyrrolidine)-5α-pregnane-11,20-dione was slightly active, whereas the quaternary steroid (1-[3α,17α-dihydroxy-11,20-dioxo-5β-pregnane-21-yl]) bromide, hydrobromide was not active. Two progesterone derivatives which gave low MIC values were progesterone, bis-(aminidinohydrazone)dinitrate, and N-[bis(dimethyl-amino)methylene]-17β-pregnane-5-ene-20-carboxamide, acetate. The former steroid indicating high activity against gram-positive and yeast organisms even had slight activity (250 μg/ml) against a gram-negative strain (*Escherichia coli*).

In the next phase of our studies, interest was focused on the activity of steroids more simple than progesterone, i.e., androgens. Nine N-androgen derivatives were screened (Table V). The simplest N-androgen was a 17 β-amino-androst-5-en-3-β-ol. This derivative was highly active and showed activity against both gram-negative strains. A derivative which was saturated and did not contain a hydroxyl group at position 3-, but rather at position 11-, was as active or more active than the original androgen.

The geometrical configuration of the amino

TABLE III

Minimal Inhibitory Concentration (μg/ml) of Homo-Azasteroids

Organism	Compound									
	PC-1	PC-2	PC-3	PC-4	PC-5	PC-6	PC-7	PC-8	PC-9	PC-10
Escherichia coli	NI[b]	NI	NI	NI	NI	NI	NI	NI	NI	NI
Pseudomonas aeruginosa	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Streptococcus faecalis (Group D)	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Streptococcus pyogenes	NI	NI	1,000	NI	NI	NI	NI	NI	NI	NI
Staphylococcus aureus	NI	NI	1,000	NI	NI	NI	NI	NI	NI	NI
Corynebacterium sp.	NI	NI	1,000	NI	NI	NI	NI	NI	NI	NI
Nocardia asteroides	NI	NI	1,000	NI	NI	NI	NI	NI	NI	NI
Candida albicans	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Saccharomyces cerevisiae	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI

[a]PC-1=3β-hydroxy-16β-morpholinandrostan-17-one, PC-2=3β-hydroxy-16β-morpholino-5-androsten-17-one, PC-3=3β-piperindoacetoxy-17α-aza-D-homo-5α-androstan-17-one, PC-4=3β-morpholinoacetoxy-17α-aza-D-homo-5-androsten-17-one, PC-5=cholestan-3',4'dimethyl[2,3-B] grinoxaline, PC-6=3β-acetoxy-5α-androstan[16,17-B]grinoxaline, PC-7=3β-morpholinacetoxy-5-androsten[16,17-B]grinoxaline, PC-8=3-keto-4-androstene[16,17:2',3']uethy-7'-iridazo(1,2-α)pyridine, PC-9=3β-pyreolidinoacetoxy-17α-aza-D-homo-5-androsten-17-one, PC-10=3β-pyreolidinoacetoxy-17α-aza-D-homo-5-androsten-17-one.

[b]NI = non-inhibitory.

group at position 17- is extremely important. While the 17 β derivative was active, the 17 α androgen was almost complete inactive.

The methylation of the 17 β-amino group, as in 17 β-dimethylamino-17-methylandrost-5-en-3-one, provided a less active compound. The conversion of the 17-amino group to an oxime almost completely obliterates antimicrobial activity. Moving a hydroxyl group to a 3 α, 11β position, or its complete removal, does not significantly alter steroid activity.

The acetylation of the 17 β-amino group in a 4-androsten-3-one derivative also drastically reduces compound antimicrobial activity.

An 11β-hydroxy-17-methyl-17-aza-13α-androst-4-en-3-one derivative was not active.

In summary, the most active compounds were either a monoazacholesterol (22-azacholesterol); progesterone, bis-(amidinohydrazone), dinitrate; or the amino androgen (17 β-amino-5α-androstan-11β-ol).

DISCUSSION

An extensive interest in the antifungal property of gonadal hormones probably originated in clinical preoccupation with the influence of pregnancy and menstruation on the incidence in severity of infectious diseases in man. As early as 1935 estrogens were reported to have antibacterial activity (7-9). There is now considerable evidence that a large number of steroids have antimicrobial effects in vitro on bacteria, molds, yeasts and protozoa (see reference 5 and 6 for review). Natural and synthetic steroids were generally shown not to be active against gram-negative bacteria.

In contrast to this lack of effect on gram-negative organisms were two reports on nitrogen-containing derivatives of six hormones which were active against gram-positive microorganisms (10-12). The MIC values for these amino steroids on *Salmonella schottmülleri, Pseudomonas aeruginosa,* and *Escherichia coli* were ca. 100-200 mg percent or 1,000-2,000 μg/ml (13). Tarbet et al. (10) found dermatophytes particularly sensitive to 20-amino-3β-hydroxy-5-pregnene monochloride (MIC=25 μg/ml).

R.F. Smith et al. (12) was the first to screen a new group of nitrogen-containing steroids that showed antimicrobial activity. The three most active azasteroids with the nitrogen primarily in position 4- were classified as quaternary salts. These aza compounds did not appear to have properties similar to those of quaternary salt disinfectants since the latter are effective against gram-negative bacteria (13) while the azasteroids were inactive.

TABLE IV

Minimal Inhibitory Concentration (μg/ml) Values for Select Progesterones[a]

Microorganisms	I	II	III	IV	V	VI	VII	VIII
Escherichia coli	NI[b]	NI	NI	NI	NI	NI	250	NI
Pseudomonas aeruginosa	NI	NI	NI	NI	NI	NI	NI	NI
Streptococcus faecalis (Group D)	NI	NI	NI	NI	1,000	NI	10	500
Streptococcus pyogenes	100	500	1,000	1,000	250	250	5	2.5
Staphylococcus aureus	NI	NI	NI	NI	500	1,000	5	25
Corynebacterium sp.	NI	250	250	250	250	100	5	25
Nocardia asteroides	NI	500	100	250	250	500	5	5
Candida albicans	NI	NI	250	NI	100	NI	5	25
Saccharomyces cerevisiae	NI	NI	250	500	100	NI	5	5

[a]I = 3-oxoPREGN-4-ENE-20β-carboxaldehyde, dioxime; II = 3-oxoPREGN-4-ENE-20α-carboxaldehyde, dioxime; III = 11β-hydroxyPREGN-4-ENE-3,20-dione, dioxime; IV = 11α-hydroxyPREGN-4-ENE-3,20-dione, dioxime, carbamate; V = 3β-(1-pyrrolidinyl)-5α-PREGNANE-11,20-dione; VI = 4-aza-1-azoniabicylclo[2.2.2]octane, 1-(3α,17α-dihydroxy-11, 20-dioxo-5β-pregnane-21-yl)- - -bromide, hydrobromide, hydrate; VII = progesterone, bis(amidinohydrazone), dinitrate; VIII = N-[bis(dimethylamino)methylene]-17β-pregn-5-3n3-20-carboxamide, acetate.

[b]NI = non-inhibitory.

TABLE V

Minimal Inhibitory Concentration (μg/ml) Values for Select Androgens

	Compound[a]								
Organism	I	II	III	IV	V	VI	VII	VIII	IX
Escherichia coli	500	100	NI	NI	NI	NI	NI	NI	NI
Pseudomonas aeruginosa	500	NI[b]	NI	NI	NI	NI	NI	NI	NI
Streptococcus faecalis (Group D)	25	25	NI	NI	NI	NI	NI	NI	NI
Streptococcus pyogenes	10	5	NI	500	NI	1,000	NI	NI	NI
Staphylococcus aureus	50	10	NI	1,000	NI	NI	NI	NI	NI
Corynebacterium sp.	10	5	1,000	250	NI	1,000	1,000	1,000	NI
Nocardia asteroides	20	50	NI	250	NI	NI	1,000	NI	NI
Candida albicans	15	5	NI	250	NI	NI	NI	NI	1,000
Saccharomyces cerevisiae	15	5	1,000	100	1,000	1,000	NI	NI	NI

[a]I = 17β-aminoandrost-5-en-3β-ol; II = 17β-amino-5α-androstan-11β-ol; III = 17α-amino-5α-androstan-11β-ol; IV = 17β-dimethylamino-17-methylandrost-5-en-3-one; V = 11β-hydroxy-5α-androstan-17-one; oxime; VI = 3α-hydroxy-5α-androstan-17-one, oxime; VII = 5α-androstan-17-one, oxime; VIII = 17β-acetamido-4-androstan-3-one; IX = 11β-hydroxy-17-methyl-17-aza-13α-androst-4-en-3-one.

[b]NI = non-inhibitory.

Holmlund and Bohonos (14) investigated the relative effects of a series of 3-dialkylaminoethoxy steroids on the growth of *Tetrahymena pyriformis*. There was good correlation between the ability of these compounds to lower serum sterol in animals and to suppress the growth of the organism. Because of the low order of activity of other steroids without the dialkylaminoethoxy group, the presence of the amine group on the steroid nucleus seems to increase the ability of the steroid to inhibit the growth of the protozoa. However, the structure of the steroid to which the dialkylaminoethoxy group is attached was also of importance in determining the activity of the compound.

It is to this latter problem of structure-function that we have addressed our own research efforts on azasteroids. These compounds have been reported to effect a diversity of biological systems. Their hypocholesteremic effect, as reported by Ranney and Counsell (15), was a great stimulus toward further interest in and development of azasteroids. Alteration of cholesterol biosynthesis by azasteroids has also been reported in animals and in man (16 and references therein). Unfortunately, the compound has dangerous side effects since the use of azasteroids has resulted in myotonia in humans and rats (17). Pronounced antifertility action of diazacholesterol and related steroids has been reported (18). Other investigators have reported antineoplastic action of a homo-azasteroid ester which was less toxic than nitrogen mustards (19). The reduction in transport of macromolecular precursors in cell culture growth was

observed with 15 azasteroid analogues (20). The glycoside tomatine is a naturally occurring azasteroid with antifungal activity (21). Since tomatine was shown to form an insoluble complex with cholesterol in a quantitative manner (22), its mode of action may well depend upon this property. Additional antifungal and antibacterial activity of nitrogen containing steroids was reported (23). Contrary to effects with steroid glycosides, action of N-steroids may be directed at transport sites in the membrane (24).

In addition to inhibitory effects of azasteroids on sterol metabolism and microorganisms, adverse effect on growth and development in insects have been extensively studied by Svoboda and Robbins (25). Insects must derive their essential cholesterol through conversion of plant sterols. Therefore, azasteroids which interfere with sterol metabolism have potential as insecticides. The results from insect research have pointed to a number of structural features that may affect the inhibitory activity of these azasteroids. The monoazasterols are the most inhibitory of the compounds tested, and of these the 25-azasterol is the most active. The tertiary nitrogen at position 25- was more inhibitory than the secondary nitrogen at either the 23- or 24- position. The 25-azacholesterol is over 30 times more potent than either the 20,25-or 22,25-diazacholesterol in blocking hornworm development, indicating that additional nitrogen apparently is responsible for some stearic effect that decreases the inhibitory action of these diazasterols. This relationship of structure to activity in the hornworm generally parallels results from studies with rats on the relative effectiveness of monoazasterols and diazasterols as hypocholesteremic agents (26). The addition of substituents larger than methyl groups at the 26- and 27-position of the side chain renders the azasterol considerably less active, particularly with respect to inhibition of growth and development. A number of such compounds tested in the hornworm quite effectively blocked the Δ^{24}-reductase system, severely reduced cholesterol formation, but had little or no effect on larvae growth. Two other azasterols with side-chains that also differ considerably from cholesterol were found to be active; both the 20,24-diaza-25-norcholesterol (with the shortened side-chain) and the 17α-epimer of 20,25-diazacholesterol were inhibitory. The 21-norderivative of the 17α-epimer was considerably less effective, indicating that the 21-methyl group is essential for maximum activity. Neither a Δ^5 bond nor a free 3β-hydroxyl group appears to be essential for azasterol activity. The saturated analog of the 22,25-diazacholesterol is approximately as active as the Δ^5 compound in both an ether and an ester derivative of 20,25-diazacholesterol; both were several times more active than the parent compound.

The differences observed in the effect of diazasterol in several species of insects warn against making broad generalizations on sterol metabolism utilization in insects. The azasterols are also apparently directly or indirectly involved in metabolic pathways other than those having to do with the formation of cholesterol from phytosterols (27).

The same caution taken for azacholesterol derivatives against insects should be applied to our studies on the effects of structure-function activity on microorganisms. Despite such limitations, a pattern of general structure-function activity has emerged. The mono derivative was more active against microorganisms than the diazacholesterol. This is similar to findings on insects. Contrary to what was found for the monoazasterol on insects, however, the 22-azacholesterol rather than the 25-azasterol was the most active mono derivative against microorganisms. The relation of structure to antimicrobial activity does not parallel the results from studies with rats on the relative effectiveness of monoazasterols and diazasterols as hypocholesteremic agents (26).

Since the 20,25-diazacholesterol was less active than the 20-aza or 25-azasterol, the additional aza group does not contribute to N-steroid antimicrobial activity. The removal of a N-methyl group from either position 20-, 25-, or the mild oxidation of the methyl group at position 20- and the removal at 25-, were without effect on steroid activity.

The conversion of the 25-azasteroid to an N-oxide derivative, a potent hypocholesterolemic drug (28), caused a precipitous loss of compound microbiological activity.

Recently, a novel group of antibiotics produced by culturing Geotrichum flavo-brunneum was isolated. The major active fraction was a 15-aza-24-methylene-D-homocholestadiene (29). The homoaza steroid was most active against pathogenic fungi, including *Candida* and *Trichophyton* species (30). Our laboratory has confirmed the high activity against *Candida*.

Homoazasteroids which were reported (19) to have antineoplastic action were screened by us for antimicrobial activity. Only the 17-azasteroid (PC-3) exhibited marginal activity and this against gram-negative organisms. In view of our previous experience on aliphatic compounds (31), these particular homoazasteroids may be too large to penetrate microbial cell walls and/or membranes.

Because size may be a factor in regard to compound activity, N-steroids more simple than N-sterols were screened. Again, as for sterols, the dioxime steroid derivatives were not active. The 3β-pyrrolidinyl derivatives were active as were the aminidinohydrazone and dimethylaminoprogesterone compounds. The basicity of a functional group seems to be more important in determining compound activity than does steroid structure. This has been nicely exploited in screening select androgens. The 17β-amino derivatives is active regardless of changes in ring saturation or position of hydroxyl group. However, when the amino group is hindered as in the 17α position, dimethylated or acetylated, the parent 17β-amino derivative is less active. The nitrogen placed in the D-ring to form an azasteroid also renders the parent compound less active.

These studies tend to support an observation made with simpler aliphatic amine derivatives (31). With similar chain length, the order of activity increases with functional group $RCOOH < RC^{=O}\text{-}NH_2 < RNH_2$. While such generalizations concerning structure-antimicrobial activity are of interest, little is known about the mechanism of action for these complex amines (N-steroids) or even the simpler (aliphatic) amines. It would be of considerable importance if the structural relationship found for antimicrobial agents could be related to effects on one or more physiological processes.

Because of the lipophilic nature of the compounds tested as antimicrobial agents, we favor the hypothesis that these substances react with membranes and cause their pharmacological effects in this manner. Similarity of effects on a variety of lipophilic agents on bacterial and mitochondrial systems has been described in recent years and is discussed in the literature (32). It is of significance that antibacterial effects of fatty acids (33), amides, and amines (31) parallel closely their effects on mitochondrial activity.

Eukaryotic organisms are generally considered to synthesize and contain sterols, whereas prokaryotic organisms are generally considered not to contain or need sterols. It is important, therefore, that certain hypocholesteremic drugs inhibit the multiplication of microorganisms which represent both cellular groups. The growth inhibition by hypocholesteremic drugs of certain bacteria, algae, fungi, and ascomycetes has previously been reported (34-38). The precedence for showing the inhibition of growth of microorganisms by both nonsteroidal (14 and references therein) and azasteroid hypocholesteremic drugs (13,38,39) has been established. The demonstration of such compounds as antibacterial agents, however, has not tempered interpretation of the mechanism of action of these agents as hypocholesteremic drugs.

Consequently, two facts, effects on oxidative phosphorylation and/or antibacterial activity, should lead to studies in which the action of these compounds on lipid metabolism is seen as secondary to effects on more basic biochemical systems. Also, the present study, as well as the work reported by Svoboda et al. (25) on insects, reveals no necessary correlation between hypocholesteremic effect and compound activity.

We believe that the activity of amino and azasteroids may be due to a mode of action more fundamental than on sterol metabolism alone, as is indicated by the preceding and following arguments.

1. Interference with respiration (39-41) and/or oxidative phosphorylation has been reported for growth inhibitory steroids.
2. Inhibition by steroids of NADH oxidation suggests that this is an important site of steroid action (42).
3. Several hypocholesteremic drugs uncouple oxidative phosphorylation in liver mitochondria (1 and references therein).
4. Inhibitors of oxidative phosphorylation block sterol synthesis in rat liver homogenates (43).
5. Azasteroids were shown to interfere with mitochondrial respiration (Kabara, unpublished data).

In review of the above, we postulate that the effect of such "hypocholesteremic" agents on sterol metabolism is secondary and that the primary effects are on oxidative phosphorylation and/or microsomal activity. The former can be supported by direct effect of other hypocholesterolemic drugs on mitochondrial metabolism (1, 44-45). More specifically, Counsell et al. (46) demonstrated the inhibition of cholesterol side-chain cleavage by azasteroids. This mitochondrial system which involves NADH and a flavoprotein is similar to that outlined above for oxidative phosphorylation. The nonorganelle transport system in bacteria is comparable to that found in eukaryotic organelles and is affected by the drugs in a similar manner (47). *Consequently, the primary effect, either directly or indirectly, of azasteroids is most probably on membrane- or enzyme-bound systems;* the consequence of this inhibition leading to decreased levels of adenosine triphosphate (ATP) and/or eventual microsomal effects. The latter is especially inferred from the mechanism of action postu-

lated for SKF-525A. This drug not only is a hypocholesteremic agent (48) inhibiting microsomal hydroxylation (49), but also inhibits the ATP requiring process (50), Other hypocholesterolemic agents, clofibrate and SaH 42-348, have indicated similar action on liver microsomes (51).

The hypothesis brought forward–"that amino and azasteroids are effectors of membranes which lead to changes in ATP levels and/or dehydrogenase activity"–is consistent with known facts concerning the drug action for these kinds of compounds. The hypothesis helps explain how hypocholesteremic drugs effect bacteria and lipid levels by a single mechanism. More experiments using simple model systems remain to be carried out in order to show the validity of this concept. This concept also emphasizes the danger of in vitro testing of lipophilic drugs as potential hypolipidemic agents since their in vitro action may be nonspecific.

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Nutrition and Metabolic Studies of Methyl Esters of Dimeric Fatty Acids in the Rat

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ABSTRACT

Methyl esters of dimeric fatty acids were prepared by fractionating a mixture of conjugated linoleic and oleic acids that was heated for 24 hr at 300 C in the absence of air. Rats fed diets containing less than 1% dimers showed no significant difference ($P<0.05$) in the growth rate, feed efficiency, liver:body weight ratio, and lipid:liver weight ratio from those fed normal diets. A lymph cannulation study using ^{14}C labeled dimers showed that ca. 0.4% of the dimers fed were absorbed within 12 hr and were transported as free acids in the lymph. Within a 28 hr period, 2% of the labeled dimers fed by gastric intubation were oxidized to $^{14}CO_2$, and 1% radioactivity was recovered from the urine. The metabolism of methyl oleate appeared normal for rats prefed diets containing dimers.

INTRODUCTION

Dimers of fatty acids are minor components of heated and oxidized fats (1-4). Used fats which have not been unreasonably abused contain less than 10% of dimers and polymers. The presence of 1-2% of dimers, however, has also been reported in freshly deodorized commercial vegetable oils (5,6). Nutritional studies (7-12) conducted on the dimer and polymer fractions of heated fats showed in some cases that rats fed high levels of dimer concentrates or polymers exhibited adverse effects such as depressed growth, diarrhea, oily and matted coats, lower basal metabolism, lower body temperature, and higher mortality rate. Although the apparent digestibility of dimers had been shown to be between 30 and 70% (10,13,14), their absorption by the rat has been questioned (15-17). In the present study, nonlabeled and ^{14}C-labeled methyl esters of dimeric fatty acids were prepared, and the absorption and nutritional effects of these dimers in the rat were investigated.

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MATERIALS AND METHODS

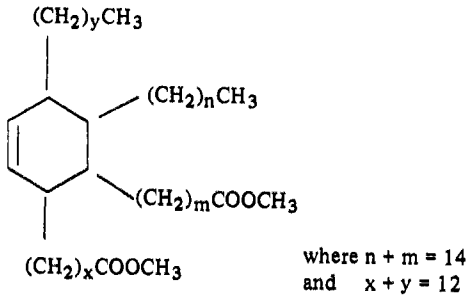
Synthesis of Dimeric Fatty Acids

Mixtures of 150 g per batch of fatty acids (Pamolyn 380, Hercules Inc., Wilmington, DE) which consisted of 76% conjugated linoleic acid, 4% linoleic acid, and 20% oleic acid were heated with 1% water in a stainless steel reactor (#406-01M, AMINCO, Silversprings, MD) at 300 C for 24 hr. After cooling to room temperature, the mixtures were converted to the corresponding methyl esters with methanol containing 3% sulfuric acid. The methyl esters of monomeric fatty acids were removed by vacuum distillation at 0.3 torr from 135 C to 190 C. The distillates were discarded, while the residues, in 20 g batches, were further fractionated with the aid of a column of silica gel, 6 cm in diameter and 40 cm in height. The residues from vacuum distillation were applied to the top of the adsorbent and eluted with cyclohexane:benzene (6:4) followed by benzene. The cyclohexane:benzene solvent system eluted the residual monomers in the residues, while benzene eluted the dimeric ester fractions. Polar compounds and polymers which remained on the column were eluted with ethyl ether. The yield of dimers obtained in this manner was 30%.

The purity of the dimer fraction obtained was monitored by thin layer chromatography (TLC), gas liquid chromatography (GLC), and gel permeation chromatography. Thin glass plates coated with 0.5 mm Silica Gel G were developed with an iso-octane:ethyl acetate (90:10, v/v) solvent system; the R_f values of monomer, dimer, and polar polymeric compounds were 0.51, 0.36, and 0.30 and lower values, respectively. A 2 ft x 1/8 in. glass column packed with 3% OV-1 coated on 80-100 mesh Supelcoport was used in the GLC of the methyl esters of the dimerized mixture. Dimers were eluted at 290 C when the column temperature was programmed from 150 C to 300 C at 8 C/min. Final determination of purity was accomplished by gel permeation chromatographic analysis (18). Quantitative determination of dimers was accomplished by using n-octacosane as an internal standard reference compound via gas chromatography.

Data obtained from infrared, nuclear magnetic resonance, and mass spectroscopy showed

that the dimers used in the present experiments were a series of structural isomers similar to those proposed by Paschke et al. (19) for thermal dimers derived from conjugated linoleates. The general structure of these isomers is shown below.



Labeled dimeric fatty acids were prepared by reacting a conjugated fatty acid mixture with 99+% pure ^{14}C uniformly labeled methyl linoleate (Dhom Products, N. Hollywood, CA). A gram of methyl esters of the conjugated fatty acids together with 150 μCi of labeled linoleate and 1% H_2O were vacuum sealed in a thick-wall glass ampule. The sealed glass ampule was placed in a steel pipe and heated in a furnace at 250 C for 12 hr. After cooling, the mixture was esterified with methanol that contained 3% sulfuric acid, and then separated into monomer, dimer, and polymer fractions with the silica gel column. The solvent systems used were those described for the large scale synthesis. Pure dimer fractions (99+% as shown by TLC, GLC, and gel permeation chromatography) were combined and used in the subsequent metabolic studies.

Experimental Animals and Diets

Male weanling SPF Albino rats (Murphy Breeding Laboratory, Plainfield, IN) (40-50 g) of Sprague Dawley descent were housed in galvanized iron wire cages with mesh bottoms. The composition of the diets used was as follows: (in g/kg diet) salt mixture (#4164 General Biochemistry, Chagrin Falls, NY), 40; vitamin premix (10 g of vitamin premix in 1 kg of diet contained vitamin A acetate, 20,000 IU; vitamin D, 3,000 IU; vitamin E, 100 IU; and the following in mg/kg of diet: inositol, 112; menadione bisulfite, 48; para-amino benzoic acid, 110; nicotinic acid, 100; calcium pantothenate, 67; riboflavin, 22; pyridoxine HCl, 22; thiamine HCl, 22; biotin, 0.45; folic acid, 2; and cyanocobalamine, 0.03), 10; choline chloride, 1.65; casein, 150; dextrose, 648.4; and corn oil containing either 0.0, 0.1, 1.0, or 5.0% methyl esters of dimeric fatty acids, 150. All diets were made up in 2 kg batches. The animals were provided with food ad libitum, fed every day, and their diets were stored under nitrogen atmosphere at 5 C between feedings. The animals were fed the different diets for 6-10 weeks.

Procedures for Lymph Cannulation and In Vivo Metabolic Studies

The collection of lymph from the thoracic duct of rats was accomplished by cannulation according to the procedures of Saldeen and Linder (20). After the surgery, the rat was fed by oral intubation ^{14}C labeled dimers (10 mg, 49 μCi/g) diluted with 500 mg of corn oil. The rat was confined in a cage, and lymph from the cannula was collected every 4 hr for a duration of 12 hr. The rat has access to 0.8% saline water and ground laboratory chow during this period.

Methyl esters of ^{14}C labeled dimeric fatty acids and uniformly labeled methyl oleate (Searle, Arlington Heights, IL) were fed individually to separate rats by gastric intubation. These rats, which were fed different diets for 6-10 weeks, were then placed in metabolic cages for 48 hr. The expired CO_2, urine, and feces were recovered and the radioactivity determined by liquid scintillation counting according to the procedures described by Iwaoka (21). The rats were sacrificed after 48 hr and their livers, stomach, small and large intestines, epididymal fat, and perirenal fat were quickly excised, blotted, weighed, and stored at 0 C.

Extraction and Characterization of Lipids from Lymph, Tissues, and Feces

The method of Folch et al. (22) was followed for the extraction of lipids from lymph and tissues of the experimental animals. Lipids were extracted from partially air dried feces by adding 25 times by weight of 30% aqueous HCl and digestion on a steam bath for 2 hr with occasional stirring. After cooling, the acid solution was extracted first with hexane followed by four diethyl ether extractions or until the ether extract was clear. The hexane and ether extracts were combined and dried over sodium sulfate. The solvents were evaporated and the lipids weighed and diluted with hexane in volumetric flasks. The radioactivities of lipids extracted from lymph, tissues, and feces were determined by liquid scintillation counting using a solution of 5 g PPO and 0.2 g POPOP in 1 liter of toluene.

Extracted lipids were separated into their respective classes by TLC with hexane:diethyl ether:acetic acid (90:10:1, v/v) as the solvent system. The fractions were visualized by iodine vapor, scraped from the thin layer plates, added

TABLE I

Weight Gain and Feed Consumption of Male Rats Fed for 6 Weeks on Diets Containing Different Levels of Dimers

Group	Dimer in oil (%)	Average weight gained (g)	Average feed consumed (g)	Feed efficiency[a]
I	0.0	196.2 ± 8.4[b]	508.9 ± 14.3[b]	0.386
II	0.1	189.4 ± 4.5	503.3 ± 6.4	0.376
III	1.0	201.1 ± 6.6	526.7 ± 10.0	0.379
IV	5.0	191.2 ± 8.4	508.6 ± 22.4	0.375

[a]Feed efficiency = $\frac{\text{weight gain}}{\text{feed consumed}}$

[b]Mean value ± SEM, for seven animals in all groups.

TABLE II

Percentage of Liver:Body Weight Ratio and Lipid:Liver Weight Ratio of Rats Fed Different Levels of Dimers for 6 Weeks

Group	Dimer in oil (%)	% $\frac{\text{Liver weight}}{\text{Body weight}}$	% $\frac{\text{Lipid weight}}{\text{Liver weight}}$
I	0.0	3.20 ± 0.10[a]	4.19 ± 0.52[a]
II	0.1	2.86 ± 0.15	4.52 ± 0.69
III	1.0	2.74 ± 0.18	4.69 ± 0.28
IV	5.0	2.82 ± 0.03	4.59 ± 0.98

[a]Mean ± SEM, for four animals.

to toluene scintillation solution, and their radioactivities were determined by liquid scintillation counting.

Statistical analyses of the data obtained in the dietary studies were calculated according to the completely randomized design and the variance analyzed by the F test (23).

RESULTS AND DISCUSSION

The effects of low levels of dimeric fatty acid methyl ester on weight gain and food efficiency of rats fed otherwise nutritionally adequate diets are shown in Table I. The levels of dimers in the diet chosen were based on reports (5,6,24) that showed the presence of 1-3% dimers in deodorized commercial vegetable oils. Heated oil often contains more than 10% dimers if the oil was badly abused (18); however, a more realistic maximum level of 5% dimers (4) in the oil was chosen for use in the feeding studies. All the rats appeared normal after the 6 week feeding period. The differences in average weight gained and feed consumed were not significant ($P<0.05$), although it seemed that the feed efficiency for the control group was slightly higher than the other three groups on diets containing dimers.

Crampton et al. (7,15), Kaunitz et al. (8), and Perkins et al. (9) showed that diets containing 12-20% dimers caused high mortality rates when fed to the rats. At 5-10% of the diet, dimers decreased growth rate and lowered food efficiencies (8,10,11). The high mortality of rats fed 12-20% dimers may have been caused mainly by malabsorption rather than the toxicity of the dimers. Diarrhea, a common symptom observed in rats fed high levels of dimers, will result in the failure of the animals to absorb essential food constituents and energy (25). Rice et al. (26) has previously demonstrated the reduction in available energy of diets containing 19% of severely heated cottonseed oil.

The decline in growth rate for rats fed diets containing 5-10% dimers may be caused in part by lower food efficiency or by lower palatability, as suggested by Rice et al. (26). Nevertheless, even if lower palatability can be demonstrated, a toxic action of heated fats resulting in slower growth rate cannot be entirely excluded (27). A maximum of 5% dimers in the oil or 0.75% in the diet did not lower the palatability of the diets nor disturb normal absorption (Table II). Food efficiencies and weight gains of rats on diets containing dimers at these levels were comparable to the control group.

The liver:body weight ratios and lipid:liver weight ratios of rats weighing 250-300 g and fed different levels of dimers are shown in Table II. There was no significant difference ($P<0.05$) in the liver:body weight ratios among the four groups. Lipid:liver weight ratios of rats

TABLE III

Percentage of Administered Radioactivity Recovered after 48 hr in CO_2 and in Urine

Group	Dimer in diet (%)	Labeled compound fed at time of experiment	Radioactivity[a] recovered in urine (%)	Radioactivity[a] recovered in CO_2 (%)
I	0.000[b]	Dimer esters	1.23	1.60
II	0.015[b]	Dimer esters	1.26	2.05
III	0.150	Dimer esters	1.25	2.22
IV	0.750[b]	Dimer esters	0.88	1.04
I	0.000[c]	Methyl oleate	0.73	31.6
II	0.015[c]	Methyl oleate	0.81	28.0
III	0.150[c]	Methyl oleate	1.26	49.4
IV	0.750[c]	Methyl oleate	0.730	28.2

[a]Mean value for two animals except for Group III fed methyl oleate, one animal.

[b]Animals were on the diets for 10 weeks.

[c]Animals were on the diets for 8 weeks.

on diets containing dimers seemed to be higher than the control group, but the differences were not significant ($P<0.05$).

Increases in the size of the liver and the amount of lipids in liver in experimental animals fed heated fats had been reported by various investigators (10,11). Friedman et al. (13) and Poling et al. (27) observed that rats on diets containing 10-20% heated fats and 18-30% protein for periods of 6 months to a year developed enlarged livers. Hemans et al. (28) fed rats diets containing 10, 20, or 30% protein and 15% heated corn oil for 126 days, and observed that both the liver:body weight ratio and the liver:lipid weight ratio of these rats were higher than those of rats on control diets containing fresh corn oil. Cyclic monomers of fatty acids when fed to rats at 0.15% level in diets containing 8, 10, or 15% protein induced fatty liver formation (21). However, the enlarged livers reported by these various authors don't necessarily contradict the results reported in Table II. The heated fats fed in those experiments were at different levels, and the fractions fed contained polar polymers or cyclic monomers and not the purified dimers employed to obtain the data in Table II.

Biological activity is dependent upon absorption of material, and accordingly the absorption of unlabeled dimers via the lympth was studied. Lipids extracted from lymph collected for 12 hr after gastric intubation of dimers were quantitatively analyzed by GLC. A total of 0.33% of the administered dimer was recovered from the lymph. A total of 88.5% of the dimers fed were found in the feces. A further lymph cannulation study using ^{14}C labeled dimers diluted in corn oil (10 mg dimers, 49 μCi/g, to 500 mg corn oil) was carried out for 12 hr. The dilution with corn oil was employed to avoid diarrhea usually caused by oral intubation of large amount of dimers. Recovery of radioactivity was 0.05% from the first 4 hr, 0.23% from the 8th hour sample, and 0.14% from the 12th hour sample. A total of 0.42% dimer was recovered in the lymph lipids.

In previous determinations of the digestibility of crude dimers carried out by balance studies of the lipid intake and fecal lipids (13), normal diets containing 10-30% dimers were fed to rats. The apparent digestibility of dimers was shown to be 60-74%. Bottino (10) reported a lower value of absorption of dimers at 32%. These values were very high when compared to the present results obtained by lumph cannulation experiments. It is possible that the absorption of dimers alone and dimers in corn oil was different from the absorption of dimers which were a small portion of a regular diet containing proteins and carbohydrates.

In comparison to the absorption of normal fatty acids, the absorption of dimers is significantly different. In a 12 hr period, Gallagher et al. (29) reported that 81.8% of uniformly labeled ^{14}C oleic acid was absorbed and 68.6% of the amount absorbed was recovered in the lymph lipids. Most of the radioactivity in the lymph lipids was in the neutral lipid fraction. Hyun et al. (30) reported that 85% of the absorbed oleic acid was transported via the lymphatic system and 15% via the portal vein in 8 hr; of the lymph lipids, 85% of ^{14}C oleic acid absorbed was incorporated into the triglyceride fraction, 7.8% in free fatty acid, 1.1% in cholesterol ester, 1.8% in the phospholipid, and 3.9% in minor lipids.

Labeled dimers were fed to rats that were prefed diets for 8-10 weeks containing different levels of nonradioactive dimers. The radioactivity recovered in the expired CO_2 and in the urine is shown in Table III. Since the absorption of dimers was much less than the oleate, the amount of $^{14}CO_2$ recovered from rats fed labeled dimer was less. The amounts of

TABLE IV

Percentage of Administered Radioactivity Recovered from Lipids of Feces and Selected Organs and Tissues after 48 Hr

Group[a]	I	II	III	IV
Dimer in diet (%)	0.000	0.015	0.150	0.750
	Percentage of recovered radioactivity[b]			
From labeled dimer				
Stomach	3.98	2.00	4.02	0.80
Small intestine	1.79	1.37	1.62	1.28
Large intestine	35.0	38.8	37.1	40.62
Feces	39.0	39.5	42.5	30.0
Liver	0.148	0.115	0.100	0.097
Epididymal fat	0.035	0.057	0.020	0.065
Perirenal fat	0.043	0.018	0.038	0.091
	79.99	81.86	85.40	72.95
From uniformly labeled oleate				
Liver	0.411	0.217	0.612	0.304
Epididymal fat	3.12	3.68	2.37	5.61
Perirenal fat	6.47	5.76	5.14	6.71

[a]Animals were on the diets for 8-10 weeks.

[b]Mean value from two animals. Except in Group III fed labeled oleate, values obtained from one animal.

radioactivity recovered in urine from rats fed labeled dimers, although very low, are significant because they were comparable or even slgihtly higher than the corresponding amounts recovered from rats fed labeled methyl oleate. A proportionately large fraction of the dimers absorbed must have been excreted through the urine as polar metabolites.

The radioactivity recovered from the gastrointestinal tract was 73-85% (Table IV). Most of the labeled dimer administered to the rats was not absorbed, and ca. 40% of the administered dimers were still retained in the gastrointestinal tract after 48 hr. Within this time period, 80-95% of normal long chian fatty acids are absorbed (31).

The incorporation of radioactivity into the lipids, mainly triglycerides, of epididymal and perirenal fat was 100 times less from labeled dimers as compared to labeled oleate, and probably originated from breakdown products such as acetyl CoA which were reincorporated into normal fatty acids and stored in the fatty tissues.

The very low percentages of radioactivity recovered from urine and the expired CO_2 of rats fed labeled dimers confirmed the poor absorption of dimers shown by the lymph cannulation studies. However, the ∿3% radioactivity recovery in urine and CO_2 indicated that absorption, although poor, was more than the 1% observed in the lymph cannulation studies.

The $^{14}CO_2$ expired by rats fed labeled dimers presumably arose from the β-oxidation or ω-oxidation of the dimeric fatty acid, followed by oxidation of acetyl-CoA in the tricarboxylic acid cycle into water and CO_2. Iwaoka (21) also showed that when labeled cyclic monomer containing a cyclohexadienyl ring was fed to the rats, $^{14}CO_2$ was expired. The cyclic portion of dimers cannot undergo β- or ω-oxidation and was probably hydroxylated or conjugated. Hydroxylation and conjugation, common detoxication mechanisms for cyclic compounds, give rise to polar metabolites easily excreted in the urine.

The present data indicated that many of the metabolites from labeled dimers fed to rats were incorporated into the phospholipid fraction of the liver (Table V). The incorporation of labeled methyl oleate into the phospholipid fraction was not favored over incorporation into the triglyceride fraction. The percentage of radioactivity in the triglyceride fraction was even higher than that in the phospholipid fraction. Increasing levels of dimers in the diet did not result in a gradual decrease in the incorporation of methyl oleate into the triglyceride fraction, indicating that the presence of a small amount of metabolites of dimers in the liver did not affect the incorporation of normal long chain fatty acids into triglycerides and phospholipids.

The radioactivity concentrated in the phospholipid fraction of livers taken from rats fed labeled dimers may not be phospholipids. It could arise from other compounds having the same polarity as the phospholipids. The ma-

TABLE V

Percentage Distribution of Raioactivity among Various Lipid Classes from Livers of Rats Fed Labeled Dimers and Methyl Oleate

Group[a]	Compound fed[b]	Lipid classes[c,d] PL	MG	DG-C	FFA	TG	CE-HC
I	D	43.6	27.7	8.9	12.4	2.8	4.6
II	D	61.2	17.2	9.6	6.9	2.2	2.4
III	D	72.0	9.8	10.7	2.8	2.8	1.8
IV	D	72.4	6.4	10.6	7.8	0.9	1.7
I	MO	31.0	4.1	2.8	6.2	52.8	3.0
II	MO	41.0	0.7	4.0	10.2	41.3	2.8
III	MO	17.1	3.1	4.0	2.6	67.3	5.7
IV	MO	36.9	1.8	3.4	13.7	41.6	2.6

[a]Animals were on the diet for 8-10 weeks.

[b]Labeled compounds fed to animals: D = dimers, MO = methyl oleate.

[c]Six fractions spearated by thin layer chromatography: PL = phospholipid, MG = monoglyceride, DG-C = diglyceride-cholesterol, FFA = free fatty acid, TG = triglyceride, CE-HC = cholesterol esterhydrocarbon.

[d]Average value from two animals in each group, except for Group III fed labeled oleate, the values were obtained from one animal.

terial designated as the phospholipid fraction shown in Table V was the fraction at the origin on the thin layer plate. Further analysis of this fraction by two-dimentional TLC was attempted, however, and because of the very low total counts, the monitoring of radioactivity was extremely difficult. No conclusion could be reached concerning the nature of the radioactivity in the phospholipid fraction.

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Inhibition of Hepatic Sterol and Squalene Biosynthesis in Rats Fed Di-2-ethylhexyl Phthalate

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ABSTRACT

Di-2-ethylhexyl phthalate (DEHP), a commonly used plasticizer, was found to be an inhibitor of the biosynthesis of hepatic nonsaponifiable lipids in the rat. The addition of DEHP at levels of 0.5% or 1.0% to a stock diet of rats resulted in a decreased conversion of acetate-1-^{14}C and mevalonate-5-^{3}H into squalene, C_{30} sterols, and C_{27} sterols by liver minces or slices, in vitro. In studies conducted with 0.5% DEHP feeding from 2 to 11 days, the degree of inhibition was found to increase with the duration of DEHP feeding; the inhibition of ^{3}H-mevalonate conversion to squalene and sterols developed more slowly, being reduced to ca. 70% of control values in 11 days, whereas ^{14}C-acetate conversion was reduced to ca. 35% of control values during the same period. ^{3}H-mevalonate conversion to sterols and squalene was, however, found to be suppressable to the same extent as ^{14}C-acetate conversion when diets containing 1.0% DEHP were fed for 18 days. The inhibitory effect of dietary DEHP on sterol and squalene biosynthesis from ^{14}C-acetate and ^{3}H-mevalonate by rat liver preparations is unlikely to be accounted for by the negative feedback of cholesterol secondary to hepatic sterol accumulation since, in these studies, hepatic total lipid and hepatic total sterol levels were simialr in control and DEHP-fed rats.

INTRODUCTION

Esters of phthalic acid are constituents of a wide variety of products which include industrial oils, cosmetics, and plastics, particularly those of the polyvinylchloride (PVC) type (1,2). As a result of their widespread usage, phthalates have become general environmental pollutants and have been found as contaminants in tissues from animals (3,4) and man (5-7) and in air (1) and water (1). Although the phthalates are considered to have a low order of acute toxicity as judged by classical toxicological studies (8-11), recent studies from this laboratory (12) and others (13) have indicated that di-2-ethylhexyl phthalate (DEHP), the most commonly used phthalate ester, is capable of modifying lipid metabolism in the rat. Stein et al. (13) found that the addition of 0.1% DEHP to rat diets supplemented with lard promoted an accumulation of hepatic total lipid, whereas Bell and Nazir (12) found that acetate-1-^{14}C incorporation into phospholipids and neutral lipids was decreased in liver slices from rats fed either 0.5% or 1.0% DEHP for 10 or 18 days, respectively.

The present studies were undertaken to specifically examine the effects of dietary DEHP on the biosynthesis of squalene and sterols (nonsaponifiable lipids) by rat liver minces and slices, in vitro. In these studies, dietary DEHP was fed at the 0.5% and 1.0% levels; a level of 0.5% DEHP was chosen since in previous toxicological studies (10,11) similar levels of DEHP were fed to rats for up to 24 months with essentially no development of toxicity.

EXPERIMENTAL PROCEDURES

Animals and Diets

Male Sprague-Dawley rats (300-325 g) were fed either a control diet of Purina Chow or Purina Chow containing 0.5% or 1.0% DEHP (12). The diets were fed ad libitum since we have not found the feeding of DEHP to rats at these levels to affect feed consumption or body weight gains (12). Based on an average feed consumption of 15 g/day, the rats received daily either 1/65th (1.0% DEHP) or 1/130th (0.5% DEHP) of their acute oral LD_{50}, which is in excess of 30g/kg (9). The DEHP was obtained commercially (Eastman Chemical Co., Rochester, NY, cat. no. 4099) and purified by column chromatography using silicic acid (AR Grade, 100 mesh, Mallinckrodt Chemical Works, Jersey City, NJ) as described previously (12,13) and the purity confirmed by thin layer chromatography (TLC) and gas liquid chromatography (12).

Tissue Preparation

The rats were killed between 9 and 10 a.m. by a blow to the head, and their livers excised immediately. All subsequent procedures were performed at 0-5 C. Liver slices were cut at 0.5 mm thickness using a Harvard tissue slicer (Harvard Apparatus Co., Inc., Millis, MA); liver

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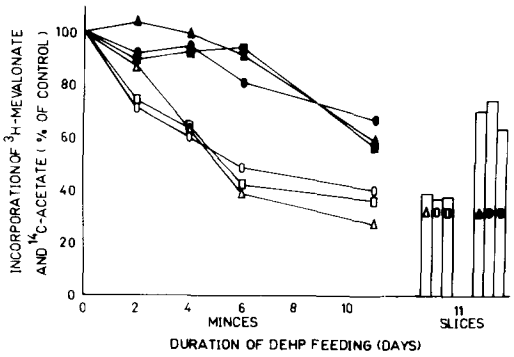

FIG. 1. Effect of duration of di-2-ethylhexyl phthalate (DEHP) feeding on the incorporation of ^{3}H-mevalonate (closed symbols) and ^{14}C-acetate (open symbols) into C_{27} sterols (△), C_{30} sterols (○), and squalene (□) by rat liver minces and slices, in vitro. Male Sprague-Dawley rats were fed Purina Chow (control) or Purina Chow containing 0.5% DEHP for up to 11 days. Liver minces and slices (500 mg) were prepared as described under Procedures and incubated 3 hr at 37 C with 4 μCi sodium acetate-1-^{14}C and 4 μCi DL-mevalonic-5-^{3}H acid in a total volume of 4.0 ml of Krebs-Ringer-Bicarbonate buffer, pH 7.4. The incubation mixtures were saponified and the nonsaponifiable lipids extracted with n-hexane and then fractionated by thin layer chromatography as described under Procedures. All data are means from two or three DEHP-fed animals expressed relative to control values obtained in parallel experiments.

minces were prepared with a Harvard tissue press.

Incubations and Analyses

Liver slices or liver minces (500 mg) were incubated in duplicate at 37 C in 25 ml Erlenmeyer flasks with either 3.7 or 4.0 ml of Krebs-Ringer-Bicarbonate buffer, pH 7.4, containing 4 μCi sodium acetate-1-^{14}C (58 mCi/mM) and 2.4-3 μCi DL-mevalonic-5-^{3}H acid, dibenzylethylene diamine salt (6.74 Ci/mM) obtained from New England Nuclear Corp., Boston, MA; penicillin and streptomycin were present at levels of 50 units and 50 μg/ml, respectively. Prior to incubation, the flasks were flushed with 95% O_2/5% CO_2 and capped. Incubations were terminated by addition of KOH and ethyl alcohol to give a final concentration of 11% and 82%, respectively, and the samples saponified 2 hr at 65 C. After saponification, 20 ml of water was added to the samples and the nonsaponifiable lipid fraction (sterols and squalene) was extracted with 3 x 20 ml of n-hexane. The hexane extract was reduced to 25 ml under N_2 and washed with 3 x 10 ml of water. In most experiments, this nonsaponifiable lipid fraction was fractionated by TLC on Silica Gel G-coated plates (20 x 20 cm) in a solvent system consisting of n-hexane:diethyl ether:acetic acid (146:50:4, v/v/v). Lipid bands corresponding to 27-carbon (C_{27}) sterols (Rf 0.12), 30-carbon (C_{30}) sterols (Rf 0.16), and squalene (Rf 0.67) were identified by co-chromatography with authentic cholesterol, lanosterol (Applied Science Laboratories Inc., State College, PA), and squalene (Sigma Chemical Co., St. Louis, MO), respectively. The lipid bands, visualized under UV light after spraying the plates with rhodamine 6 G (0.05% in ethanol), were scraped from the plates and assayed for radioactivity by liquid scintillation counting, employing double-labeled techniques to separate the ^{14}C and ^{3}H radioactivity in each sample (14). In two experiments, squalene eluted from thin layer plates was enriched by carrier squalene and purified by formation of the hexahydrochloride derivative (15), which was recrystallized three times. In each case, the hexahydrochloride derivative reached constant specific activity with one recrystallization, thus confirming the identity of the squalene biosynthesized.

The ^{3}H and ^{14}C activity co-chromatographing with authentic squalene was 96% and 98%, respectively, convertible to the hexahydrochloride.

Total hepatic lipid was obtained from liver samples by extraction with chloroform:methanol (2:1, v/v) according to Folch et al. (16). A portion of the extracts was placed in preweighed planchets, dried to constant weight at 100 C, and total lipid determined gravimetrically. Another portion of the lipid extracts was evaporated to dryness under N_2 and the lipid redissolved in isopropanol and asssayed for total cholesterol by an automated technique (17).

RESULTS

The effect of 0.5% DEHP feeding on the incorporation of ^{14}C-acetate and ^{3}H-mevalonate into sterols and squalene by rat liver minces is shown as a function of the duration of DEHP feeding over the period 2-11 days (Fig. 1); data obtained with liver slice preparations from the 11-day fed rats are included in the same figure for comparison with the results obtained in liver minces. Incorporation of isotopes into the sterols and squalene (dpm/g wet wt) is expressed relative to control values obtained in parallel incubations using liver minces or slices from rats not receiving dietary DEHP. In mince preparations (Fig. 1), incorporation of ^{14}C-acetate and ^{3}H-mevalonate into C_{27} sterols, C_{30} sterols, and squalene decreased steadily with the duration of DEHP feeding. Although the inhibitory effects of DEHP feeding were, in general, reflected equally among the three frac-

tions studied, the inhibition of ^{14}C-acetate incorporation into C_{27} and C_{30} sterols and squalene developed more rapidly than the inhibition of ^{3}H-mevalonate incorporation. The decrease in acetate incroporation into sterols and squalene was evident after 48 hr of DEHP feeding; the decrease amounted to ca. 27% for squalene and C_{30} sterols and ca. 14% for C_{27} sterols. In contrast, mevalonate incorporation into squalene and C_{30} sterols decreased ca. 10% during the same period but without an apparent decrease in incorporation into C_{27} sterols. After 11 days of DEHP feeding, acetate incorporation into C_{27} and C_{30} sterols and squalene was ca. 30-40% of control values, whereas mevalonate incorporation was equivalent to ca. 60-65% of control values.

A comparison of the incorporation of ^{14}C-acetate and ^{3}H-mevalonate by liver slices vs. liver minces in animals fed 0.5% DEHP for 11 days (Fig. 1) indicates that the mince preparations are as suitable as slice preparations for the type of studies presented here.

The results of Table I indicate that the incorporation of mevalonate into squalene and sterols, as shown in Fig. 1, can eventually be suppressed to the same extent as acetate incorporation by the feeding of DEHP. After feeding 1.0% DEHP to rats for 18 days, the incorporation of both ^{14}C-acetate and ^{3}H-mevalonate into sterols and squalene was reduced to ca. 25% of control values (Table I)

The rate of incorporation of ^{14}C-acetate and ^{3}H-mevalonate into the nonsaponifiable lipid fraction of liver minces from rats fed diets with and without 1.0% DEHP for 18 days is shown in Figure 2. Irrespective of diet, incorporation of the isotopic precursors showed a steady increase over the 3 hr period in which all the studies were performed; the effect of DEHP feeding on biosynthesis was apparent during the first hour of incubation.

In view of the observation that liver weight: body weight ratios increase with DEHP feeding (10-12), the expression of the data on a dry tissue weight basis rather than on a wet tissue weight basis may have been preferable. It should be pointed out, however, that even in rats fed 0.5% DEHP for 11 days, absolute liver weights (16.5 ± 0.9 g, n = 6) differed from control values (14.6 ± 0.7 g, n = 6) by only ca. 13%; such differences, however, did reach ca. 35% in rats fed 1.0% DEHP for 18 days (19.4 ± 0.7 g vs. 14.3 ± 0.8 g, n = 6). Nevertheless, these changes in liver weight with DEHP feeding are less than the magnitude of the changes in sterol biosynthesis observed with the two levels of DEHP feeding reported here.

TABLE I

Effect of 1.0% Dietary DEHP on Incorporation of Acetate-1-^{14}C and DL-Mevalonic-5-^{3}H Acid into Sterols and Squalene by Rat Liver Minces, In Vitro[a]

Diet	Precursor	Incorporation of isotope (dpm/g wet wt)				Percent of Control
		C_{27} Sterols	C_{30} Sterols	Squalene	Total	
Control	^{3}H-Mevalonate	171,937 ± 5,561[b]	57,358 ± 12,861	206,025 ± 28,825	435,320 ± 44,082	—
1.0% DEHP	^{3}H-Mevalonate	38,385 ± 8,823[c]	24,117 ± 2,257[d]	44,398 ± 4,102[e]	106,846 ± 12,312[e]	24.5
Control	^{14}C-Acetate	2,147 ± 287	664 ± 110	1,154 ± 198	3,965 ± 551	—
1.0% DEHP	^{14}C-Acetate	629 ± 182[e]	182 ± 64[f]	307 ± 72[f]	1,118 ± 217[e]	28.2

[a]Male Sprague-Dawley rats were fed Purina Chow (control) or Purina Chow containing 1.0% di-2-ethylhexyl phthalate (DEHP) for 18 days. Liver minces (500 mg) were prepared as described under Procedures and incubated 3 hr at 37 C with 4 μCi sodium acetate-1-^{14}C and 2.4 μCi DL-mevalonic-5-^{3}H acid in a total volume of 3.7 ml of Krebs-Ringer-Bicarbonate buffer, pH 7.4. The incubation mixtures were saponified and the nonsaponifiable lipids extracted with n-hexane and then fractionated by thin layer chromatography as described under Procedures.

[b]Values are means ± SEM of three animals.

[c,d,e,f] Significiantly different from control values by Student's independent t-test (c, $P<0.001$; d, $P<0.05$; e, $P<0.01$; f, $P<0.02$).

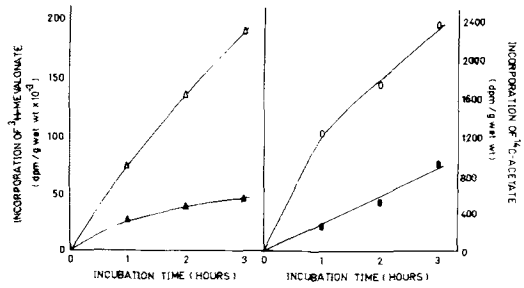

FIG. 2. Effect of dietary di-2-ethylhexyl phthalate (DEHP) on the rate of incorporation of ^{14}C-acetate and ^{3}H-mevalonate into nonsaponifiable lipids of rat liver minces, in vitro. Liver minces (500 mg) from male Sprague-Dawley rats fed Purina Chow (control, open symbols) or Purina Chow containing 1.0% DEHP (closed symbols) for 18 days, were incubated for up to 3 hr at 37 C with 4 μCi sodium acetate-1-^{14}C and 2.4 μCi DL-*mevalonic*-5-^{3}H acid in a total volume of 3.7 ml of Krebs-Ringer-Bicarbonate buffer, pH 7.4. The incubation mixtures were saponified and the nonsaponifiable lipid fraction extracted with n-hexane as described under Procedures; the hexane extract was assayed for radioactivity. Values are the means of two animals.

DISCUSSION

The studies presented here indicate that DEHP feeding to rats inhibits the biosynthesis of sterols and squalene from acetate and mevalonate by liver, in vitro.

Cholesterol, the principal C_{27} sterol of liver, is formed from acetyl CoA units through a squence of 20 or more intermediates (18,19), which include 3-hydroxy-3-methylglutaryl CoA (HMG CoA), mevalonic acid, squalene (a C_{30} hydrocarbon), and lanosterol (a C_{30} sterol). The conversion of HMG CoA to mevalonate by HMG CoA reductase (EC 1.1.1.34) is the primary rate limiting step in the formation of squalene and sterols (20), while secondary sites of control beyond mevalonate formation have been described (21,22). The activity of hepatic HMG CoA reductase is under negative feedback control by cholesterol (23-26) but can also be influenced by the administration of certain chemical agents such as clofibrate (α-p-chlorophenoxyisobutyryl ethyl ester) (27) and Triton WR-1339 (oxyethylated t-octyl phenol) (28).

In the studies reported here, 0.5% DEHP in the diet resulted in a decrease in ^{14}C-acetate incorporation into sterols and squalene that was observed within 48 hr of administering the DEHP-containing diet to the rats; this inhibition of acetate incorporation occurred prior to the formation of mevalonic acid formation since ^{3}H-mevalonate incorporation showed little change from control values during the same 48 hr period. In fact, ^{3}H-mevalonate incorporation was similar to control values for up to 6 days of DEHP feeding, after which time inhibition of mevalonate incorporation also occurred.

The time lag in the development of inhibition of hepatic sterol biosynthesis from mevalonate compared to inhibition of biosynthesis from acetate has been reported previously (22). One possible explanation is that one or more of the enzymes between mevalonate and squalene are induced by substrate and thus decline in activity as mevalonate production decreases (22).

The study in which 1.0% DEHP was fed for 18 days (Table I) establishes the fact that, while inhibition of mevalonate incorporation into hepatic sterols and squalene develops slowly relative to acetate incorporation (Fig. 1), mevalonate incorporation can eventually be suppressed to at least the same extent as acetate incorporation. The 18 day DEHP feeding trial reported in Table I represents the longest time study examined thus far. Subsequent studies of longer duration would be important to determine whether or not the degree of inhibition (ca. 70%) of sterols and squalene observed in the 18 day trial reflects a maximum response to DEHP.

In the studies reported here, it was necessary to examine the possibility that the effect of DEHP on hepatic sterol biosynthesis was secondary to hepatic sterol accumulation because (a) hepatic cholesterol biosynthesis in the rat is under feedback control by cholesterol (21,23-26) and (b) it has been reported that hepatic lipid can accumualte under certain conditions in rats fed dietary DEHP (13). Analysis of livers from six control rats and six rats fed 0.5% DEHP for 10 days indicated no significant ($P>0.05$) differences in hepatic total lipid (36.8 ± 1.2 vs. 39.7 ± 2.3 mg/g wet wt) or hepatic total cholesterol (2.6 ± 0.1 vs. 2.5 ± 0.2 mg/g wet wt) between the groups; on this basis, inhibition of sterol biosynthesis in DEHP-fed rats is not attributable to hepatic sterol accumulation. Furthermore, the time course study of the incorporation of acetate and mevalonate into hepatic nonsaponifiable lipids (sterols, squalene) demonstrates that the differences between liver from control and DEHP-fed rats (Fig. 1, Table I) do not merely reflect unique features of 3-hr incubations or differences in tissue survival but represent a defect in lipid biosynthesis attributable to DEHP feeding.

In a previous report from this laboratory (12), we found that dietary DEHP fed to rats at levels of 0.5% and 1.0% for 10 and 18 days, respectively, resulted in a significant ($0.02>P<0.01$) decreased in ^{14}C-acetate incorporation into total lipid by liver slices, in

vitro; the decreased incorporation was reflected in the phospholipids as well as the triglycerides, free fatty acids, and combined fractions consisting of sterol esters plus hydrocarbons, and sterol plus diglycerides. These differences in acetate incorporation between the two groups were not the result of changes in endogenous acetate pool sizes in liver (12), nor did they represent a general tissue response to DEHP feeding inasmuch as the incorporation of ^{14}C-acetate into total lipid by heart and kidney slices was similar to control values (12).

The present study is an extension of the previous work (12) and confirms that phthalate esters, in this case DEHP, are capable of modifying lipid metabolism in the rat. The significance of these results and the extent to which other species will respond similarly to phthalates is unknown; similarly, the relationship between the levels fed and those likely to be encountered environmentally is not known. However, the possible cumulative nature of the phthalates must be considered since DEHP has been reported at levels as high as 27 mg/100 g dry wt in human tissue (29) and found to represent as much as 60% of the lipid of the triglyceride fraction isolated from heart mitochondria of slaughterhouse beef cattle (4). The widespread use of phthalate-containing products by man and the contamination of the environment by phthalates emphasizes the need for further studies on the biochemical effects of phthalates in living systems.

ACKNOWLEDGMENTS

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Modulation by Ketone Bodies of the Rate of Fatty Acid Synthesis in Mammary Gland Slices from Lactating Rats

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ABSTRACT

The absolute rate of fatty acid synthesis was measured in slices of mammary gland from lactating rats by incubation in $[^3H]_2O$-labeled medium containing 10 mM D-3-hydroxybutyrate or acetoacetate alone and paired in combination with 10 mM glucose, lactate, or pyruvate. When compared with our previous studies, the ketone bodies alone supported significant fatty acid synthesis; the rate of synthesis from either ketone body and lactate was higher than that from pyruvate and lactate, and that from pyruvate and glucose; the rate of synthesis from D-3-hydroxybutyrate and lactate was the highest we have observed in the absence of an exogenous substrate for the hexose monophosphate pathway. This study confirms our previous contention that, in rat mammary gland, substrates formed in the mitochondria can be utilized in the cytosol to provide some of the NADPH necessary for fatty acid synthesis.

INTRODUCTION

Mammary gland from lactating rats is noted for its ability to form fatty acids in vitro (1-12). We (13) have shown in experiments with glucose, pyruvate, and l-lactate, alone and in combinations, that the extent of fatty acid synthesis can be modulated by the substrates presented to slices of lactating gland. We postulated that the availability of NADH in the cytosol along with a source of NADPH allowed the combination of glucose and lactate as exogenous substrates to maintain the highest rate of synthesis (13). Paradoxically, lactate alone is not converted to fatty acid (11,13,14). We (13) and others (11,14) have suggested that it is the lack of a mechanism for rapidly disposing of NADH produced in the cytosol that inhibits the conversion of carbon from lactate to fatty acid. Pyruvate enhanced the conversion of carbon from lactate to fatty acid as well as maintaining an intermediate rate of absolute fatty acid synthesis by itself (13). These results provided evidence of involvement of mitochondria in these phenomena (13). Utilization of pyruvate in the mitochondria would provide citrate for transfer to the cytosol. Subsequent action by citrate lyase (EC 4.1.3.8) would provide acetyl-CoA and oxaloacetate (OAA). The utilization of the OAA in the malate dehydrogenase (EC 1.1.1.37) reaction and oxidation of the malate produced in the reaction catalyzed by malate enzyme (EC 1.1.1.40) would provide, respectively, a means of utilizing the NADH produced during lactate oxidation and a source of NADPH to support fatty acid synthesis. Utilization of the pyruvate produced in these reactions completes a cycle between the mitochondrial and cytosolic compartments.

To test this mitochondrial involvement further, we employed the ketone bodies, acetoacetate and D-3-hydroxybutyrate, as exogenous substrates in addition to those tested previously. In mammary gland, D-3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) is limited to the mitochondrial compartment (15). Hence, utilization of either ketone body would yield acetyl-CoA as well as NADH solely within that compartment.

The interpretation of our previous results (13) provides a basis for predicting the influence of ketone bodies on fatty acid synthesis in lactating gland. If production and utilization of NADH in the cytosol is a factor in the maintenance of the maximal synthesis observed from glucose and lactate, then either ketone body and glucose should support a rate of fatty acid synthesis approaching the observed maximum. The rate of transport of NADH or its equivalent to the cytosol would be the only additional factor in using ketone bodies as the second exogenous substrate with glucose. Either ketone body replacing pyruvate as the other substrate with lactate should maintain synthesis at least equivalent to that with pyruvate and lactate; it might exceed this rate since these substrates would not have the disadvantage inherent in exogenous pyruvate, namely, decreasing the availability of NADH in the cytosol. We would expect the ketone bodies alone to support a higher rate of fatty acid synthesis than lactate under the same conditions if overproduction of NADH in the cytosol inhibits lactate conversion to fatty acid (11,13,14).

EXPERIMENTAL PROCEDURE

Tissue Preparation

Mammary glands from rats of the Long-

TABLE I

Absolute Rate of Fatty Acid Synthesis in the Presence of Ketone Bodies in Mammary Gland Slices from Lactating Rats[a]

Second substrate	Incorporation of $[^3H]_2O$ into fatty acid with:[b]	
	3-Hydroxybutyrate	Acetoacetate
None	182 ± 21[c]	81 ± 12
Glucose	1247 ± 92	1085 ± 29
Pyruvate	435 ± 19	413 ± 53
1-Lactate	878 ± 41	418 ± 12

[a]A precisely weighed portion (80-150 mg) of slices of mammary gland from lactating rats was incubated in 1 ml of Krebs-Henseliet bicarbonate buffer (17), pH 7.4, containing 10 mM D-3-hydroxybutyrate or acetoacetate and 10 mM of the substrates as listed in the table for 30 min at 37 C. The medium was labeled with tritiated water (3.5 μCi/mmol).

[b]Results are presented as nmol of $[^3H]_2O$ converted to fatty acid/100 mg fresh tissue/30 min. See text and reference 13 for other experimental details.

[c]Each value is the mean ± S.E. of six separate incubations.

Evans strain in the 10th to 16th day of lactation were removed as described previously (13). Slices (0.4 mm thick) were prepared with a mechanical chopper (16) and washed repeatedly with Krebs-Henseleit bicarbonate buffer (17) until milk no longer appeared in the wash solution. After gently blotting the slices with coarse filter paper, a precisely weighed portion (80-105 mg) was transferred to a 25 ml Erlenmeyer flask containing medium labeled with tritiated water and the unlabeled substrates as indicated in the table. The concentration of all added substrates was 10 mM. 3-Hydroxybutyrate was added as the DL-isomers at 20 mM so that effective concentration of D-3-hydroxybutyrate was 10 mM. The concentrations were verified by specific enzymatic assays. The flasks were gassed with a mixture consisting of 95% O_2 and 5% CO_2 prior to closure with a self-sealing rubber serum cap and incubated for 30 min, with shaking, in a water bath maintained at 37 C. At the end of that time, the tissue was inactivated by injecting 0.5 ml of 1 N H_2SO_4.

Analytical Procedures

The contents of the incubation flasks were transferred to conical centrifuge tubes. The slices were separated from the medium by centrifugation in the cold. The supernatant fraction was saved for assay of glucose, L-lactate, pyruvate, acetoacetate, and D-3-hydroxybutyrate. The slices were washed seven times with copious amounts of water and reisolated each time by centrifugation. Fatty acids in the washed slices were saponified and extracted into hexane (18). A portion of the hexane extract was assayed for radioactivity.

All measurements of radioactivity were done in a liquid scintillation spectrometer (Searle Analytic, Inc., Des Plaines, IL). The liquid scintillation medium was 10 ml of a 2:1 (v/v) mixture of toluene and 2-ethoxyethanol containing 98 mg of 2,5-diphenyloxazol (PPO) and 2 mg of p-bis-(o-methylstyryl)-benzene (Bis-MSB). All samples were adjusted to the same counting efficiency by the use of quench factors derived from the external standard ratio.

Glucose was determined by a glucose oxidase method (Glucostat, Worthington Biochemical, Freehold, NJ) in which 0.1 M phosphate buffer, pH 7.2, was substituted for water in making up the reaction mixture. L-Lactate was determined with lactate dehydrogenase in hydrazine-glycine buffer, pH 9.5 (19). Pyruvate was also determined with lactate dehydrogenase (20). Acetoacetate and D-3-hydroxybutyrate were determined enzymatically with D-3-hydroxybutyrate dehydrogenase (21).

Significance was calculated on the basis of Student's t-test (22). A random probability of $< 1\%$ was considered statistically significant.

Materials

Tritiated water (5 Ci/gm) was purchased from Amersham/Searle Corp., Arlington Heights, IL). With the exception of the glucose oxidase kit (Worthington Biochemical), enzymes and cofactors utilized in the substrate assays were purchased from Calbiochem (San Diego, CA) and were of the highest purity possible. Solutions containing pyruvate were made up the morning of the experiment.

RESULTS

With two exceptions, acetoacetate and D-3-hydroxybutyrate supported similar rates of tritium incorporation from $[^3H]_2O$ into fatty acid with all the substrate combinations tested (Table I). The rates of fatty acid synthesis with

3-hydroxybutyrate alone and in combination with lactate were more than twice those with acetoacetate under the same substrate conditions. When compared to our previous results (13), the rates of synthesis with either ketone body in combination with glucose approached that observed from glucose alone (1700 nmol/30 min/100 mg tissue), exceeded that from glucose with pyruvate (790 nmol/30 min), but was lower than that from lactate and glucose together (2590 nmol/30 min) (Table I). The rates of synthesis in combination with pyruvate were almost identical to that reported previously from pyruvate and lactate, ca. 400 nmol/30 min. In contrast, D-3-hydroxybutyrate and lactate supported a rate of synthesis over twice this rate (Table I). This rate was equivalent to that from glucose and pyruvate (790 nmol/30 min) and approached that with glucose and D-3-hydroxybutyrate together (Table I). This high rate of synthesis in the absence of exogenous substrate for the hexose monophosphate pathway is particularly striking.

The uptakes of D-3-hydroxybutyrate and acetoacetate were unaffected by the presence of other exogenous substrates. The overall means were 1.2 ± 0.06 μmol of D-3-hydroxybutyrate and 1.4 μmol of acetoacetate taken up by 100 mg tissue during the incubation period. Neither ketone body appeared to significantly influence the uptake of the other substrates.

DISCUSSION

It is now generally accepted that the incorporation of tritium from tritiated water into fatty acid represents the total extent of synthesis from both exogenous and endogenous substrates (23,24). We (13) have shown that in mammary gland, this incorporation reflects the absolute rate of fatty acid synthesis because the pathway of synthesis is the same from all substrates and synthesis is dependent upon the addition of substrates.

The results presented here show once again that the extent of fatty acid synthesis can be modulated by the substrates presented to the gland, in this case in combination with either D-3-hydroxybutyrate or acetoacetate. Since the uptakes were not altered by the exogenous substrates present, any differences in the extent of fatty acid synthesis must be due to changes in substrate utilization within the cell.

That mammary gland from lactating rats can utilize ketone bodies was first suggested by Page and Williamson (25) on the basis of enzyme assays. Later, Hawkins and Williamson (26) demonstrated that rat mammary gland does indeed utilize D-3-hydroxybutyrate and acetoacetate. Williamson and his coworkers (27) have also shown that the utilization of acetoacetate by the gland can alter the utilization of glucose, including that used for fatty acid synthesis.

For the most part, the results conform to those predicted on the basis of previous experiments (13). The rate of fatty acid synthesis from either ketone body and glucose was, however, lower than that predicted. As suggested above, the rate of transfer of NADH or its equivalent in the form of a reduced substrate to the cytosol may limit the rate of synthesis in this case.

We did not expect that D-3-hydroxybutyrate, together with lactate, would support a rate of fatty acid synthesis in excess of twice that from lactate and acetoacetate or from lactate and pyruvate. While D-3-hydroxybutyrate does yield one equivalent more of NADH than acetoacetate, there may be other reasons for D-3-hydroxybutyrate's enhanced support of fatty acid synthesis. The dehydrogenase responsible for oxidizing this substrate to acetoacetate resides in a unique site for a dehydrogenase, on the inner mitochondrial membrane (28). It is possible that NADH produced at this site escapes immediate oxidation in the electron chain so that it can be utilized in transhydrogenation reactions involving the cytosolic and mitochondrial compartments. Since the fatty acid synthetase of rat mammary gland requires almost exclusively NADPH for the reductive steps of synthesis (29), such transhydrogenation may be essential for fatty acid synthesis to continue in the absence of hexose monophosphate activity. Two reactions, both utilizing substrates derived from the mitochondria, are candidates for an alternate source of NADPH in the cytosol: the oxidation of isocitrate to 2-oxoglutarate and the conversion of malate to pyruvate. We favor the latter reaction catalyzed by malic enzyme for a number of reasons. First of all, we have shown that citrate is the carrier across the mitochondrial membrane for at least a portion of the acetyl-CoA utilized in fatty acid synthesis in rat mammary gland (30). Reduction of the oxaloacetate, derived from the cleavage of citrate, to malate would provide the substrate for NADPH production. Secondly, the activity of malic enzyme increases markedly with the onset of lactation and decreases with weaning (31,32). During the same period, the activity of isocitrate dehydrogenase in the cytosol is unchanged (31,32).

Utilizing the oxaloacetate produced from the cleavage of citrate to convert indirectly

NADH to NADPH would yield only one-half of the reductive power required for converting the acetyl-CoA, produced in the same cleavage, to fatty acid. Hence, NADPH must also be generated (a) from malate derived from some other source or (b) from the isocitrate dehydrogenase reaction (EC 1.1.1.42). Increased availability of NADH in the mitochondria would tend to increase the level of malate and isocitrate and, thereby, their chances for transfer to the cytosol. It has been shown in other tissues (33-35) that these substrates are transported to the cytosol by specific carriers. Direct measurement of organic anion transport across the mitochondrial membrane might help to determine which of these reactions is crucial to maximal fatty acid synthesis in mammary gland of lactating rat under these substrate conditions. Even without the direct measurements, these results provide additional evidence of mitochondrial cytosolic compartments interacting in support of fatty acid synthesis in this gland.

ACKNOWLEDGMENTS

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Lipid Composition of *Neurospora crassa*

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ABSTRACT

The lipids of *Neurospora crassa*, isolated in pure form from freeze-dried mycelium, were found to contain squalene, sterol esters, triglycerides, free fatty acids, geranylgeraniol, free sterols, carotenoids, cardiolipin, phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl serine, and phosphatidic acid. The above compounds were isolated in pure form by column and thin layer chromatography and were characterized by infrared spectroscopy and chromatographic mobilities. Fatty acid moieties were characterized by gas liquid chromatographic retention times of their methyl esters relative to those of authentic standards. The fatty acid composition of the triglycerides was found to be similar to that of phosphatidic acid, cardiolipin, and lecithin.

INTRODUCTION

The carotenoid (1-7) as well as the sterol (8-12) composition of *Neurospora crassa* has been extensively investigated in recent years. However, relatively little is known about the phospholipid composition (12, 13), and no report has yet appeared on the relative distribution of neutral and polar lipids and their subclasses, as well as the fatty acid composition of these lipid classes from *N. crassa*. In the present study, the nonpolar and polar lipids from this organism have been isolated and identified and their fatty acid composition determined.

MATERIAL AND METHODS

Cells of wild-type *N. crassa* were grown in a culture medium described elsewhere (7). Total lipids were extracted from freeze-dried cells according to Folch et al. (14), and total lipids were stored at −70 C in chloroform solution. Total lipids were fractionated on a silicic acid column as described by Sweeley et al. (15). Neutral lipids were eluted with 10 column volumes of chloroform (fraction I), glycolipids with 3 column volumes of 10% methanol in acetone (fraction II), and phospholipids with 10 column volumes of pure methanol (fraction III). Carotenoids in the total lipids were separated and identified as described elsewhere (7).

Sterol esters, triglycerides, and free fatty acids were purified from fraction I by thin layer chromatography (TLC) on Silica Gel H using the solvent system hexane:diethyl ether:acetic acid (85:15:1, by vol); geranylgeraniol and sterols (ergosterol + episterol) using the solvent system hexane:diethyl ether:acetic acid (65:35:1, by vol). Fraction II contained no lipid material. Cardiolipin (CL), phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), phosphatidyl serine (PS), and phosphatidic acid (PA) were purified from column fraction III on TLC using the solvent system chloroform: methanol:conc. ammonium hydroxide (65:35:5, by vol). PS was further purified by TLC using the solvent systems chloroform:

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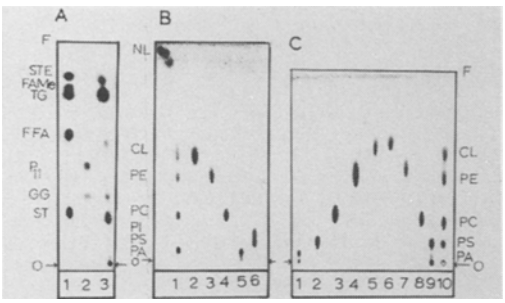

FIG. 1. Thin-layer chromatogram on Silica Gel H of lipids from *Neurospora crassa*. A: Nonpolar lipids in solvent system hexane:diethyl ether:acetic acid (65:35:1, by vol). B: Total polar lipids. C: Isolated polar lipid components in solvent system methanol: chloroform:conc. NH_4OH (65:35:5, by vol). Material chromatographed: (A) 1 = mixture of authentic cholesterol (ST), free fatty acid (FFA), triglyceride (TG), fatty acid methyl ester (FAMe), sterol ester (STE); 2 = mixture of authentic geranylgeraniol (GG) and undecaprenol (P_{11}); 3 = total lipids from *N. crassa;* (B) 1 = total lipids from *N. crassa;* 2-5 = authentic samples of cardiolipin (CL), phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), and phosphatidic acid (PA), respectively; 6 = mixture of authentic phosphatidyl serine (PS) and phosphatidyl inositol (PI); (C) 1-5 = isolated samples of PA, PS, PC, PE, and CL, respectively, from *N. crassa*; 6-9 = authentic samples of CL, PE, PC, and PS, respectively; 10 = mixture of 6-9. NL = neutral lipids; F = solvent front, O = origin.

TABLE 1

Lipid Analysis and Fatty Acid Composition of Individual Lipids from *N. crassa*

Lipid component	% by wt	Fatty acids (% by wt)						
		14:0	16:0	16:1	18:0	18:1	18:2	18:3
Sterol esters	0.6	0.2	10.7	3.0	3.8	17.1	58.7	1.7
Free fatty acid	1.5	1.1	38.3	5.4	3.8	13.0	35.2	0.9
Triglyceride	62.0	1.1	28.4	4.9	4.4	9.1	46.5	1.7
Phosphatidic acid	4.0	0.9	30.2	6.4	2.4	8.6	49.3	1.1
Cardiolipin	1.0	1.1	25.0	4.2	2.9	12.0	41.8	0.8
Phosphatidyl choline	10.0	0.4	21.0	5.6	3.4	12.2	55.2	1.5
Phosphatidyl serine	1.0	0.8	44.4	3.8	0.7	6.8	41.0	0.8
Phosphatidyl ethanolamine	5.0	0.2	40.1	4.7	1.5	8.9	41.0	0.8
Geranylgeraniol	2.0							
Sterols	4.0							
Squalene	<0.5							
Carotenoids[a]	0.2							

[a]Includes phytoene, phytofluene, and carotenes (see ref. 7).

methanol:water (65:25:4, by vol) and chloroform:acetone:methanol:acetic acid:water (6:8:2:2:1, by vol). Lipid spots were detected by specific spray reagents (16): ninhydrin for amino phosphatides, $(NH_4)_2MoO_4/HClO_4$ for phosphatides, iodine vapor for neutral lipids, and conc. H_2SO_4:ethanol (1:1, v/v) followed by charring for detection of all lipids. All lipid classes were identified by cochromatography on TLC with authentic standards and by infrared spectroscopy (16, 17).

The fatty acid composition of the isolated lipid classes was determined after transesterification with HCl-methanol (5% by wt) using a Hewlet-Packard Model 5830A gas chromatograph equipped with flame ionization detectors. Glass columns, 180 cm x 2 mm (ID), were used coated with 10% SP-222 (Supelco Inc., Bellefonte, PA). Peaks were identified by comparing their retention times to those of authentic standards and quantitated by a digital integrator.

RESULTS AND DISCUSSION

The total lipid extracted from the freeze-dried cells of *N. crassa* was 8.3% by wt, of which neutral lipids comprised 78% and polar lipids 21%. The major components of the neutral lipids fraction were triglycerides (62%), with smaller amounts of free sterols, sterol esters, geranylgeraniol, squalene, carotenoids, and free fatty acids; mono- and diglycerides were not detected (Fig. 1A, Table I). The sterols, shown previously (9,10) to be ergosterol and episterol, were not further investigated.

Several phospholipids were identified by TLC and infrared spectroscopy in the polar lipid fraction (CL, PA, PS, PC, and PE), of which PC and PE were the major components, accounting for 48% and 24%, respectively, of the total phospholipids (Fig. 1B,C, Table I). PS and CL were minor components (5% each), but the content of PA was unusually high (20% of phospholipids), similar to that reported for thermophilic fungi (18). Except for the high PA content, the phospholipid composition is similar to that for fungi in general (12,13).

The fatty acid composition of all lipid classes is shown in Table I. The major fatty acids of all subclasses were palmitic (16:0) and linoleic (18:2) acids, with intermediate amounts of oleic acid (18:1) and smaller amounts of myristic (14:0), palmitoleic (16:1), stearic (18:0), and linolenic (18:3) acids. The sterol esters contained low levels of 16:0 and high levels of 18:1 and 18:2 compared to all other subclasses. Free fatty acids, on the other hand, contained high levels of 16:0 and lower levels of 18:2. The relative proportions of fatty acids were remarkably similar in TG, PA, CL, and PC, with 16:0 and 18:2 being major fatty acids. The fatty acid compositions of PS and PE were very similar, the major acids being 16:0 and 18:2; the levels of 16:0 were much higher than in the other polar lipids.

In general, little is known about the fatty acid distribution of individual polar and nonpolar lipid components of fungi (12,19). However, the fatty acid composition of *N. crassa* appears to conform to the general fatty acid distribution of those fungi studied (12). Linoleic acid was the major fatty acid in sterol esters, as was reported previously for *Tricholama nudum* (20), while palmitic acid predominated in the free fatty acids, as reported also for *Choanephora cucurbitarum* (21). However, the similarity in the fatty acid composi-

tion of triglycerides of *N. crassa* to that of the phospholipids PA, CL, and PC was unusual since in triglycerides from most fungi the concentrations of 18:1 and 18:2 differ from those in the polar lipids (12). Also, in *N. crassa* only low concentrations of 18:3 were present both in polar lipids and neutral lipids, in contrast to other fungi in which 18:3 is higher in the polar lipids (12).

The similarity of the fatty acid composition of TG with that of the polar lipids, particularly PA, is surprising and would imply that triglycerides are derived from PA by dephosphorylation and incorporation of fatty acyl groups with composition similar to that of PA or the free fatty acid.

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Further Studies of the Saturated Methyl Branched Fatty Acids of *Vernix Caseosa* Lipid[1]

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ABSTRACT

By the method of capillary gas chromatography-mass spectrometry, we have identified 35 monomethyl and 46 dimethyl branched acids in the saturated acids of vernix caseosa lipid with chain lengths ranging from C_{11} to C_{18}. Many other mono-, di-, and trimethyl branched acids have been partially identified. All methyl branches were found to be on the even numbered C-atoms except for some terminal iso methyl groups. Three types of dimethyl branched acids were found: those with a terminal iso structure, those with a terminal anteiso structure, and those with neither iso nor anteiso structures. The 4-methyl branch predominated for all types of branched acids. Equivalent chain length data for di- and trimethyl branched acids were determined on a Pentasil (nonpolar) wall coated capillary column and checked by calculation from monomethyl branched acid data. Mass spectral identification was performed with and without the aid of a data system. A possible mode of formation of these acids is discussed.

INTRODUCTION

In an earlier study of the wax ester fatty acids of vernix caseosa lipid (VCL) it was reported that human sebaceous glands excrete a series of monomethyl and dimethyl branched acids (1). In that study, the positions of the methyl groups on the chains of the monomethyl branched acids were determined. The 12 ft packed gas chromatography (GC) column used, however, could not resolve the numerous components present, so that the location of more than one methyl group in a molecule could not be determined. We have now reexamined the saturated acids from a sample of total acids of VCL, i.e., not simply those derived from the wax esters (as was done in the earlier study) but also those derived from the sterol esters and the triacyl glycerols, the other main ester components of this sample. The branched acids were enriched by urea adduct removal of the straight chain material. We used gas chromatography-mass spectrometry (GC-MS) but with capillary columns having efficiencies of 150,000 to 200,000 theoretical plates. This enabled us A) to confirm the structures of the monomethyl branched acids previously reported and to report some new ones, B) to determine the position of both methyl groups of many of the dimethyl branched acids, C) to obtain evidence for the presence of very small amounts of trimethyl branched acids, and D) to obtain quantitative data on most of the monomethyl and some of the dimethyl isomers. We now present and discuss these findings. We also present a possible mode of formation of these acids.

MATERIALS AND METHODS

A sample of total saturated fatty acids of VCL (as methyl esters), and a fraction of these, enriched in the branched chain material, were prepared as described in detail in an earlier study (2). The samples were sealed in glass ampules and stored at -20 C till used. The work-up consisted of extraction of the VCL lipid with chloroform/methanol, 2:1 v/v, saponification, methylation of the total acids with BF_3 in methanol, and separation of the saturated from the unsaturated methyl esters by argentation chromatography. Scrupulous care was taken to avoid external contamination. A portion of the total saturates was sealed and stored. We used this stored sample in the present study to determine the distribution of fatty acids by quantitative GC (triangulation, Fig. 1A) after rehydrogenating it to remove a trace of unsaturated substances present (4). The saturates comprise 46% of the total acids of VCL. In the earlier study, another aliquot of the total saturated methyl esters was treated with urea to concentrate the branched chain material. Isothermal GC of this stored sample (also rehydrogenated as above) provided the equivalent chain length (ECL) data needed in the present study for

[1] For supplementary material to this manuscript, write to Micrographics Department of the University of Southern California, Library, University Park, Los Angeles, CA, 90007. Enclose a money order of $3.50 for postage and handling.

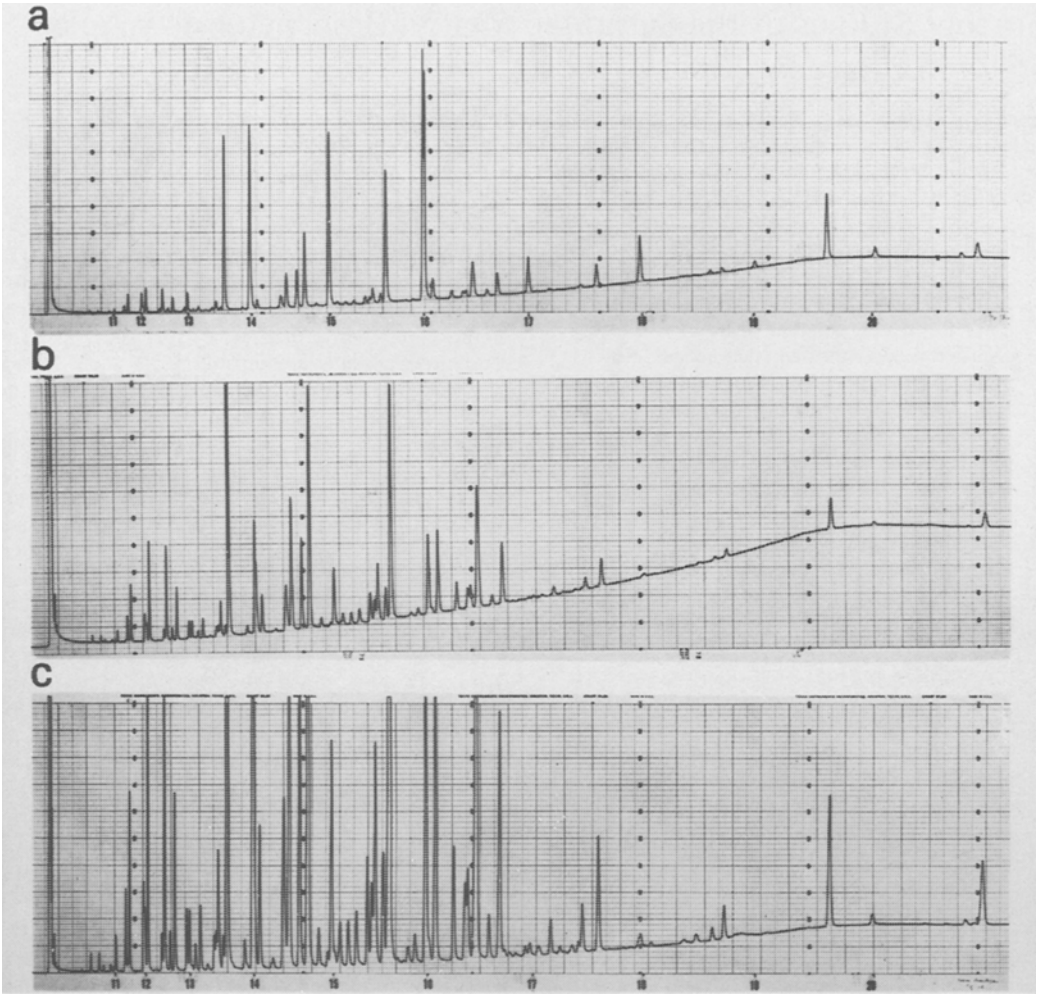

FIG. 1. A. Total saturated fatty acids (as methyl esters) of vernix caseosa lipid. B. Branched chain enriched saturated fatty acids (as methyl esters) of vernix caseosa lipid. C. Same as middle chromatogram except under high load. Gas chromatography (GC) conditions: Instrument, Beckman GC-4 modified as in (3); column, 1000 ft x 0.030 in. stainless steel coated with Pentasil (3); carrier gas, ultra high pure He at a flow rate of 8 cc/min; temperature, programmed from 170 C to 210 C in 160 min and constant thereafter; inlet and detector temperature 225 C; chart speed 10 in./hr.

structural assignments (3). This sample was analyzed by GC-MS using two procedures.

Procedure 1: To simplify the GC-MS analysis, we divided the total branched methyl esters into five fractions by preparative GC. This enabled us to increase the concentration of some of the minor components relative to the major ones and to choose GC conditions that allowed sufficient time for MS scanning of each component. Also, by not collecting the entire large amounts of iso and anteiso compounds, we avoided overloading the capillary column with these substances and thus diminished the extent of their tailing into subsequently emerging components.

The capillary GC-MS system used has been described in detail (3). Conditions for the GC are essentially those of Figure 1 except that we used a 500 ft rather than a 1000 ft capillary column coated with Pentasil, a nonpolar phase. Specific MS conditions used were: ion source 1×10^{-6} Torr, mass range scanned 27 to 400 amu, scan speed 2 sec/decade, slit width .030 in., oscillograph chart speed 16 in./sec, frequency response 300 hertz, jet line 240 C, ion source 130 C, probe 140 C.

Procedure 2: Procedure 2 was carried out at the duPont Applications Laboratory, Monrovia, CA. The total branched chain sample, not divided into fractions, was analyzed by the

same 1000 ft capillary column as used in Figure 1. The GC was a Varian model 2700 equipped with a flame ionization detector and temperature programmer. It was interfaced through a glass jet separator to a duPont mass spectrometer (MS-21-491) with a duPont data system (21-094). After the solvent peak appeared, repeated MS scans were taken of the entire chromatogram with the data stored on magnetic tape. The time of an MS scan cycle, scanning downward from 500 to 42 amu and resetting the magnet, was approximately 9 sec. The MS data was addressable via cathode ray tube and teletypewriter terminals. A Tektronix hard copier provided an additional output format. The MS data of a scan was presentable either as a bar graph (as in the light beam oscillographic tracings of Procedure 1) or in tabular format. The latter listed the total ion counts of the base peak and the percent of the base peak value for each m/e peak. To detect a minor component that was covered up by a large component, the computer could subtract from any other MS scan, either on an unadjusted ion count basis or on a percentage basis.

Structure assignments were based on A) GC retention time of each substance expressed as ECL, and B) its fragmentation pattern as it emerged from the capillary column and was fed into the MS. We started with a complete series of standard methyl monomethyloctadecanoates where the methyl branch varied in its location from the 2-position to the 17-position on the C_{18} chain. From the fragmentation patterns of each of these isomers (mass spectra and diagnostic ions are given in ref. 3), we were able to deduce the structures of the corresponding monomethyl branched isomers of shorter chain lengths. However, as the chain size decreased from C_{18}, the fraction of the ECL (attributable to the methyl branch at a specific position in the fatty acid chain) (FCL) for all the 4-Me isomers on chain lengths of C_{18} to C_{11} ranged from 0.47 to 0.51, and the 6-Me isomers ranged from 0.38 to 0.47 for these same chain lengths.

It is noteworthy that even on the 1000 ft capillary columns in some cases the ECL of 2 different monomethyl position isomers were very close together. For example, for the C_{12} straight chain length, the 4-Me and the 8-Me isomers have ECL values of 12.51_0 and 12.51_4, respectively. The subscript portions of these values represent uncertainty but indicate the order of emergence as found by the multiple ion detection (MID) technique as described in (3). In this technique, we plotted the intensity of the diagnostic ions (including the molecular ion) for successive scans over a single GC peak. From the position of the maximum of these plots, we determined the relative order of emergence of each component, and from the areas under these curves, we could make a rough approximation as to the amount of each component present.

We found that we could calculate the ECLs of the dimethyl isomers if we chose the corresponding FCLs of the series of monomethyl isomers having the same total number of C-atoms as in the dimethyl acid. For example, for 4,8-diMe-C_{13}, the total number of C-atoms of the fatty chain is 15. The calculated ECL would be 13.00 for the straight chain contribution plus FCLs of 0.49 and 0.43 for the rounded off values of the contributions of 4-Me and 8-Me, respectively, of the C_{15} monomethyl series (Ref. 3, Table 1) making a total of 13.92. This compares favorably with the value actually found by GC, namely, 13.90.

When we used this technique to calculate the ECLs of the trimethyl branched acids, we found the values to be generally too high. This was especially true for the isomers where the methyl branches were grouped together as in 2,4,6-triMe-C_{14}. It appears that in cases like these, the full retention effect of the methyl branches do not register as they do in the mono- and dimethyl branched acids. We found, however, that we could fit most of the ECL data of the trimethyl branched acids to those experimentally found if we assumed that the contribution of each methyl branch at any specific position was that which was found in the series of monomethyl branched acids where the total number of C-atoms was one more than that of the isomer in question. These were the values used to calculate the FCLs of the trimethyl branched acids. These acids were present in very small amounts and were usually buried in large amounts of other acids. This fact, together with the low observed ECLs mentioned above, makes their proposed structures tentative. The appearance of the appropriate molecular ion in an otherwise bare area of the mass spectrum, however, provides strong evidence for a trimethyl branched structure.

Except for the iso methyl group of iso acids, the position of the methyl branch was always located on an even number of C-atoms from the carboxyl carbon, despite our persistent efforts to find evidence for any branch on an odd numbered C-atom. Considering this, the FCLs of the monomethyl branched acids of VCL range from 0.27 to 0.73 for acids with a total carbon content of 12 to 19 C-atoms. Likewise, the FCLs of the two methyl branches of the dimethyl acids range from 0.63 (as in 2,8-diMe-C_{17}) to 1.23 (as in 4,8-diMe-C_{10}) if one calculates as above.

TABLE I

Monomethyl Branched Normal Fatty Acids of Vernix Caseosa Lipid Identified by Gas Chromatography-Mass Spectrometry (GC-MS)

Normal chains		Normal chains with a methyl branch at positions indicated											
		2-Me		4-Me		6-Me		8-Me		10-Me		12-Me	
	Wt %[a]	ECL[b]	Wt %	ECL	Wt %	ECL	Wt %	ECL	Wt %	ECL	Wt %	ECL	Wt %
C_{11}	.01[c]	-	-	11.51	.11	11.47	.01	11.55	.02				
C_{12}	.7	-	-	12.51	.42	12.45	.06	12.51+[d]	.21				
C_{13}	.6	-	-	13.50	.36	13.43	.07	13.47	.08	13.58	.08		
C_{14}	7.0	14.28	.01	14.50	1.2	14.40	.31	14.41	.62	14.50	.27		
C_{15}	6.5	15.28	.21	15.48	.82	15.40	.06	15.40	.38	15.45	.33	15.57	.47
C_{16}	18.6	16.28	.41	16.48	1.2	16.40+	.02	16.40	.25	16.42	.32	16.48+	1.2
C_{17}	2.0	17.28	.03	17.50	.20	17.40	.01	17.40+	.02	17.40++	.02	17.45	.04
C_{18}	2.5	-	-	18.49	.04	18.39	.02	-	-	-	-	-	-
sum	37.9		.66		4.35		.56		1.58		1.02		1.71
other	5.3												
total	43.2							9.88					

[a]All percents are weight percent of the total saturated fatty acids of vernix caseosa lipid (VCL). The saturates comprise 46% of all acids of VCL.

[b]Equivalent chain length.

[c]Identified by GC only.

[d]Order of emergence in the gas chromatogram of esters with the same ECL is ECL, ECL+, ECL++, etc.

[e]Includes n-C_9 < .001%, n-C_{10} .001%, n-C_{11} .01%, n-C_{19} .4%, n-C_{20} .7%, n-C_{21} .2%, n-C_{22} .7%, n-C_{23} .6%, n-C_{24} 1.7%, n-C_{25} .6% and n-C_{26} .4%, all identified by GC only.

TABLE II

Monomethyl Branched Iso Fatty Acids of Vernix Caseosa Lipid Identified by Gas Chromatography-Mass Spectrometry (GC-MS)

Iso acids with no further branches		Position of the additional methyl branch on the iso chain									
		2-Me		4-Me		6-Me		8-Me		10-Me	
ECL[a]	Wt %[b]	ECL	Wt %	ECL	Wt %	ECL	Wt %	ECL	WT %	ECL	Wt %
11.62	.40	–	–	12.13	.59	12.05	.07				
12.62	.06	–	–	13.12	.05	13.05	.01				
13.62	6.1	13.92	.01	14.12	.37	14.03	.03	14.03	.11		
14.62	1.6	14.89	.01	15.10	∿.12	15.02	.02				
15.62	6.4	15.89	.14	16.10	1.1	– ?	–	15.93 ?	<.01	∿16.05	∿1.0
16.62	.15	16.90 ?[c]	<.01	17.10	.02	17.00 ?	<01	16.95	.02		
17.62	1.3					17.99 ?	.10				
18.62	..28										
19.62	4.1										
sum	20.39		.16		2.25		2.23		.13		1.0
other[d]	10.61										
	31.00						3.77				

[a]Equivalent chain length.

[b]All percents are weight percent of the total saturated fatty acids of vernix caseosa lipid (VCL).

[c]A question mark between the ECL and percent columns indicates some doubt as to its identity or presence.

[d]Includes 9.60 .004%, 10.60 .001%, 20.60 .4%, 21.60 3.7%, 22.60 .007%, 23.60 4.7%, 24.62 .7%, and 25.60 1.1%, all identified by GC only.

TABLE III

Monomethyl Branched Anteiso Fatty Acids of Vernix Caseosa Lipid Identified by Gas Chromatography-Mass Spectrometry (GC-MS)

Anteiso acids with no further branches		Position of the additional methyl branch on the anteiso chain							
		2-Me		4-Me		6-Me		8-Me	
ECL[a]	%[b]	ECL	Wt %	ECL	Wt %	ECL	Wt %	ECL	Wt %
10.71	< .01			11.20	.05				
12.72	.49			13.20	.16	13.12	.01		
14.72	3.2	14.99	~.4	15.19	.18	15.10	~.07		
16.72	1.2	17.00 ?[c]	~.01	17.20	.13	17.10	~.02	17.10	~.01
18.72	.31								
sum	5.20		.41		.52		.10		.01
other[d]	4.61								
total	9.81						1.04		

[a]Equivalent chain length.

[b]All percents are weight percent of the total saturated fatty acids of Vernix caseosa lipid (VCL).

[c]A question mark between the ECL and percent columns indicates some doubt as to its identity or presence.

[d]Includes 10.71 .008%, 20.70 1.9%, 22.70 1.2% and 24.70 1.5%, all identified by GC only.

In the literature, one frequently finds as assignment of the iso or anteiso structure solely on the basis of retention data, i.e., FCL = 0.62 for iso and 0.72 for anteiso. Since two methyl groups on a fatty chain can also give each of these retention times, such assignments could be entirely in error. Even a normal acid could be confused with an acid having two methyl groups in the chain whose FCLs add up to 1.00, e.g., a 2,anteiso-dimethyl branched acid would have FCLs of .28 + .72 = 1.00 for many chain lengths. Other combinations adding up to 1.00 can also be found. Pitfalls such as these can be avoided by GC-MS, for the possible branched chain alternative structures will always have a higher molecular ion.

The mass spectral data obtained from both the light beam oscillograph and from computer printouts which were used for the assignment of the structures of the numerous compounds of this study (some 18 pages), together with the figures where the scans were made in each GC run, are available on microfiche (See title footnote 1). Additionally, a paper will be submitted for publication of the structures of the saturated branched chain fatty acids of adult human skin surface lipid which contains mass spectral and GC data similar to those used to support the structures reported here (5).

The monomethyl branched methyl esters for chain lengths other than the methyl monomethyloctadecanoates (Me-C_{18}'s), showed mass spectra very similar to those of the Me-C_{18}'s, and we were able to use the corresponding homologous diagnostic ions tabulated for the Me-C_{18}'s (3). These ions also provide a basis for assigning structures to the dimethyl compounds. Especially useful in this regard were the *a* ions, i.e., the methoxycarbonyl ion, $CH_3-O-\overset{O}{\overset{\|}{C}}-(CH_2)_n^+$ where the fatty chain is split on the ester side of the chain just before the methyl branch, the *a* + 1, and *a* + 2 ions (6); the *b* ions, $CH_3O-\overset{O}{\overset{\|}{C}}-(CH_2)_n-\overset{CH_3}{\overset{|}{C}}H+$, where the chain is split on the other side of the methyl branch to form a secondary ion 28 amu greater than the *a* ions; the *c* ions, which is *b* minus the loss of CH_3OH; and the *d* ions, which is the *c* ion minus H_2O (7,8). These ions could include an additional methyl branch between the point of the split in the fatty chain and the methoxycarbonyl group. Also useful were m/e = 88 and 87, the base peaks for 2-Me and 4-Me, respectively. When a 2-Me branch preceded a 6-Me branch, the useful M-76 ion for the 6-Me branch then shifted to M-90. This ion was very useful in identifying the 2,6-diMe compounds. The mass spectra of many dimethyl branched fatty acid methyl esters have been published, see (9) and references to G. Odham's earlier work therein, and (10) which lists many references to earlier work of J. Jacob and his collaborators.

RESULTS AND DICUSSION

The saturated fatty acids of VCL show a striking variety of skeletal types. Besides normal chains, containing odd and even members (43%), there are large amounts of iso (31%) and anteiso (9.8%) compounds. These percentages include relatively large amounts of homologues above C_{18}, especially iso acids (Tables I-III). This study shows that the interior of the chains of these basic skeletal

TABLE IV

Dimethyl Branched Fatty Acids of Vernix Caseosa Lipid other than Monomethyl Iso or Monomethyl Anteiso Acids Identified by Gas Chromatography-Mass Spectrometry (GC-MS)

	Me positions	ECL[a]	Wt %[b]	Me positions	ECL	Wt %	Me positions	ECL	Wt %	Me positions	ECL	Wt %
C_{11}				4,8-	12.03	.24						
C_{12}				4,8-	12.96	.15						
C_{13}	4,6-	13.92	<.01	4,8-	13.90	.10	4,10- ?[d]	14.01	~.06			
C_{14}	4,6-	14.85	.07	4,8-	14.85$_+$[c]	.07	4,10-	14.99	~.5			
C_{15}	4,6- ?	15.82	~.03	4,8- ?	15.82	~.06	4,10-	15.93	<.01	4,12-	~15.95	<.01
C_{16}										4,12-	16.95	.02
Total			.10			.62			.56			.02
C_{14}	2,6-	14.7	~.01	2,8-	14.7	~.01						
C_{15}	2,6-	15.7	~.01	2,8- ?	15.7	~.01				2,12- ?	15.77	?
C_{16}	2,6-	16.7	.04	2,8- ?	16.7	<.01	2,10- ?	16.7	<.01	2,12- ?	~16.73	~.06
Total			.06			.02						.06

[a]Equivalent chain length.

[b]All percents are weight percent of the total saturated fatty acids of vernix caseosa lipid (VCL).

[c]Order of emergence in the gas chromatogram of esters with the same ECL is ECL, ECL$_+$, ECL$_{++}$, etc.

[d]A question mark between the ECL and percent columns indicates some doubt as to its identity or presence.

TABLE V

Tentative Structures of Some Trimethyl Branched Fatty Acids of Vernix Caseosa Lipid Identified by Gas Chromatography-Mass Spectrometry (GC-MS)

	Observed ECL[a]	Calculated ECL[b]	Wt %
2,6,12i-triMe-C_{13}	14.28	14.30	<.01
4,8,12i-triMe-C_{13}	14.48	14.50	<.01
2,4,6-triMe-C_{14}	15.10	15.15	<.01
4,8,14i-triMe-C_{15}	16.42	16.47	<.01

[a]Equivalent chain length.

[b]See text in Materials and Methods as to how the calculations were made.

TABLE VI

Some Fatty Acids of Vernix Caseosa Lipid Detected but not Identified

ELC[a]	Amount	Possible structure[b]	Remarks[c]
9.12	.001	4,7i-diMe-C_8	n.s.
9.50	.002	4-Me-C_9	n.s.
9.79	.001	a diMe-C_9	n.s.
9.88	.001	a diMe-C_9	n.s.
10.12	.03	4,7i-diMe-C_9	n.s.
10.27	.001	2-Me-C_{10}	n.s.
10.51	.03	4-Me-C_{10}	n.s.
10.71	.008	8ai-Me-C_{10}	n.s.
11.12	.006	4,8i-diMe-C_{10}	n.s.
11.33	.004	?	n.s.
12.28	.003	2-Me-C_{12}	n.s.
12.33	.003	?	n.s.
13.32	.03	?	n.s.
13.72	.009	a 2,X-diMe-C_{13}	n.s.
14.31	.04	?	buried
14.50	?	a triMe-C_{13}	buried
14.95	.06	a diMe-C_{14}	n.s.
15.05	.009	a diMe-C_{14}	n.s.
15.32	.005	?	n.s.
15.45	?	a triMe-C_{14}	buried
15.50	?	a triMe-C_{14}	buried
15.72	.002	2,10-diMe-C_{15}	n.s.
15.78	.009	a diMe-C_{15}	n.s.
16.30	.002	?	n.s.
16.42	?	a triMe-C_{15}	buried
16.46	?	a triMe-C_{15}	buried
16.77	.01	a diMe-C_{16}	buried
16.82	.02	a diMe-C_{16}	n.s.
17.25	?	a triMe-C_{16}	buried
17.33	?	?	n.s.
17.60	.01	14-Me-C_{17}	n.s.
17.72	.001	a 2,X-diMe-C_{17}	n.s.
17.77	.001	a diMe-C_{17}	n.s.
17.82	.003	a diMe-C_{17}	n.s.
17.90	.004	a diMe-C_{17}	n.s.
18.10	.02	4,16i-diMe-C_{17}	n.s.
18.12	.001	?	n.s.
18.42	.003	a Me-C_{18}	n.s.
18.82	.001	a diMe-C_{18}	n.s.
18.92	.002	a diMe-C_{18}	n.s.
18.95	.001	a diMe-C_{18}	n.s.
18.97	.001	a diMe-C_{18}	n.s.
18.99	.002	a diMe-C_{18}	n.s.
19.28	.001	2-Me-C_{19}	n.s.
19.33	.001	?	n.s.
19.37	.002	a Me-C_{19}	n.s.
19.42	.001	a Me-C_{19}	n.s.
19.51	.002	a Me-C_{19}	n.s.
19.71	.001	a 2,X-diMe-C_{19}	n.s.
19.90	.001	a diMe-C_{19}	n.s.
20.05	.002	a diMe-C_{19}	n.s.
20.38	.003	a Me-C_{20}	n.s.
20.48	.003	4-Me-C_{20}	n.s.
20.66	.005	a 2,X-diMe-C_{20}	n.s.
20.93	.001	a diMe-C_{20}	n.s.
21.08	.01	a diMe-C_{20}	n.s.
21.40	.002	a Me-C_{21}	n.s.
21.48	.001	4-Me-C_{21}	n.s.
21.55	.003	triMe-C_{20}	n.s.
21.70	.007	a 2,X-diMe-C_{21}	n.s.
21.87	.003	a diMe-C_{21}	n.s.
21.89	.001	a diMe-C_{21}	n.s.
21.97	.02	a diMe-C_{21}	n.s.
22.04	.01	a diMe-C_{21}	n.s.
22.10	.004	4,20-diMe-C_{21}	n.s.
22.35	.001	?	n.s.
22.40	.001	a Me-C_{22}	n.s.
22.48	.004	4-Me-C_{22}	n.s.
22.97	.004	a diMe-C_{22}	n.s.
23.04	.001	a diMe-C_{22}	n.s.
23.42	.001	a Me-C_{23}	n.s.
23.48	.001	4-Me-C_{23}	n.s.
23.55	.004	a triMe-C_{22}	n.s.
23.70	.001	2,X-diMe-C_{23}	n.s.
23.90	.003	a diMe-C_{23}	n.s.

[a]Equivalent chain length

[b]i = iso; ai = anteiso; X = a methyl group at an unknown position.

[c]n.s. = not scanned; "buried" means that this compound occurs with a much larger amount of another substance.

types can contain one or more additional methyl branches. Although these internally branched compounds amount to only about 16% of the total saturates, they represent numerous species. Some 90 such compounds were identified here (Tables I-V). An additional 75 compounds, not identified by MS because they were either too small in amount or buried in a large amount of another component, showed, in many cases, GC retention data similar to those of the methyl branched compounds reported here.

The structures of these branched acids can perhaps best be comprehended by considering the manner in which they could be biologically synthesized (11). In this process, the fatty chain is built up by the successive addition of a number of "C_2-units" to a molecule called the starter or primer. Each "C_2-unit" is actually a sequence of four reactions occurring in the enzyme complex, fatty acid synthetase, where the first reaction is the addition of malonyl-CoA to the starter. Different starters, built up with C_2-units, form the basic carbon skeletons of the major fatty acids of VCL:

starter	type of chain
acetyl-CoA	straight even
propionyl-CoA	straight odd
isobutyryl-CoA	iso even
isovaleryl-CoA	iso odd
α-methylbutyryl-CoA	anteiso odd

In the buildup of the fatty chain, if a molecule of methylmalonyl-CoA substitutes for a molecule of malonyl-CoA, then a methyl branch will be placed at the point of substitution. Thus, if one molecule of methylmalonyl-CoA is inserted in a chain started either by acetyl-CoA or by propionyl-CoA and built up with additional molecules of malonyl-CoA, we would get the structures listed in Table I. If this inserted methylmalonyl-CoA molecule was the one which also terminated the chain, then the branch would be a 2-Me branch. If the methylmalonyl-CoA insertion was the next to the last molecule to extend the fatty chain, then the methyl branch would be on the fourth C-atom from the carboxyl carbon. Thus, the methyl branch will always occur on an even number of C-atoms from the terminating carboxyl carbon. Similarly, we can account for the buildup of the iso fatty chains of Table II if we start with isobutyryl-CoA or isovaleryl-CoA and proceed with the insertion of a molecule of methylmalonyl-CoA at the proper place. Table III lists the corresponding structures we would get if we started with α-methylbutyryl-CoA, i.e., the anteiso acids and the monomethyl branched anteiso acids.

When one methylmalonyl-CoA molecule is used with any of the last three starters listed above, we end up with a dimethyl acid because the second methyl branch is in the starter itself. However, if acetyl-CoA or propionyl-CoA is the starter, we can get a dimethyl fatty chain only if two molecules of methylmalonyl-CoA substitute for malonyl-CoA. Such structures do occur (Table IV). They constitute about 1% of the total VCL saturated acids. In like manner, two methylmalonyl-CoA molecules inserted into a chain begun with a methyl branched starter will result in a trimethyl compound. Three of the four molecules listed in Table V have this type of structure. The fourth—2,4,6-triMe-C_{14}—is one which, if it exists, would exemplify three methylmalonyl-CoA substitutions. The 75 compounds of Table VI amount to only about 0.5% of the total saturates of VCL. Many of these appear to be di- and trimethyl compounds.

Thus, in summary, the main bulk (90%) of the internally branched acids are monomethyl branched acids. They constitute about 14.5% of the total saturates of VCL. They can originate by substitution of one molecule of methylmalonyl-CoA for malonyl-CoA in the buildup of the fatty chains beginning with any one of five different starters. However, some dimethyl branched acids occur where both branches can come from methylmalonyl-CoA. This category would also include some trimethyl branched acids where the third methyl branch was in the starter. A minute amount of a trimethyl branched acid occurs where possibly all three branches originate from methylmalonyl-CoA.

If one compares the relative amounts of monomethyl branched acid with the amount of unbranched acid of a particular chain type in order to determine whether there is any preference of internal branching, one notes a slight preference as follows: straight odd > straight even = iso > anteiso. However, the preference is not remarkable, and, considering the accuracy of the data, the process could be an entirely random one. It appears that for many chain lengths an internally branched fatty acid molecule is formed for every three to five molecules with no internal branching.

In our earlier study (1), we could not find evidence for any 2-methyl branched acids. With the greater resolution provided by the capillary columns, we have found that 2-methyl compounds occur for many chain types, although in relatively small amounts. The major internally branched acid for all chain types is the 4-methyl compound, as was previously reported (1). For the straight chain monomethyl branched compounds, the 8-methyl and 12-methyl appear to be the next most abundant; the 2-methyl and the 6-methyl are the least abundant. Consistent with this order, the 4,8-dimethyl compounds are the most abundant of the internally branched dimethyl compounds. This order of abundance is not followed for the monomethyl branched compounds with branched starters. The latter seems to have a deterring effect for further methyl branching toward the ω-end of the fatty chain. The 4-methyl isomer, however, still predominates even for these acids.

The large variety of chain types present in VCL is a remarkable feature. However, the extent of the range in chain lengths is also remarkable. Here we report a range of C_9 to C_{26}, but these are not the actual limits at either end. Components below C_9 would be very volatile, and, since we made no special effort to collect these during the analysis of the major bulk of methyl esters, they would be lost. At the higher end, we have additional GC evidence that decreasing amounts of acids occur up to C_{30} and beyond. One wonders what is the functional significance of so many acids.

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Lipid Accumulation in Cells Derived from Porcine Aorta and Grown Under Anaerobic Conditions

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ABSTRACT

Fibroblast-like cells, derived from porcine aorta, were cultured under aerobic and anaerobic conditions. Light and electron microscopic examinations, lipid composition measurements, and incorporation of radioactive precursors into lipids of these cells were performed. Anaerobically grown cells accumulated oil red O stainable droplets and within 6 hr the triacylglycerol content increased to 4 times the level determined in cells grown under aerobic conditions. This ratio remained constant throughout an additional 12 hr of growth. The fatty acid composition of the triacylglycerols which accumulated under anaerobic conditions differed from the composition of fatty acids in the triacylglycerols present in the growth medium. The cellular unesterified fatty acids of the anaerobically grown cells differed only slightly in composition from the fatty acids in the growth medium, while the unesterified fatty acids of aerobically grown cells differed to a greater extent from those of the growth medium.

INTRODUCTION

Studies with inhibitors of oxidative metabolism (1,2) have shown that the lipid composition of cultured cells was altered in the presence of such compounds. Morphological studies of cells that had been treated with such inhibitors, or had been subjected to anaerobic growth, revealed granular cytoplasms and accumulation of lipid droplets within the cells (3.4). Because of the possible relevance of such phenomena to processes occurring within the thickened atherosclerotic intima (5-7), we have investigated the lipid composition of a line of cells cultured from porcine aortic intima grown under standard and anaerobic conditions. [Anaerobic conditions, produced by replacing air with prehumidified nitrogen do not preclude presence of low oxygen tension (3).]

MATERIALS AND METHODS

A cell line derived from an explant of aortic intima was kindly supplied by the Pathology Department of Albany Medical College of Union University. Certain characteristics of early generations of this cell line have been presented elsewhere (8,9).

The cells were grown on glass surfaces in BME-Hanks medium which was supplemented with porcine serum, 20 vol %. The medium and serum were obtained from the Grand Island Biochemical Co., Grand Island, NY. HEPES buffer, (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid), was present at 0.01 M, pH 7.4 for additional buffering capacity. Penicillin and streptomycin were present in the medium, each at a concentration of 100 units per ml. The medium was freshly prepared for each cell passage and was filtered under pressure through a 0.2 μ Gelman Metracel Filter. Passage of the cells was accomplished every 3 days by treating the cell culture with a 0.25% trypsin solution in BME-Hanks medium and subsequent suspension of the sloughed-off cells into fresh medium. In order to maintain optimal growth and to prevent propagation of any contamination, only those cultures exhibiting good growth were selected for further passage. Occasional samples of the medium were streaked on blood-agar plates to establish the absence of bacterial contamination. For the first 16 hr after passage, the cells were grown at 42 C, a step which has been cited by Hayflick (10) as a means of suppressing mycoplasma infection. All other manipulations of cells were performed in a walk-in incubator maintained at 37 C.

The high purity nitrogen used in this study was prehumidified in a water-filled washing bottle and passed through a Millipore Swinnex Filter with 0.2 μ pore size. The cells to be rendered anaerobic were grown out in Blake bottles for two days and then provided with fresh medium. Anaerobic conditions were achieved by bubbling nitrogen through the medium until bubbles reached the neck of the bottle at which time a silicone rubber stopper was aseptically inserted.

Isolation of Lipids

After trypsinization, the cells were washed

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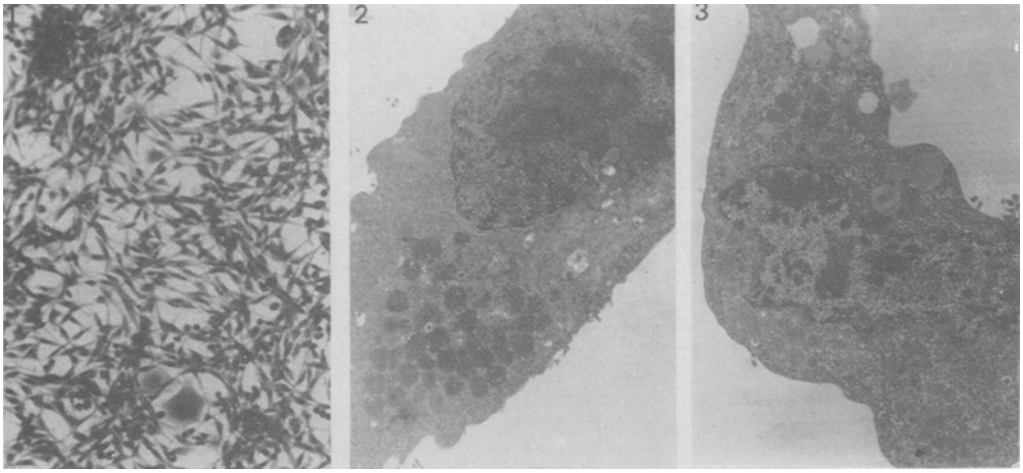

FIG. 1. Cultured cells grown under aerobic conditions. This field was selected because of the variety of cell forms present; haematoxylin-eosin (150x).

FIG. 2. Electron micrograph of aerobically cultured cell. The dense cellular bodies are clearly visible but mitochondria, several of which are present, are not well distinguished from the background; fixed in glutaraldehyde, stained with osmic acid in potassium dichromate (10,700x).

FIG. 3. Electron micrograph of an anaerobically cultured cell. Dense cellular bodies, also seen in aerobically cultured cells, are present but in addition, large droplets of low electron density are observed, two of which appear to have left their original locations. Fixing and staining same as in Figure 4 (10,700x).

by centrifugation (250 g), first with ice cold serum-containing medium and then with serum-free medium. The washed cells were lysed by brief agitating with a volume of water equal to 6.7 times the volume of packed cells and then by mixing well with 13.3 volumes of ethanol. Centrifugation at 1000 g yielded an ethanol/water extract which was set aside while the pellet was extracted in sequence with 20 volumes of ethanol/water (2:1 v/v), then twice with 20 volumes of chloroform/methanol (2:1 v/v). The ethanol/water extracts were combined and shaken with an equal volume of diethyl ether. The resulting homogeneous mixture was then thoroughly mixed with a volume of water equal to one-half the amount of ether employed in the previous step. The upper layer from the resulting two phase system was removed and the lower phase again extracted with diethyl ether. The chloroform/methanol extracts were combined, partitioned, and washed with the upper phase mixture described by Folch et al. (11). The chloroform and ether extracts were combined, taken to dryness, and redissolved in chloroform. The ethanol/water and methanol/water phases from the above extractions were combined and contained the water soluble lipid precursors. The final pellet from these extractions contained nucleic acids and protein. Nucleic acids were further purified by extraction with hot sodium chloride, alkaline hydrolysis, and perchloric acid precipitation (12).

Lipids obtained from 0.4 ml of packed cells were applied to a 3 g Unisil column (Clarkson Chemical Corp., Williamsport, PA), which had been previously washed with 40 ml of chloroform, 80 ml of acetone, and 40 ml of methanol in a manner similar to the procedure of Rouser et al. (13). The resulting fractions contained neutral lipids, glycolipids, and phospholipids in that order of elution.

The neutral lipids were separated by two-dimensional thin layer chromatography (TLC) on 20 x 20 cm glass plates to which had been applied 0.5 mm layers of Silica Gel G (Merck, Darmstadt). The plates were activated overnight at 110 C prior to use. Development in the first dimension was carried out with chloroform. After drying, the plates were developed at right angles to the first direction using butyl acetate/formic acid (95:5 v/v). When this solvent system had risen one-third of the way up the plates, they were removed and dried, and development was continued in the same direction using hexane/diethyl ether/formic acid (85:15:1 v/v/v). The lipids were located using iodine vapor and appropriate regions were transferred to centrifuge tubes. The lipids were recovered from the silica gel by elution with chloroform, followed by chloroform/methanol/formic acid (50:38:1 v/v/v) and finally twice more with chloroform.

Individual phosphatides were isolated using a

TABLE I

Composition[a] of Cells and of Growth Medium

	Aerobically grown cells	Anaerobically grown cells	Growth medium
DNA-phosphorus	6.7 ± 1.2 (4)[b]	7.6 ± 1.1 (4)	-
RNA-phosphorus	19.2 ± 2.5 (4)	17.4 ± 2.8 (4)	-
Inorganic phosphorus	8.4 ± 2.4 (5)	7.7 ± 0.9 (5)	1.05[c] ± 0.08 (9)
Organic phosphorus	11.3 ± 1.3 (3)	7.1 ± 1.3 (3)	-
Phospholipid phosphorus	19.6 ± 2.0 (9)	23.6 ± 3.6 (8)	0.24 ± 0.02 (5)
Monoacylglycerol	0.4 ± 0.2 (6)	0.4 ± 0.3 (6)	-
Diacylglycerol	0.6 ± 0.2 (6)	1.5 ± 0.8 (6)	-
Alkyldiacyl glycerol	0.2 ± 0.0 (2)	0.6 ± 0.2 (2)	-
Unesterified cholesterol	3.0 ± 0.5 (7)	4.1 ± 0.8 (7)	0.11 ± 0.01 (3)
Esterified cholesterol	0.5 ± 0.3 (7)	0.6 ± 0.6 (7)	0.44 ± 0.04 (3)
Triacylglycerol	1.9 ± 0.5 (7)	9.8 ± 2.3 (7)	0.19 ± 0.07 (3)
Unesterified fatty acid	0.5 ± 0.1 (3)	1.1 ± 0.1 (3)	-

[a]All results expressed in μmol ± standard deviation.

[b]Bracketed numbers represent the number of determinations performed in separate experiments of pooled cells. Values for both aerobically and anaerobically grown cells are expressed per 2.8 ± 0.8 x 10^8 cells packed at 250 x g for 15 min.

[c]Determinations expressed per ml of growth medium.

modification of the two dimensional system of Rouser et al. (14). Recovery of the phosphatides from the silica gel was accomplished by three elutions with methanol. The glycolipid fraction was not examined.

Analysis of Fatty Acids in Lipids

Aliquots of the isolated lipids were methylated (15) in a nitrogen atmosphere for ten minutes at 100 C with 2.5 ml of 14% boron trifluoride in methanol (Applied Science Labs, State College, PA). An internal standard of methyl heptadecanoate was added to each tube and the methyl esters were separated by hexane extraction and dried over anhydrous sodium sulfate. Analysis of fatty acids in each fraction was performed with a Barber-Coleman Model 10 Gas Chromatograph using a column packing of 15% HiEff-1-BP coated on 80-100 mesh Chromosorb-W (Applied Science Labs, State College, PA). The temperature of the column was 160 C while the argon ionization detector was maintained at 210 C. Nonlinearity of detector response was compensated for by comparison with experimental peak heights obtained with quantitatively injected methyl ester standards. The identification of methyl esters was established using retention times as compared to authentic standards and using James plots (16).

Additional Methodology

Triacylglycerols were determined by the method of Van Handel and Zilversmit (17), cholesterol by the procedure of Zak et al. (18), and phosphorus according to the method of Bartlett (19). DNA content was determined by direct phosphorus analysis or by the procedure of Burton (20).

Light microscopy of cells grown on cover slips was preceded by formalin fixation, staining with alum-haematoxylin and eosin counterstaining. Selected cover slips were stained with oil red 0 after fixation and counter-stained with alum-haematoxylin. For electron microscopic studies, cells in the milk dilution bottles were fixed in glutaraldehyde and cacodylate buffer, pH 7.2, scraped off the glass surface and pelleted by centrifugation, and then stained with 1% osmic acid in potassium dichromate.

"Student's" t test was used whenever the statistical significance of differences was determined.

Methods Pertaining to the Identification of Alkyldiacyl Glycerol

The neutral lipid migrating ahead of triacylglycerol after two-dimensional TLC was tentatively identified as alkyldiacyl glycerol. This substance, upon rechromatography employing the solvent system described by Snyder et al. (21), migrated to a region which was distinct from triolein and adjacent to palmityldioleoylglycerol, both of which were chromatographed in parallel lanes. When the cells were grown in the presence of [1-^{14}C]-hexadecanol and [9,10-^{3}H] palmitic acid, the suspected alkyldiacyl glycerol possessed a $^{3}H/^{14}C$ ratio much lower than that of the isolated triacylglycerol pool. A similar observation has been reported by Lumb and Snyder (22) for the preferential incorporation of fatty alcohols into the glyceryl ether lipids of certain tumor cells. Preliminary results for our laboratory indicated that this

TABLE II

Phospholipid Composition[a] of Cells and of Growth Medium

	Aerobically grown cells	Anaerobically grown cells	Growth medium
Lysophosphatidylcholine	1.2 ± 0.6 (7)[b]	1.5 ± 1.0 (8)	14.7 ± 7.6 (3)
Sphingomyelin	9.9 ± 1.5 (9)	9.0 ± 1.3 (9)	17.1 ± 3.6 (3)
Phosphatidylcholine	52.9 ± 3.2 (9)	51.9 ± 2.9 (9)	66.9 ± 5.3 (3)
Lysophosphatidylethanolamine	3.0 ± 2.2 (4)	1.4 ± 1.7 (4)	-
Phosphatidylserine	3.1 ± 1.0 (9)	2.5 ± 1.4 (9)	-
Phosphatidylinositol	3.7 ± 1.7 (9)	3.1 ± 2.1 (9)	tr.
Unknown	1.4 ± 1.7 (6)	3.3 ± 2.2 (8)	-
Phosphatidylethanolamine	22.3 ± 2.4 (9)	23.3 ± 2.1 (9)	tr.
Cardiolipin	2.6 ± 1.0 (9)	3.0 ± 2.6 (9)	-
Lysobisphosphatidic acid	1.2 ± 0.2 (5)	1.1 ± 0.2 (9)	-

[a]Results expressed as percentage of total lipid phosphorus ± standard deviation.

[b]Bracketed numbers represent the number of determinations performed in separate experiments on pooled cells.

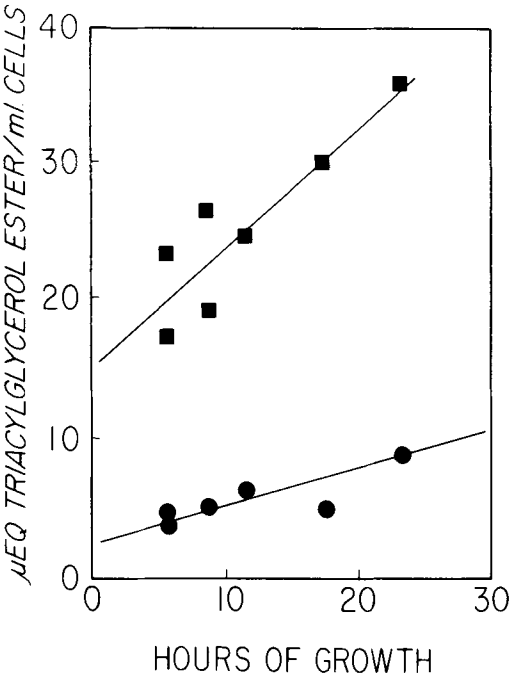

FIG. 4. Triacylglycerol accumulation during aerobic growth (•) and anaerobic growth (■). The triacylglycerols were isolated and determined as described in Methods section.

compound, identified as alkyldiacyl glycerol, was probably synthesized via the dihydroxyacetone pathway (23).

RESULTS

Cells grown under standard and anaerobic conditions are depicted in Figures 1-3. Almost all of the cells were spindle-shaped, fibroblast-like cells with a granular cytoplasm. Aerobically grown cells contained cytoplasmic granules as well as the usual complement of organelles (Fig. 2). In preliminary experiments, we determined that these cells had a doubling time of 21 hr, converted glucose to lactic acid quantitatively at an aerobic rate which was one-half of that observed anaerobically, incorporated linoleic acid into lipids at a rate at least one hundred times greater than conversion of linoleic acid to arachidonic acid, and lacked the ability to convert phosphatidylethanolamine to phosphatidylcholine via direct methylation.

Upon imposing anaerobiosis, characteristic morphological changes occurred which are illustrated in Figure 3. The vacuoles, observable by light and electron microscopy, were stainable by oil red 0. In electronmicrographs, these vacuoles were larger and of lower electron density than the cytoplasmic granules which were still present and also seen in aerobically grown cells. The anaerobic cultures sometimes grew at a rate comparable to that of controls but at other times simply failed to grow. The presence of 1 mM cyanide in the growth medium always resulted in stationary culture populations. Accumulation of lipid in the cultures invariably was observed whether cell division had or had not occurred. Phenol red indicator, present in the culture medium, and direct measurements of pH with a glass electrode were used to establish the fact that vacuole formation occurred at pH values from 7.2 to 7.8.

The overall composition of the cultured cells is presented in Table I while their phospholipid composition is listed in Table II. The unknown phosphatide listed in Table II migrated between phosphatidylserine and phosphatidylinositol but was not always completely resolved from these two phosphatides. Preliminary studies indicated that the unknown phosphatide possessed a fatty acid pattern similar to that of

TABLE III

Fatty Acid Composition[a] of Selected Cellular and Growth Medium Lipids

Lipid source	Fatty acids						
	14:0	16:0	16:1	18:0	18:1	18:2	20:4
Aerobic cells							
Diacylglycerol (1)[b]	1.7	21.0	1.4	30.4	34.2	11.1	tr.
Triacylglycerol (4)	3.7 ± 0.5	22.4 ± 1.8	4.0 ± 0.8	26.2 ± 2.5	36.8 ± 1.9	6.7 ± 2.5	0.2 ± 0.2
Unesterified fatty acids (6)	5.0 ± 1.6	35.6 ± 4.9	5.5 ± 1.4	29.9 ± 3.4	18.8 ± 3.4	5.3 ± 1.8	tr.
Anaerobic cells							
Diacylglycerol (3)	1.5 ± 0.3	15.7 ± 1.2	2.7 ± 1.9	32.2 ± 3.6	26.2 ± 2.8	12.0 ± 5.8	7.2 ± 6.4
Triacylglycerol (4)	2.8 ± 0.7	24.2 ± 3.5	3.5 ± 0.3	17.2 ± 3.7	38.8 ± 3.6	11.6 ± 3.7	2.1 ± 1.5
Unesterified fatty acids (6)	4.9 ± 3.7	34.1 ± 3.8	4.5 ± 1.3	25.7 ± 5.0	24.1 ± 7.5	6.5 ± 4.9	tr.
Growth medium							
Diacylglycerol[c]	4.1	22.0	2.2	11.8	50.8	9.0	-
Triacylglycerol	1.4	29.3	1.7	9.0	52.7	5.9	-
Unesterified fatty acids	3.1	34.7	3.1	23.0	32.9	3.4	tr.

[a]Results are expressed as percentage of total fatty ester, ± standard deviation.

[b]Bracketed numbers signify the number of determinations performed in separate experiments on pooled cells.

[c]Analysis of growth medium containing 20 vol % porcine serum.

phosphatidylserine. In cells grown in the presence of [2-^{3}H]- and [2-^{14}C]-glycerol, the unknown lipid exhibited a $^{3}H/^{14}C$ ratio higher than any other lipid isolated from the culture.

The only significant effects of anaerobiosis on lipid composition were the observed increases in triacylglycerol content and unesterified fatty acid concentrations (Table I). The ratio of anaerobic triacylglycerol to aerobic triacylglycerol was 4.3 ± 0.5 at all time intervals studied after anaerobiosis was initiated (Fig. 4). A similar but less striking relationship was also observed for the level of unesterified fatty acids, with the ratio of anaerobic to control levels being 1.6 ± 0.2.

Fatty acid compositions were determined for lipids in some experiments and the results are presented in Table III. The fatty acid patterns of the triacylglycerols, isolated from cells grown aerobically or anaerobically, were different than the fatty acid composition of triacylglycerols in the growth medium. This was most pronounced with regard to the ratio of oleic acid to stearic acid, which was 5.9 in the growth medium, 2.3 under anaerobic conditions, and 1.4 when the culture was grown aerobically. The composition of cellular unesterified fatty acids, either isolated from cells grown aerobically or anaerobically, more closely resembled the fatty acid pattern of the growth medium. The ratio of oleic to stearic acid was 1.4, 0.9, and 0.6 in unesterified fatty acid fractions of the growth medium, anaerobic, and aerobic cells, respectively.

DISCUSSION

The chemical composition of the cells in this study was similar to those reported for other cell lines. Cholesterol content, as well as the ratio of esterified to nonesterified cholesterol, fell within the range reported by Rothblat and Kritchevsky (24). The levels of unesterified fatty acids were also comparable to those of other cell lines analyzed by Howard and Kritchevsky (25). Total phosphatide and RNA were similar to the values found by Tsao and Cornatzer (26) for several cell lines and for other mammalian cells (27). DNA phosphorus values, although consistent, were lower than values listed in the literature because of the isolation technique employed.

Mackenzie et al. (28) reported that rabbit cells, when grown in the presence of rabbit serum, accumulated lipid droplets and the radioactive palmitic acid that was incorporated into the lipid droplets attained a specific activity only 80% of the specific activity of palmitic acid in the growth medium. More recently, Nelson et al. (29), studying the same rabbit system, estimated that one-third of the triacylglycerol in the rabbit cells was derived from serum lipoproteins in the growth medium. Brenneman and Spector (30), working with Ehrlich ascites tumor cells, reported that the Ehrlich cells utilized triglycerides contained in very low density lipoproteins and their data indicated that a portion of the triglycerides were taken up intact. In contrast to these findings, Bailey et al. (31) observed very little uptake of isotopic tripalmitoylglycerol by cell cultures, although it was not established in their study whether the radioactive compound was associated with serum lipoproteins. This group of investigators also reported that the palmitic acid in the cellular triacylglycerol pool attained a specific activity which was only 85% of the specific activity of palmitic acid in the medium. It would appear, from the results of other workers as well as our own, that exogenous triacylglycerols may be incorporated intact into the lipids of cell cultures.

The accumulation of triacylglycerols in various cell types was reported by Mackenzie et al. (32) to be associated with increased levels of extracellular fatty acids. These workers (33) had previously shown that cells cultured in a medium with a pH below 6.8 also accumulated lipid droplets. Similar effects of reduced pH on Ehrlich ascites cells have been reported by Spector (34) who also correlated such findings with an elevation of cellular fatty acid concentrations, and who further found this observation to be dependent upon the presence of albumin in the medium. In our study, we observed an increase in cellular fatty acids which approached the composition of unesterified fatty acids in the growth medium. It is possible that cellular anaerobiosis led to the elevation in cellular fatty acids by decreasing fatty acid oxidation (35).

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Changes in the Cholesterol and Phospholipid Content of Mouse Spleen after Rauscher Leukemia Virus Infection

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ABSTRACT

The effect of Rauscher Leukemia Virus (MuLV-R) infection on the lipid composition of mouse spleen from BALB/c mice was investigated. Drastic changes in the lipid composition of the spleen as a result of tumor growth induced by the virus could be demonstrated at 21 days after infection. The molar ratio of cholesterol to phospholipids was found to be low, while a shift within the choline containing phospholipid classes resulted into a lower sphingomyelin and a higher phosphatidyl choline content of the MuLV-R infected spleen. The cholesterol ester content increased more than two-fold during tumor growth, and shifts in the fatty acid patterns of the lipids were demonstrated.

INTRODUCTION

Infection of BALB/c mice with Rauscher Leukemia Virus (MuLV-R) causes a rapidly developing erythroblastosis in the spleen (1) in parallel with the reduction of mature elements. The general picture of the disease becomes that of an undifferentiated blastcell leukemia (2,3), resulting in an increase in number of undifferentiated cells in the infected spleens, which we will further refer to as "Rauscher cells." During the infection, the spleen increases enormously in size and, at 21 days after infection, most of the spleen consists of Rauscher cells (90-95%).

Also reported have been such changes in the peripheral blood as an increased number of normoblasts and reticulocytes (4). The latter may reflect a compensation for the severe anemia which occurs during the disease. Recently it has been proposed that different forms of leukemia are associated with changes in the lipid composition of white blood cells and plasma of man and also in lymphocytes and spleen of mice (5,6). It was suggested that lipid changes in cell membranes may lead to stimulation or reduction in cell growth (5,7,8).

Lipids appear to play a major role in determining the physicochemical properties of the cell surface membranes. Inbar and Shinitzky (5) have reported changes in membrane viscosity coincident with lipid changes of leukemic cells. The major membrane lipids, cholesterol and phospholipids, therefore are not only important building stones in the architecture of the cell membrane (9), but they are also important in determining the functional behavior of cell membranes. In fact, Inbar and Shinitzky have even proposed that these constituents of membranes may act as bioregulators for cell growth. We reported here that the MuLV-R induced erythroblastosis in the spleen of infected mice is accompanied by a marked alteration in the lipid composition of the tissue.

MATERIALS AND METHODS

Animals

Female BALB/c mice, 6-8 weeks old, received 0.2 ml (4 mg) of a purified MuLV-R preparation intraperitioneally (10). Mice were sacrified 21 days after inoculation by prolonged ether anaesthesia and spleens were collected,

TABLE I

The Choiesterol and Phospholipid Content of Control Mouse Spleen and Mouse Spleen at 21 days after MuLV-R-Infection[a]

	Cholesterol			Molar ratio
	Free	Esterified	Phospholipids	Cholesterol/Phospholipid
Control spleens[b]	3.20 ± 0.37	0.27 ± 0.07	14.64 ± 1.83	0.44 ± 0.02
MuLV-R spleens[c]	1.98 ± 0.06	0.65 ± 0.03	16.60 ± 1.23	0.24 ± 0.02

[a]Expressed as mg per gr wet weight ± S.E.M.

[b]20 animals per sample, 3 samples analyzed in duplicate

[c]8 animals per sample, 3 samples analyzed in duplicate (Data corrected for increased water content at 21 days of MuLV-R infection).

after inspection of their size, being the main parameter of infection (3). Spleens were then blotted with filter-paper, weighted, and used for lipid extraction.

Lipid extraction and analysis

Three groups of 20 spleens from noninfected BALB/c mice were analyzed as a control. For the analyses of spleens from MuLV-R infected mice, three groups of 8 spleens each were studied.

Extraction of lipids was carried out according to Folch et al. (11). The different phospholipids were separated by thin layer chromatography (TLC) on Silica Gel G (Merck, Darmstadt, Germany) and the solvent system used was chloroform:methanol:ammonia:water (70:30:2:3, by volume). Separation of phosphatidylserine from sphingomyelin was achieved using TLC on plates coated with a 3% (w/w) magnesium acetate-Silica Gel H (Merck, Darmstadt) mixture, using chloroform:methanol:ammonia (65:25:5, by volume) as a solvent system. The phospholipids were scraped off the plates after staining with iodine, and lipid phosphorus was determined according to Fiske and Subbarow (12).

Chromatographic separation of cholesterol, cholesteryl esters, and free fatty acids from the phospholipid fraction was carried out on Silica Gel G-plates using a mixture of petrolether: ether:formic acid (60:40:1.5, by volume) as solvent system. Rhodamine-6G was used to visualize the lipids in case of fatty acid analysis by gas chromatography. Cholesterol was estimated according to Ferro and Ham (13). Reference substances (Supelco, Inc. Bellefonte, PA) were used to identify the lipids.

Gas chromatography

The lipids were saponified with 15 ml methanolic HCl containing 26 g HCl/liter for 2 hr at 70 C and additionally with 2 ml of 8 M NaOH. After acidification with 8 M H_2SO_4 and extraction with pentane, the fatty acids were converted into their respective methyl esters by freshly prepared diazomethane.

For fatty acid analyses, a N_2-atmosphere was used to prevent oxidation. Gas liquid chromatography was carried out in a Hewlett-Packard instrument equipped with a flame ionization detector and a 10% polyethyleneglycol succinate column at 170 C.

RESULTS

The cholesterol, cholesteryl ester, and phospholipid composition of normal and MuLV-R infected spleens is given in Table I. After 21 days of infection, the free cholesterol content of MuLV-R infected spleens is decreased by 38%. Concomitantly, the phospholipid content is increased by 13%, resulting in a reduction of the molar ratio of free cholesterol to phospholipids by about 50%. The amount of esterified cholesterol is a more than two-fold increase compared to control spleens. The percent-distribution of the individual phospholipids is also different after viral infection (Table II).

TABLE II

The Percentage Distribution of the Major Phospholipid Classes in Control and MuLV-R Infected Mouse Spleens[a]

Phospholipid classes	Controls[b]	MuLV-R[c]
LysoLecithin	3.1 ± 0.2	1.9 ± 0.9
Phosphatidylserine	13.8 ± 0.9	9.1 ± 4.2
Phosphatidylinositol	2.2 ± 0.6	4.2 ± 2.0
Sphingomyeline	9.0 ± 0.9	4.5 ± 0.2
Phosphatidyl choline	44.3 ± 0.8	52.0 ± 1.3
Phosphatidyl ethanolamine	27.5 ± 0.5	28.4 ± 0.5

[a]Mean ± standard error.

[b]20 animals per sample, 3 samples analyzed in duplicate

[c]8 animals per sample, 3 samples analyzed in duplicate

Both the relative and absolute content of sphingomyelin of the MuLV-R infected spleens is reduced (controls: 132 ± 17 mg/100 gr wet weight; MuLV-R: 68 ± 5 mg/100 gr wet weight). This reduction contrasts with an increase in the phosphatidylcholine content (controls: 649 ± 81 mg/100 mg wet weight; MuLV-R: 787 ± 58 mg/100 gr wet weight). The amount of the choline containing phospholipids as percentage of the total phospholipid fraction remains rather constant after MuLV-R infection (56-58%). The molar ratios of sphingomyelin to lecithin that can be calculated are 0.21 ± 0.05 for control spleens and 0.09 ± 0.01 for MuLV-R spleens at 21 days after infection. Differences in the fatty acid patterns of the lipids of control and MuLV-R infected mouse spleens are shown in Table III.

The total lipids of MuLV-R spleens show a slight increase in plamitic acid (16:0) while the stearic acid (18:0) content is slightly reduced. The total amounts of saturated fatty acids remain fairly constant. The changes in palmitic acid and stearic acid as shown for the total lipids are also apparent in the isolated total phospholipid fraction of the MuLV-R infected spleens. Whereas the oleate (18:1) content of the total spleen lipids is clearly reduced, and the phospholipids of MuLV-R infected spleens contain relatively more oleic acid. The opposite is the case for arachidonic acid (20:4); i.e., the

TABLE III
The Major Fatty Acids of the Total Lipids and Phospholipids of Control Mouse Spleen and 21 Days after MuLV-R-Infection (% Total)

Total lipids	14:0	16:0	16:1	18:0	18:1	18:2	20:3	20:4	Others[a]	Unsaturated/Saturated Fatty Acid Ratio
Control[b]	+[d]	29.9 ± 2.3[e]	+	10.1 ± 0.6	20.8 ± 1.1	9.6 ± 0.4	3.1 ± 0.3	16.1 ± 2.3	10.4 ± 1.3	–
MuLV-R[c]	0.4 ± 0.3	34.5 ± 0.5	+	7.6 ± 0.6	13.3 ± 1.2	7.7 ± 0.9	1.5 ± 0.5	20.7 ± 1.9	14.3 ± 2.1	–
Phospholipids										
Control[b]	+	27.0 ± 0.4	+	14.3 ± 1.0	12.7 ± 0.5	4.6 ± 2.6	1.3 ± 0.7	26.0 ± 1.5	14.1 ± 0.1	1.1
MuLV-R[c]	+	30.6 ± 1.4	+	7.6 ± 2.5	17.7 ± 1.9	6.6 ± 0.4	1.6 ± 0.6	21.0 ± 0.8	14.9 ± 2.5	1:2

[a]Include 20:5 20:6 22:0 22:5 22:6 24:0 24:1
[b]20 animals per sample, 3 samples analyzed in duplicate
[c]8 animals per sample, 3 samples analyzed in duplicate
[d]+ = detectable level of <0.1%
[e]Mean ± standard error

total lipids of MuLV-R infected spleens contain more 20:4, while the isolated phospholipids contain less 20:4 than control spleens.

DISCUSSION

The analyses of infected mouse spleen, as described in this paper, demonstrate substantial changes in the lipid composition as a result of MuLV-R infection. Our data on the lipids of normal mouse spleen agree with the findings of Rouser et al. (14). With respect to the lipid composition of the MuLV-R spleens, our data differ from those of Redai et al. (6) probably due to the fact that the latter results are expressed per mg of protein. This manner of expression may obscure differences because the protein content of the MuLV-R infected spleen increased two-fold during MuLV-R infection (unpublished results).

The most important change in the lipid composition of mouse spleen after 21 days of MuLV-R infection was the reduction in free cholesterol. There was also a less pronounced increase in phospholipids, giving rise to a very low molar ratio of cholesterol to phospholipids. Concomitantly, the cholesteryl esters are more than doubled. Our results agree with the low molar ratios of free cholesterol to phospholipids described by Inbar and Shinitzky (5) for leukemic cells. From Table III, it can be seen that the ratio of unsaturated to saturated fatty acids remains fairly constant despite the drastic change in cholesterol to phospholipid ratio. Mainly because of a decrease in cholesterol content after MuLV-R infection, the overall membrane fluidity is expected to increase. In this respect, it is not clear what might be the effect of the observed increase of oleic acid and decrease of arachidonic acid in the phospholipids on the membrane fluidity.

The linoleic acid (18:2) content of the phospholipids has been reported to increase during aging of the erythrocyt (15), while significant differences have been reported between the various animal species (15, 16). However, no marked differences in the linoleic acid content of the red blood cell phospholipids of newborns and neonates with Rh-erythroblastosis have been found (17). Our studies on the fatty acid pattern of the total phospholipid fraction from mouse spleen and MuLV-R infected mouse spleen, which is very rich in large immature erythroblasts, also show no substantial differences in linoleic acid.

The phospholipid composition of red blood cells enriched with erythroblasts is not much different from adult erythrocytes and red blood cells from neonates (18). The relative decrease

of sphingomyelin of the mouse spleen after 21 days of MuLV-R infection agrees with the results by Gottfried (19) for blast cells in acute leukemias. In the erythrocytes of various animal species, the sum of the choline-containing phospholipids has been shown to be rather constant, though great differences in sphingomyelin and phosphatidyl choline content for the different animals have been demonstrated (15). The total amount of lysolecithin, sphingomyelin, and phosphatidylcholin, as presented in Table II, is rather constant for the control spleens (56.4%) as compared to the MuLV-R infected spleens (58.4%). To what extent the differences in cholesterol and phospholipids, in particular sphingomyelin, reflect the immature cell population rather than virus transformed cells remains to be determined.

In our laboratory, a method has been worked out to isolate pure Rauscher cells (Van 't Hull et al., to be published). Lipid analyses of these cells and their plasma membranes reveal the same lipid composition as described here for whole spleens after MuLV-R infection of the host (Montfoort et al. to be published).

These results are the first analyses of the lipid composition of an almost pure population of erythroblasts of the mouse. The extent to which the lipid composition of these cells is a consequence of the viral induced transformation or simply a reflection of the stage of development represents a problem that is common to many comparative studies of normal and transformed tissues.

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Effect of Early Postnatal Dietary Sterculate on the Fatty Acid Composition of Rat Liver and Brain Lipids

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ABSTRACT

Pregnant rats were fed a high carbohydrate diet containing either 1% trilinolein or 1% trilinolein with 0.2% methyl sterculate from 18 day gestation to 21 day postpartum. The pups were weaned at 21 days and continued on the same diet for an additional 10 days. The microsomal stearyl CoA desaturase activities of the liver were effectively inhibited. Liver triglycerides showed increases in the saturated fatty acids concentrations at the expense of the corresponding monoenes. The concentration of *cis* 6-7 octadecenoic acid was elevated. In liver phospholipids, the concentration of stearic acid was increased without a corresponding decrease in the oleic acid content. A drastic decrease in the nervonic acid (24:1, n-9) concentration of liver sphingomyelin was observed. The lipids of the brain did not contain sterculic acid, and brain desaturase activity was unaffected. There was no significant change in the concentration of monoenoic acids from 16:1 to 22:1. However, nervonic acid was decreased by 32%. These results suggest that brain nervonic acid may be derived from a precursor other than oleic acid.

INTRODUCTION

Studies on bacterial unsaturated fatty acid auxotrophs have shown that membrane permeability as well as the activities of certain membrane-bound enzymes change according to the degree of unsaturation of the fatty acids of the phospholipids (1-3). Manipulation of the degree of unsaturation of fatty acids in mammalian cells has been concentrated mainly on dietary restriction of linoleic and linolenic acids (4,5). Cyclopropene fatty acids are known to reduce the tissue concentration of oleic acid (6,7) and should also reduce the concentration of nervonic acid (24:1, n-9), since this acid is presumably produced by elongation of oleic acid (8,9). Nervonic acid constitutes about one-fourth to one-third of the total fatty acids in sphingomyelin of the liver and in the sphingomyelin, cerebrosides, and sulfatides of the brain. A procedure of manipulation of this acid in brain might be especially useful because myelin deficient mutant mice such as Quaking and Jimpy are known to be deficient in nervonic acid (10-12), and a model might thus be developed for the study of the function of nervonic acid in myelinogenesis.

The present study was undertaken to explore to what degree the fatty acid composition of liver and brain lipids can be modified by the early postnatal administration of dietary sterculate.

EXPERIMENTAL PROCEDURES

Two groups of pregnant rats (CDF strain, Charles River) were fed a fat-free high carbohydrate diet (General Biochemicals, Chagrin Falls, OH) containing 1% trilinolein or 1% trilinolein with 0.2% methyl sterculate from 18 day gestation to 21 day postpartum. Fresh diet was prepared and fed every day in order to avoid intake of oxidized fatty acids. The number of pups per dam was adjusted to 8 in order to ensure uniform growth. At 21 day postpartum, the pups were weaned and continued on their respective dams diet for an additional 10 days.

At the end of the feeding period, the animals were sacrificed and brain and liver were analyzed for microsomal stearyl CoA desaturase activity and fatty acid composition. Desaturase activities were determined by a procedure described elsewhere (13). Like brain microsomes, the liver microsomes gave high activity at pH 6.00, and there was no effect of addition of glycerophosphate.

Extractions of lipids from tissues were carried out as described elsewhere (14). Phospholipids and triglycerides were separated by thin layer chromatography (TLC) on Silica Gel H plates with hexane:diethyl ether:acetic acid (70:30:1.5) as developing solvent. Sphingomyelin was isolated by TLC on Silica Gel H plates using a solvent system consisting of chloroform:methanol:acetic acid and water (50:20:6:1). Glycolipids from total lipid extract from brain were isolated by Unisil column chromatography (15). Phospholipids were determined by phosphorus estimation after separating the lipids by two dimensional TLC, essentially as described by Nelson (16).

TABLE I

Stearyl CoA Desaturase Activities of Liver and Brain Microsomes of Rats Fed Methyl Sterculate

	Liver (pmol/min/mg protein)	Brain (pmol/min/mg protein)
Control	5600 ± 1100[a]	132 ± 12
Sterculate fed	1800 ± 130	152 ± 17

[a]Each value represents the mean ± SD for four different rats.

Fatty acid methyl esters were prepared from the isolated TLC bands by transesterification with 2% H_2SO_4 in methanol in a nitrogen atmosphere at 70-75 C for 1 hr for triglycerides and 16 hr for other lipids. Methyl esters were analyzed on a Perkin Elmer model 900 gas chromatograph equipped with a diethylene glycol succinate support-coated open-tubular column (150 ft x 0.02 in. inside diameter). Details of operating conditions, identification of fatty acids, and quantitation are given elsewhere (14).

Protein determinations were done by the procedure of Lowry et al. (17) with bovine plasma albumin as standard.

Materials

Methyl sterculate was prepared by the urea adduct procedure of Kircher (18) from *Sterculia foetida* seed oil. Analysis of the product by gas chromatography after treatment with silver nitrate in methanol (19) showed a purity of about 88% with the rest being mainly linoleic acid. Trilinolein of about 99% purity was obtained from NuChek Prep (Elysian, MN). [1-^{14}C] stearyl CoA, obtained from New England Nuclear Corp. (Boston, MA), was used without purification since paper chromatography using butanol:acetic acid:water (5:2:3) system showed 97% of the radioactivity in the long chain CoA spot, and gas radiochromatographic analysis of the methyl ester (Packard gas proportional counter model 894 attached to a Varian model 920 gas chromatograph column; 6 ft x ¼ in. packed with 10% diethylene glycol succinate on Gas Chrom P) showed all the radioactivity in methyl stearate. Unlabeled stearyl CoA (80%) NADH and albumin were purchased from Sigma Chemical Company (St. Louis, MO); fat-free diet (fat-free diet with added vitamin and zinc TD 72218; sucrose 58.45% casein 21.1% w/w) from General Biochemicals (Chagrin Falls, OH). We did not analyze the diet for its composition.

RESULTS

The final weight of the animals on cyclopropene fatty acid diet was about 15% lower than that of the control group (95.2 ± 8 vs. 81.5 ± 7 gm, mean ± SD for eight rats per group). However, there was no change in either brain or liver weights. The stearyl CoA desaturase activities of brain and liver microsomes are shown in Table I. The brain desaturase activity was considerably low in comparison to the liver. Dietary cyclopropene fatty acids did not alter it while liver desaturase activity was severely reduced.

TABLE II

Cyclopropene Content of Tissue Lipids of Rats Fed Methyl Sterculate[a]

	Cyclopropene fatty acids, in weight percent of total lipid
Brain	–[b]
Liver	0.15%
Adipose tissue	1.47%
Stomach contents[c]	0.53%

[a]Determined by Halphen Test (20).
[b]Limit of detection, > 0.03%.
[c]7 day old pups.

Lack of suppression of the desaturase activity suggested that no sterculate had entered the brain. This conclusion was supported by the estimation of cyclopropene fatty acids in brain, liver, adipose tissue, and stomach content of the pups (Table II). Even though appreciable amounts of cyclopropene fatty acids were detected in liver, adipose tissue, and stomach content of the young pups, no cyclopropene fatty acids could be found in the brain. That young pups do receive cyclopropene fatty acids through the milk is demonstrated by the analysis of stomach contents. Later experiments in which as much as 250 mg of methyl sterculate was fed by stomach tube to 15 day old rats failed to show any of it in the brain even after three days.

Since liver desaturase activity is severely reduced in sterculate-fed animals, liver lipid fatty acid composition should be affected. The fatty acid composition of liver triglycerides is shown in Table III. As expected, all the satu-

TABLE III

Fatty Acid Composition of Liver Triglycerides of Rats Fed Methyl Sterculate

Fatty acid		Control (mol %)	Experimental (mol %)
14:0		1.46 ± 0.23[a]	1.64 ± 0.26
15:0		0.16 ± 0.03	0.21 ± 0.03
16:0		26.54 ± 0.77	36.20 ± 0.16
16:1	(n-9)	1.03 ± 0.08	0.98 ± 0.04
16:1	(n-7)	9.50 ± 1.59	2.63 ± 0.30
17:0		0.24 ± 0.04	0.34 ± 0.06
18:0		2.63 ± 0.35	16.88 ± 0.76
18:1	(n-12)	0.13 ± 0.04	1.14 ± 0.21
18:1	(n-9)	46.32 ± 1.27	32.20 ± 1.34
18:1	(n-7)	5.80 ± 0.35	2.21 ± 0.30
18:2	(n-6)	6.18 ± 0.90	4.56 ± 0.54

[a]Each value represents mean ± SD for three different rats.

TABLE IV

Fatty Acid Composition of Liver Phospholipids of Rats Fed Methyl Sterculate

Fatty acid		Control (mol %)	Experimental (mol %)
14:0		0.33 ± 0.04[a]	0.23 ± 0.02
15:0		0.08 ± 0.01	0.06 ± 0.0
16:0		20.59 ± 0.32	19.66 ± 0.54
16:1	(n-9)	0.30 ± 0.04	0.37 ± 0.02
16:1	(n-7)	2.90 ± 0.53	0.68 ± 0.17
17:0		0.19 ± 0.02	0.15 ± 0.01
18:0		19.58 ± 2.14	27.84 ± 1.99
18:1	(n-12)	0.06 ± 0.04	0.47 ± 0.17
18:1	(n-9)	9.57 ± 1.18	7.79 ± 0.71
18:1	(n-7)	3.81 ± 0.43	1.33 ± 0.20
18:2	(n-6)	8.47 ± 0.47	12.47 ± 0.99
20:0		0.09 ± 0.01	0.14 ± 0.03
20:2	(n-9)	0.06 ± 0.06	0.23 ± 0.01
20:2	(n-6)	1.46 ± 0.52	2.47 ± 0.27
20:3	(n-9)	0.34 ± 0.06	0.87 ± 0.05
20:3	(n-6)	1.37 ± 0.13	2.28 ± 0.08
20:4	(n-6)	22.33 ± 0.43	16.43 ± 1.57
22:0		0.25 ± 0.03	0.39 ± 0.06
22:3	(n-9)	0.09 ± 0.01	0.10 ± 0.01
22:3	(n-6)	0.09 ± 0.01	0.14 ± 0.06
22:4	(n-6)	0.50 ± 0.05	0.29 ± 0.04
22:5	(n-6)	5.80 ± 1.43	3.83 ± 0.74
24:0		0.52 ± 0.03	0.63 ± 0.15
24:1	(n-9)	0.44 ± 0.06	0.17 ± 0.02
22:6	(n-3)	0.80 ± 0.08	0.83 ± 0.03

[a]Each value represents mean ± SD for three different rats.

rated fatty acids were increased at the expense of the corresponding *cis* 9 monoenes. The 16:1 (n-9) concentration was unaffected by dietary sterculate. An eighteen carbon *cis* monoenic fatty acid (deductions from hydrogenation studies and behavior on silver ion TLC) was increased several-fold in sterculate fed animals. This fatty acid ester eluted between stearate and oleate and is tentatively identified as 18:1 (n-12). *Cis* vaccenic acid, 18:1 (n-7), an elongation product of palmitoleic (16:1, n-7) acid was also reduced in sterculate fed animals.

Table IV shows the fatty acid composition of total liver phospholipids. The concentration of stearic acid was consistently higher in females than in males by about 10%, and this trend was observed in control and experimental animals. Values for females only are given in the table. Concentrations of palmitoleate and its elongation product, *cis* vaccenic acid, were reduced while the oleate concentration remained unchanged. Palmitate level remained the same but the level of stearic was increased by 43%. Linoleic acid concentration was increased with a decrease in its major desaturation and elongation product, 20:4 (n-6). Nervonic acid (24:1, n-9) concentration was reduced drastically in sterculate fed animals.

Since nervonic acid is primarily localized in liver sphingomyelin, an analysis of this lipid was carried out (Table V). Nervonic acid concentration was reduced by 65%. This decrease was compensated by an increase in stearic acid. Table VI shows that the reduction in nervonic acid content did not change the concentration of sphingomyelin among the liver lipids.

The fatty acid composition of the combined brain lipids, except gangliosides, is given in Table VII. In general, the brain fatty acids showed little change compared to liver lipids

TABLE V

Fatty Acid Composition of Liver Sphingomyelin of Rats Fed Methyl Sterculate

Fatty acid		Control (mol %)	Experimental (mol %)
14:0		0.38 ± 0.14[a]	0.35 ± 0.01
16:0		14.80 ± 2.03	16.97 ± 1.88
16:1	(n-9)	0.10 ± 0.03	0.23 ± 0.08
16:1	(n-7)	0.46 ± 0.18	0.20 ± 0.11
17:0		0.26 ± 0.02	0.37 ± 0.18
18:0		10.92 ± 0.84	29.02 ± 1.96
18:1	(n-9)	2.49 ± 0.55	1.89 ± 1.40
18:1	(n-7)	0.37 ± 0.04	0.26 ± 0.14
18:2	(n-6)	0.92 ± 0.65	0.70 ± 0.33
19:0		0.15 ± 0.03	0.24 ± 0.06
20:0		3.06 ± 1.40	5.59 ± 0.77
21:0		0.29 ± 0.11	0.18 ± 0.03
22:0		12.99 ± 0.65	14.52 ± 1.10
22:1	(n-9)	0.25 ± 0.07	
22:1	(n-7)	0.74 ± 0.05	0.18 ± 0.15
23:0		5.48 ± 0.28	3.63 ± 0.48
24:0		24.48 ± 1.38	17.05 ± 1.17
24:1	(n-9)	20.50 ± 0.41	7.48 ± 2.70
24:1	(n-7)	1.38 ± 0.15	1.14 ± 0.84

[a]Each value represents mean ± SD for three different rats.

TABLE VI

Liver Phospholipid Composition of Rats Fed Methyl Sterculate

Phospholipids	Control (mol %)	Experimental (mol %)
Diphosphatidyl glycerol	5.0[a]	4.5
Ethanolamine phosphoglycerides	21.9	22.6
Choline phosphoglycerides	57.7	55.3
Inositol phosphoglycerides	7.2	9.8
Serine phosphoglycerides	3.2	3.1
Sphingomyelin	3.4	3.5
Others[b]	1.6	1.2

[a]Each value represents mean for four different rats.

[b]Includes phosphatidic acid, lysophosphatidyl choline and lysophosphatidyl ethanolamine.

except for about a 30% reduction in the nervonic acid content.

Nervonic acid was reduced in sphingomyelin, cerebrosides, and sulfatides, with the least effect in cerebrosides (Table VIII). A decrease in lignoceric acid was also noted. Changes in the 24 carbon fatty acids were mainly compensated by an increase in palmitic and stearic acids.

DISCUSSION

Liver

The analysis of the effects of dietary sterculate on the fatty acid composition and desaturase activities confirms and extends previously published data (6,7,21-23). In liver triglycerides, the decrease in the concentration of palmitoleate and *cis* vaccenate is almost quantitatively compensated by the increase in palmitic acid. The decrease in oleic acid is compensated by an increase in stearic acid. Lack of change in the 16:1 (n-9) shows that this fatty acid is formed by a pathway not involving the cyclopropene-sensitive desaturase system. Synthesis of the n-12 isomer of 18:1 is stimulated in sterculate fed animals. In brain, the existence of a *cis* 6 desaturase system for saturated fatty acids, especially for palmitate, has been reported (23). Perhaps such a system is operative in liver for stearate. Thus, sterculate is effective in inhibiting the *cis* 9 desaturase and not the *cis* 6 desaturase of the liver.

The direct quantitative relationship of increase in saturated fatty acids with decrease in the corresponding *cis* 9 desaturated product as found in liver triglycerides is not seen in liver phospholipids. It is not clear why the palmitate level remained the same when palmitoleate and *cis* vaccenate levels are reduced. The increase in stearic acid is not compensated by a decrease in oleate. An alternate route for the biosynthesis of oleic acid which is not inhibited by cyclopropene fatty acid and provides oleic acids preferentially to phospholipids has been reported (25). This could explain the lack of significant reduction in the oleate concentration even though stearate level is increased.

TABLE VII

Fatty Acid Composition of Brain Lipids of Rats Fed Methyl Sterculate

Fatty acid		Control (mol %)	Experimental (mol %)
14:0		0.26 ± 0.01[a]	0.26 ± 0.02
15:0		0.06 ± 0.01	0.06 ± 0.02
16:0		20.99 ± 0.10	21.34 ± 0.46
16:1	(n-9)	0.32 ± 0.02	0.28 ± 0.10
16:1	(n-7)	0.63 ± 0.04	0.44 ± 0.03
17:0		0.16 ± 0.01	0.16 ± 0.02
18:0		20.36 ± 0.32	20.93 ± 0.27
18:1	(n-9)	17.05 ± 0.16	16.69 ± 0.87
18:1	(n-7)	4.52 ± 0.10	3.75 ± 0.04
18:2	(n-6)	0.51 ± 0.02	0.83 ± 0.07
20:0		0.59 ± 0.06	0.65 ± 0.14
20:1	(n-11)	0.07 ± 0.01	0.11 ± 0.01
20:1	(n-9)	1.20 ± 0.12	1.13 ± 0.07
20:1	(n-7)	0.42 ± 0.05	0.35 ± 0.01
20:2	(n-6)	0.20 ± 0.02	0.16 ± 0.01
20:3	(n-9)	0.17 ± 0.04	0.21 ± 0.03
20:3	(n-7)	0.26 ± 0.02	0.21 ± 0.10
21:0		0.11 ± 0.02	0.06 ± 0.02
20:3	(n-6)	0.44 ± 0.02	0.56 ± 0.01
20:4	(n-6)	11.16 ± 0.29	11.89 ± 0.15
22:0		0.68 ± 0.08	0.54 ± 0.09
22:1	(n-11)	0.06 ± 0.02	0.08 ± 0.05
22:1	(n-9)	0.17 ± 0.02	0.12 ± 0.03
22:1	(n-7)	0.14 ± 0.01	0.09 ± 0.02
22:3	(n-9)	0.11 ± 0.03	0.09 ± 0.04
23:0		0.07 ± 0.05	0.05 ± 0.02
22:4	(n-6)	3.29 ± 0.05	3.58 ± 0.12
22:5	(n-6)	6.02 ± 0.97	4.86 ± 0.33
24:0		0.74 ± 0.13	0.60 ± 0.04
24:1	(n-9)	1.55 ± 0.05	1.08 ± 0.05
24:1	(n-7)	0.12 ± 0.05	0.13 ± 0.04
22:6	(n-3)	7.51 ± 0.39	8.57 ± 0.24
24:4	(n-6)	0.19 ± 0.03	0.17 ± 0.05

[a]Each value represents mean ± SD for three different rats.

TABLE VIII

Fatty Acid Composition of Brain Sphingomyelin, Cerebroside, and Sulfatides of Rats Fed Methyl Sterculate

	Sphingomyelin		Cerebrosides		Sulfatides	
Fatty acid	Control	Experimental	Control	Experimental	Control	Experimental
14:0	0.51 ± 0.03[a]	0.20 ± 0.03	0.39 ± 0.03	1.32 ± 0.12	0.48 ± 0.15	1.31 ± 0.24
16:0	5.00 ± 0.04	6.50 ± 1.49	3.52 ± 0.33	4.96 ± 0.90	0.95 ± 0.26	8.68 ± 1.15
18:0	68.33 ± 0.35	73.44 ± 2.08	11.61 ± 0.98	15.89 ± 2.03	10.34 ± 2.60	31.52 ± 4.00
18:1 (n-9)	3.36 ± 0.71	1.51 ± 0.59	1.30 ± 0.27	2.05 ± 0.21	0.96 ± 0.21	2.93 ± 0.38
18:1 (n-7)	0.39 ± 0.12	0.23 ± 0.06	0.26 ± 0.06	0.44 ± 0.10	--	0.23 ± 0.03
20:0	4.23 ± 0.12	4.61 ± 0.57	4.50 ± 0.82	6.89 ± 1.26	3.68 ± 0.33	5.22 ± 0.59
22:0	3.87 ± 0.24	3.36 ± 0.85	12.94 ± 0.02	12.20 ± 1.17	12.89 ± 0.55	10.51 ± 1.18
22:1 (n-9)	--	--	0.18 ± 0.10	0.34 ± 0.09	0.10 ± 0.02	0.17 ± 0.02
22:1 (n-7)	0.62 ± 0.01	0.26 ± 0.04	1.43 ± 0.45	1.40 ± 0.29	1.15 ± 0.06	0.78 ± 0.09
23:0	0.37 ± 0.09	0.28 ± 0.05	1.80 ± 0.10	1.55 ± 0.23	1.50 ± 0.69	0.54 ± 0.06
24:0	3.98 ± 0.13	2.90 ± 0.97	25.09 ± 2.22	21.88 ± 0.77	31.29 ± 1.73	18.26 ± 2.32
24:1 (n-9)	9.00 ± 0.76	6.55 ± 0.36	34.82 ± 1.59	29.35 ± 1.73	34.72 ± 1.07	19.61 ± 2.24
24:1 (n-7)	0.40 ± 0.16	0.15 ± 0.07	2.15 ± 0.92	1.73 ± 0.37	1.96 ± 0.42	0.24 ± 0.03

[a]Each value represents mean ± SD for three different rats. All values are expressed as mol %.

Since the 16:1 (n-9) concentration is unchanged, it is difficult to assess whether this acid could act as a precursor for oleate synthesis by the alternate route. Of course, the possibility exists the 16:1 (n-9) is not a biosynthetic product but a partial oxidation product of oleate.

The increase in linoleic acid and decrease in the major desaturation and elongation products of linoleic acid, 20:4 (n-6), suggest that sterculate inhibits one or more of the desaturation steps. Decrease in the product of arachidonic acid resulted in an increased formation of 20:3 (n-9), a phenomenon similar to that observed in essential fatty acid deficiency condition (26). It appears that lack of arachidonic acid, not of linoleic acid, stimulated the production of 20:3 (n-9).

The nervonic acid concentration of liver sphingomyelin from sterculate fed animals was drastically reduced even though the liver phospholipid oleate concentration remained unchanged. This suggests that the alternate route does not provide oleate for nervonic acid biosynthesis.

Brain

Lack of inhibition of the brain microsomal stearyl CoA desaturase activity, absence of any cyclopropene fatty acids in the brain, and lack of any major change in the saturated and monoenoic fatty acids of brain lipids show that there are effective barriers for the passage of sterculate into the brain. This is surprising since fatty acids such as linoleic and linolenic enter the rat brain even in the adult (27,28). Blood brain barrier is generally considered to be developed after the weaning period (29). Even in the brains of 7 day old pups, no cyclopropene fatty acids were detected, suggesting that sterculate entry into brain is obstructed by a mechanism different from that of the classical blood-brain barrier phenomenon.

The most striking effect of dietary sterculate is the reduction in the proportions of 24:0 and 24:1 in brain sulfatides. Even though cerebrosides and sulfatides have very similar fatty acid composition, the effect on cerebrosides fatty acids is small. Myelin-deficient mutant mice show decreases in long chain fatty acids and increases in 16:0 and 18:0 in both cerebrosides and sulfatides (11,12). The observed changes in the fatty acid composition of sulfatides of sterculate fed animals may thus reflect mild changes in the myelin metabolism. It will be interesting to see whether myelin content and sphingolipid levels are affected by dietary sterculate.

The decrease in the nervonic acid concentration in sphingolipids of the brain is difficult to explain, since no cyclopropene fatty acids were detected in the brain. The lack of change in the oleate concentration suggests that oleate may not be the precursor for nervonate. The possibility cannot be excluded that the nervonic acid of the brain is in part supplied from liver, the decreased level of this acid in brain sphingolipids thus being the result of the drastically reduced level in the liver. Whatever the mechanism, the present study shows that dietary sterculate can alter brain sphingolipid fatty acid composition. Higher dietary levels and more prolonged periods of feeding could probably produce marked changes.

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Retarding Effects of DNA on the Autoxidation of Liposomal Suspensions

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ABSTRACT

Deoxyribonucleic acid (DNA) is associated with the cell membrane of prokaryotes and the inner nuclear membrane of eukaryotes. The unsaturated fatty acids of phospholipids, which constitute the bilaminar structure of membranes, undergo autoxidation in the presence of O_2. Calf thymus DNA was incubated with methyl archidonate-enriched phosphatidyl choline liposomes in order to study the effect of DNA upon the oxidation of phospholipids while present in their natural in vivo bilayer configuration. DNA retarded the rate of lipid oxidation as monitored by both diene conjugation and the TBA test, but it did not alter the induction period. These results suggest that DNA is scavenging free radicals produced within the phospholipid bilayer.

INTRODUCTION

Free radical oxidation of lipid has been studied in varying degrees of ordered complexity: in pure solutions (1), aqueous emulsions (2), micelles (3), and membrane bilayers (liposomes) (4,5). The free radical reactions in these various systems may not necessarily be identical, since it has been suggested that ordering lipids in a bilayer may present a physicochemical environment favoring certain reaction pathways (6). The addition of other molecular species to emulsions and bilayers can alter both the time of onset and/or the rate of lipid oxidation, as well as the type and yield of final products (7-10). Oxidation of the unsaturated fatty acids of membrane phospholipids can therefore be modulated by the local chemical environment, in addition to the structural features of the membrane.

The major constituents of a biomembrane include phospholipids and proteins. The latter are arrayed as a loose matrix throughout the bilayer and may penetrate through the membrane itself, having chains of hydrophobic amino acids in that portion of the protein molecule that crosses the hydrophobic midzone (11). Others have shown that the types of amino acids that cross through the hydrophobic midzone can inhibit free radical reactions (12). Hence, in addition to the types of phospholipids in the micro-environment of the membrane, the presence of macromolecules, like proteins, can add potent chemical and structural modulating factors.

Another type of macromolecule, deoxyribonucleic acid (DNA), although not intrinsic to membrane structure, can be closely membrane-associated (13-15). In view of the reported role of this association in bacteria, and the finding that associations exist between DNA and the nuclear envelope in eukaryotes, although no function has been found, it seemed important to determine the consequences of DNA interaction with model membranes undergoing free radical damage.

For this purpose, incubation mixtures of liposomes and calf thymus DNA were studied. Since liposomes are highly organized bilayers, they provide a model of in vivo membrane phospholipid configuration (16,17). The content of polyunsaturated fats was increased by enrichment with methyl arachidonate in order to enhance the rate of lipid oxidation in the bilayered system. This procedure avoids the hazards of using agents such as metals and ultraviolet (UV) irradiation which, in addition to enhancing the rate of lipid oxidation, would also directly damage the DNA. Lipid oxidation was monitored colorimetrically by the thiobarbituric acid method (5) and spectroscopically by diene conjugation (18). The results suggest that DNA can modulate the kinetics of oxidizing liposomal suspensions. The effect of this lipid oxidation on the biological activity of *Bacillus subtilis* DNA was also studied and will be published separately.

MATERIALS AND METHODS

L-α-ovolecithin (PC) was obtained from Grand Island Biological Co. (Grand Island, NY); methyl arachidonate (MA) was from Sigma Chemical Co. (St. Louis, MO); methyl esters of linoleic, oleic, stearic, palmitic and myristic acid were obtained from Supelco, Inc. (Bellefonte, PA). Sonication was performed in a bath type sonicator from Heat Systems, Ultrasonics, Inc. (Plainview, NY). The Rinco flash evaporator was from Rinco Instrument Co., Inc. (Greenville, IL). The desk top sorvall angle centrifuge was from Ivan Sorvall, Inc. (Nor-

walk, CT), and the desk top model CL International clinical centrifuge was from International Equipment Co. (Needham, MA). Water was 18 megaohm and 2,6-ditertbutylphenol was from ICN–K+K Lab., Inc. (Plainview, NY). Centrifuge tubes were cellulose nitrate ½ in. diameter x 2 in. obtained from Beckman (Palo Alto, CA). Ribonuclease-free density gradient grade sucrose was obtained from Mann Research Laboratories (New York, NY). Type 5 G glass cuvettes from Precision Cells Inc. (Hicksville, NY) were used for all spectrophotometric assays in the visible range, while Suprasil quartz cuvettes from the same manufacturer were used for all absorbance measurements in the ultraviolet region. 2-Thiobarbituric acid was from Sigma Chemical Co. Calf Thymus DNA (Type V) was obtained from Sigma Chemical Co. Cellulose Dialysis Tubing (5/8 in. diameter) was obtained from Arthur H. Thomas (Philadelphia, PA). Sephadex G10 was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). All solvents and chemicals were reagent grade.

Liposome Preparation

Liposomes were prepared from L-α-ovolecithin (PC) alone or from PC and methyl arachidonate (MA), in proportions of 82 mol percent PC and 18 mol percent MA (PCMA). Final total lipid concentration was 10 mM in both cases. The lipids were added to a 100 ml round bottom flask and the organic solvents (chloroform for PC and cyclohexane for MA) were evacuated on a Rinco flash evaporator at 33 C, resulting in a dry film of lipid coating the flask wall. Then 0.05 M sodium phosphate buffer, pH 7.5, was added and the resulting suspension was vortexed for 2 min and then sonicated 20 min.

Gas Liquid Chromatography

Aliquots of each liposomal suspension (0.5 ml) were added to 10 ml chloroform and 5 ml methanol containing 25 mg 2,6-ditertbutylphenol in a screw cap test tube and allowed to stand for 20 min at room temperature. A solution of 3×10^{-2} N HCl (10 ml) was layered on top and the tube inverted carefully allowing the methanol to enter the top aqueous phase. The sample was then centrifuged at 2000 rpm in a desk top sorvall angle centrifuge for 30 min to obtain a distinct separation of layers. The chloroform layer was removed and evaporated to dryness under a nitrogen stream. A 9% H_2SO_4 in MeOH solution containing 0.1% benzene was added (0.1 ml) and the tube flushed with N_2, capped and incubated overnight at 65 C. Water (1.0 ml) was then added and the methyl esters extracted with 2 ml petroleum ether, concentrated and stored at -20 C under N_2. Approximately 5 μl samples were applied to a stainless steel Silar 10c column 6 ft by ¼ in. inner diameter, contained in a Hewlett Packard 7620 A gas chromatograph equipped with a Hewlett Packard integrator. The injection port and flame ionization detector temperatures were set at 270 C and the N_2 carrier gas flow rate was 27 ml/min. The oven temperature was programmed as follows: initial temperature 200 C for 2 min, then an increase of 1 C per min up until 230 C and held at this temperature for 10 min. Methyl esters were identified by comparison with retention times of authentic standards.

Sucrose Density Gradient Centrifugation

A 4.5 ml sucrose gradient in water (0-5%) was layered over a 20% sucrose cushion (0.5 ml) using a Buchler Density Gradient Maker. Samples of 0.5 ml were gently layered over the gradient. Centrifugation for 3½ hr at 35,000 rpm was performed on a Beckman Model L centrifuge equipped with a SW 39 rotor. Fractions of 11 drops were collected by puncturing the bottom of the centrifuge tubes with a needle. Each fraction was assayed for optical density at 540 nm (PC and PCMA) or 300 nm (MA emulsion) on a Beckman DU spectrophotometer.

DNA Preparation

Dialysis: A stock solution of DNA (2 mg/ml) in 0.05 M sodium phosphate buffer, pH 7.5, was prepared. An aliquot of this solution (15 ml) was then dialyzed sequentially against four 1-liter quantities of 0.05 M sodium phosphate buffer, pH 7.5, at 4 C. The dialysate was mixed continuously by magnetic stirrer.

Sephadex column chromatography: A stock solution of DNA (0.5 mg/ml) in 0.05 M sodium phosphate buffer, pH 7.5, was prepared. An aliquot of this solution (2 ml) was passed through a column packed with Sephadex G-10 (total bed volume, 15 ml). The excluded sample volume was collected and used as a purified DNA sample.

Liposomal Incubation

Liposomes as described above were incubated alone or with calf thymus DNA added in rubber stoppered 25 ml erlenmeyer flasks in a shaking water bath at 37 C. The DNA was used as received from manufacturer, purified by dialysis, or purified by sephadex G-10 column chromatography. Each flask contained 4 or 6 ml liposomal DNA suspension, depending upon subsequent assays to be performed.

TABLE I

Gas Liquid Chromatographic Analysis of Fatty Acids in PC[a] and PCMA[b] Liposomal Suspensions

Fatty chain[c]	PC %[d]	PCMA %
14:0	1.0	0.9
16:0	36.4	32.8
18:0	11.2	10.1
18:1	30.5	27.5
18:2	16.9	15.2
20:4	4.0	13.5
Percent Polyunsaturation	20.9	28.7

[a]L-α-ovolecithin.

[b]PC and methyl arachidonate.

[c]The designation 18:0 refers to a fatty chain 18 carbons long with no double bonds; while 18:2 is also 18 carbons long but contains 2 double bonds.

[d]All components constituting less than 1% of total in PC are not included in either profile.

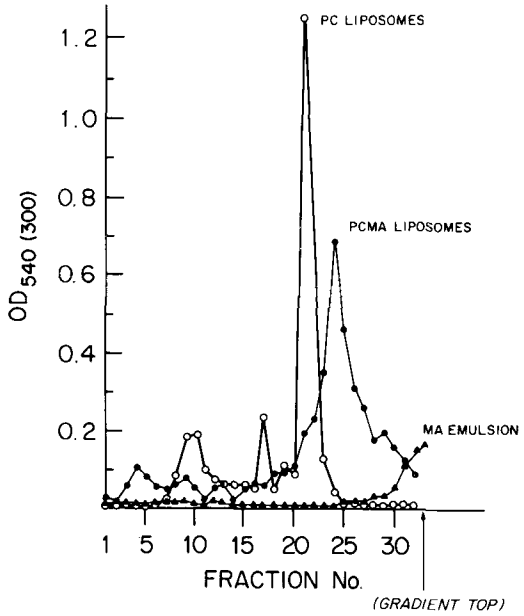

FIG. 1. Sucrose density gradient centrifugation of L-α-ovolecithin (PC) and PC and methyl arachidonate (PCMA) liposomes. Centrifugation was for 3½ hr at 35,000 rpm. All preparations utilized H_2O as the aqueous phase. Samples —o— 0.5 ml PC; •- 0.5 PCMA; ▲- 0.5 ml MA emulsion. OD 540 plotted for PC and PCMA (expanded scale - 10X); OD 300 plotted for MA emulsion.

Determination of Lipid Oxidation

Diene conjugation: A modification of Klein's method (18) was used to follow oxidation in the liposomal suspensions. Aliquots of liposomes (0.01 ml) were added to 5 ml 95% ethanol and vortexed. The UV absorption at 215 nm and 233 nm were read in quartz cuvettes with a Beckman DU Spectrophotometer using a 95% ethanol blank. The absorbency ratio at 233 nm/215 nm is a measure of lipid oxidation and was plotted against the time of incubation.

Thiobarbituric acid test: A modification of the procedure utilized by Leibowitz and Johnson (5) was used to follow oxidation in the liposomal suspensions. Aliquots of liposomes (0.2 ml) were added to 3 ml trichloacetic acid (20%) and 1 ml of a saturated 2-thiobarbituric acid in 7% perchloric acid solution and mixed by vortexing. This mixture was then incubated at 100 C for 20 min and centrifuged 15 min at 2500 rpm in a desk top model CL International clinical centrifuge temperature. The supernatant was read at 532 nm on a Beckman DU Spectrophotometer using a reagent blank. Absorbance at 532 was plotted against incubation time.

RESULTS

Characterization of Liposomal Suspensions

Gas chromatography: The fatty acid composition of PC and PCMA liposomes is shown in Table I. Whenever the values of the more labile polyunsaturated chains differed significantly from those shown (>15%), the sample was considered partially oxidized and not utilized in any study.

Sucrose density gradient centrifugation: Figure 1 shows the sucrose density gradient centrifugation characteristics of PC and PCMA liposomes; an emulsion of MA is included for comparison. The emulsion remains at the gradient top while both PC and PCMA liposomes migrate into the gradient, PC more deeply. The inclusion of MA into the PC bilayers seemingly results in a decreased buoyant density of PCMA liposomes compared to PC liposomes.

Oxidation kinetics of liposomal suspensions: Figure 2 shows the oxidation kinetics of PC and PCMA liposomal suspensions obtained in a typical experiment. In each suspension, oxidation is monitored by production of conjugated dienes (A 233/215) and TBA-reactive product (A 532). As shown, both PC and PCMA liposomes undergo "autoxidation." The induction period for each suspension is the same, in this experiment about 10 hr. This equivalence of induction period was a constant feature in each experiment where PC and PCMA were studied in parallel, even though the induction period varied within the limits of 10 to 21 hr from one experiment to another. This variation was

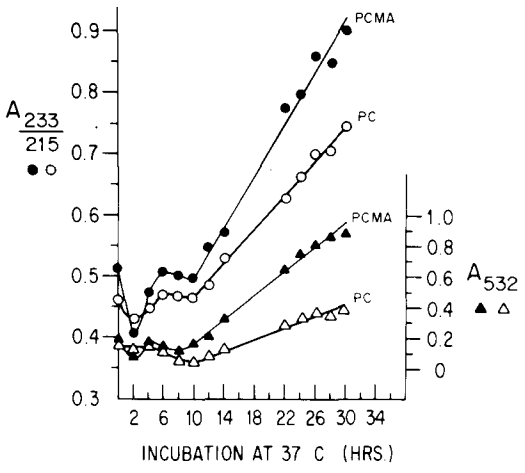

FIG. 2. Oxidation kinetics of L-α-ovolecithin (PC) and PC and methyl arachidonate (PCMA) liposomal suspensions. Liposomes were suspended in 0.05 M sodium phosphate buffer, pH 7.5. Total lipid concentration was equal to 10 mM. Each point plotted represents the average of readings taken from two identically prepared liposomal incubation samples during a typical experiment. These results have been repeated three times. PCMA - ●, ▲; PC - ○, △.

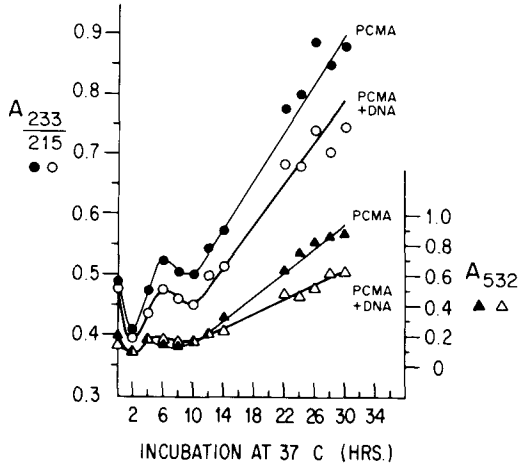

FIG. 3. Oxidation kinetics of L-α-ovolecithin and methyl arachidonate (PCMA) liposomal suspensions containing 4.5 μgm/ml calf thymus DNA. Liposomes were suspended in 0.05 M sodium phosphate buffer pH 7.5. Total lipid concentration was equal to 10 mM. DNA concentration in DNA containing samples was 4.5 μgm/ml. Each point plotted represents the average of reading taken from two identically prepared liposomal incubation samples during a typical experiment. PCMA - ●, ▲; PCMA + 4.5 μgm/ml DNA - ○, △.

probably related to variations in water impurities, lipid samples, and other uncontrolled factors. The rate of oxidation, however, is greater for PCMA, in parallel to the enrichment of methyl arachidonate in these liposomes.

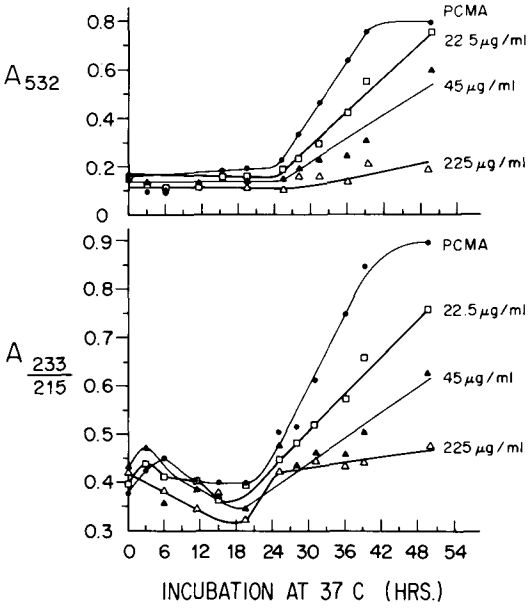

FIG. 4. Oxidation kinetics of L-α-ovolecithin and methyl arachidonate (PCMA) liposomal suspensions containing various concentrations of calf thymus DNA. Liposomes were suspended in 0.05 M sodium phosphate buffer pH 7.5. Total lipid concentration was equal to 10 mM. Each point plotted represents the average of readings taken from two identically prepared liposomal incubation samples during a typical experiment. PCMA - ●; PCMA + 22.5 μgm/ml DNA - □; PCMA + 45 μgm/ml DNA - ▲; PCMA + 225 μgm/ml DNA - △.

Examination of the line slopes in Figure 2 indicates that PCMA is more efficient than PC in forming TBA reactive product relative to measurable conjugated dines.

Oxidation Kinetics of PCMA Liposomal Suspensions Containing 4.5 μgm/ml Calf Thymus DNA

Since PCMA liposomes had a greater rate of oxidation than PC and therefore would maximize the possibility of eliciting intermediate responses to modulating conditions, they were chosen as the main system for further study. Figure 3 shows the oxidation kinetics of PCMA liposomes with and without 4.5 μgm/ml calf thymus DNA studied in parallel during the same experiment. The DNA does not affect the length of the induction period even though, as mentioned previously, this length varies from experiment to experiment (Fig. 4). The DNA does, however, retard the rate of lipid oxidation in the liposomes as shown by both A 233/215 and A 532 measurement. These results have been repeated twice with calf thymus DNA prepared directly from the sample received from the manufacturer,

TABLE II

Protection of 18:2 and 20:4 Fatty Acid by DNA in Oxidizing PCMA[a] Liposomes[b]

Fatty chain	PCMA %[c]	PCMA+DNA %
14:0	1.8[d]	1.2
16:0	47.0	40.3
18:0	18.7	15.3
18:1	32.5	34.2
18:2	0	6.8
20:4	0	2.2

[a]L-α-ovolecithin and methyl arachidonate.

[b]Gas liquid chromatographic analysis of fatty acids in PCMA and PCMA+DNA suspensions after 44 hr incubation.

[c]All components constituting less than 1% of total are not included in either profile.

[d]The apparent increase in the remaining fatty acids, compared with values in Table I, reflects the fact that the values shown are percent of fatty acids present.

twice with DNA dialysed extensively, and twice with DNA passed through a column packed with Sephadex G-10. The two latter procedures were performed to eliminate any possible impurities.

Table II shows the gas chromatographic analysis of fatty acids in PCMA and PCMA+DNA suspensions under the experimental conditions shown in Figure 3. The polyunsaturated fatty acids 18:2 and 20:4 have disappeared in PCMA while there is no substantial change in the percent of the monounsaturate 18:1 (see Table I for comparison). The presence of DNA inhibits the disappearance of the polyunsaturated fatty acids in agreement with the results of Figure 3.

Oxidation Kinetics of PCMA Liposomal Suspensions Containing Various Concentrations of Calf Thymus DNA

Figure 4 shows the effect of 22.5 μgm/ml, 45 μgm/ml, and 225 μgm/ml calf thymus DNA on the oxidation kinetics of PCMA liposomal suspensions as measured by A 233/215 and A 532. None of the DNA concentrations utilized change the length of the induction period, which in this experiment is approximately 21 hr. The DNA does, however, retard the rate of lipid oxidation in a concentration dependent manner. These results have been obtained three times.

DISCUSSION

The addition of MA to PC liposomes alters several of their characteristics, particularly the buoyant density and the rate of oxidation. The addition of calf thymus DNA to PCMA liposomal suspensions results in a decreased rate of lipid oxidation, but it does not change the time of onset. Therefore, DNA, in these experiments, has antioxidant activity; compounds that can alter the rate, but not the time of initiation, of lipid oxidation are referred to as free radical acceptors or trappers (19,20).

Inclusion of MA into the PC bilayers results in a decreased buoyant density of PCMA liposomes compared to PC liposomes (Fig. 1). In accordance with their enrichment in MA, the PCMA liposomal suspensions contain a larger percentage of their total fatty acids as polyunsaturates (28.7% in PCMA vs. 20.9% in PC). This property was chosen to characterize the oxidation capabilities of the liposomal suspensions since the autoxidation rate of polyunsaturated fatty chains (i.e., containing doubly allylic hydrogens) is 40 to 300 times faster than the autoxidation rate of monounsaturated fatty chains (not containing doubly allylic hydrogens) (21); in addition, most of the loss of the polyunsaturates, 18:2 and 20:4, precedes any noticeable decrease in 18:1 during the oxidation of the liposomal suspensions studied in this paper (Table II).

In agreement with their higher degree of polyunsaturation, PCMA liposomes exhibit a greater oxidation rate than PC liposomes as measured by both A 233 nm/215 nm and A 532 nm (Fig. 2). Furthermore, there exists a quantitative difference between the two suspensions in their ability to generate TBA-reactive product relative to measurable conjugated dienes. PCMA is more efficient than PC in forming TBA-reactive product relative to conjugated dienes. This greater relative efficiency in forming TBA-reactive product is also reflective of the MA enrichment in PCMA, since 20:4 fatty acyl chains are the main species capable of forming TBA-reactive product in either suspension (22,23). On the other hand, both 18:2 and 20:4 fatty chains will contribute significantly to conjugated diene formation. Therefore, the enrichment in PCMA of species that form TBA-reactive product is greater (13.5% 20:4 in PCMA vs. 4.0% 20:4 in PC) than the enrichment in species that form conjugated dienes (13.5% 20:4 plus 15.2% 18:2, total 28.7% in PCMA vs. 4.0% 20:4 plus 16.9% 18:2, total 20.9% in PC).

While the addition of calf thymus DNA does not alter the temporal onset of lipid oxidation (i.e., induction period), it retards the oxidation rate of PCMA liposomal suspensions in a concentration dependent manner (Fig. 3,4). DNA is therefore an antioxidant in this system. Comparable results have also been obtained

using incubation mixtures of DNA and PC liposomes (unpublished observations).

Antioxidants can be grouped into 2 classes: inhibitors and retarders (19,24). Inhibitors lengthen the induction period but have no effect on the rate of oxidation. Retarders have no effect on the induction period but decrease the oxidation rate. DNA can therefore be termed a retarder. Retarders are substances which serve as free radical acceptors or trappers (19,24). The precise nature of the radicals trapped or accepted by the DNA is unknown. The main chain-propagating species in autoxidizing lipid are the hydroxyl radical (•OH), the lipid alkoxy (RO•), and lipid hydroperoxy (ROO•) radicals (25,26). The hydroxyl radical is known to react with DNA at a high rate constant (27) and the formation of oxidizing lipid-nucleotide adducts has been reported (28). These results, in addition to the high efficiency with which DNA retards liposomal oxidation (Fig. 4), suggest that DNA is capable of neutralizing most, if not all, chain propagating radicals produced in these membrane models.

In agreement with the data presented in this paper, the interaction between autoxidizing methyl linoleate and DNA results in the production of DNA free radicals, as monitored by electron spin resonance (29; Schaich, K.M., and D.C. Borg, Brookhaven National Laboratory, Upton, N.Y., personal communication). This finding, taken together with the antioxidant activity of DNA described above, would suggest that during its retardation of liposomal oxidation, alterations occur in the DNA. In fact, we have recently found that biologically significant alterations in *B. subtilis* DNA are produced during its interaction with oxidizing liposomal suspensions (unpublished observations).

ACKNOWLEDGMENTS

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SHORT COMMUNICATIONS

High Pressure Reverse Phase Liquid Chromatography of Fatty Acid *p*-Bromophenacyl Esters

ABSTRACT

High pressure reverse phase liquid chromatography has been employed to rapidly separate saturated and unsaturated fatty acids as the corresponding *p*-bromophenacyl esters. Through the use of a highly efficient C_{18} reverse phase column packing, it has also been possible to distinguish among geometrical and positional isomers of the unsaturated acids. The use of ultroviolet-sensitive esters has permitted the detection of low (nanogram range) concentrations of fatty acids. The time required for analysis has been further reduced by employing a novel and rapid method for the preparation of the esters.

INTRODUCTION

The analysis of fatty acids by high pressure liquid chromatography (HPLC) has been limited somewhat by the detection capabilities of commercially available instruments. It has, for example, been demonstrated (1-4) that refractive index detectors are relatively insensitive to fatty acid concentrations at the nanogram level. Ultraviolet (UV) detectors can be quite sensitive to such low concentrations, but most fatty acids do not absorb UV radiation at the wavelength (254 nm) employed in many HPLC instruments. Consequently, conversion of the fatty acids to UV-sensitive derivatives is required. Suitable derivatives include the corresponding benzyl (5), *p*-bromophenacyl (6,7), 2-napthacyl (8), *p*-nitrobenzyl (9-11), *p*-nitrophenacyl (7,12), and phenacyl (11,13) esters.

We have been able to separate a number of important long chain fatty acids as their *p*-bromophenacyl esters. By employing both a novel method for the preparation of the esters (6,14) and a highly efficient 5μ particle size C_{18} chemically-bonded reverse phase column packing, we were able to rapidly analyze mixtures of representative saturated, monoenoic, dienoic, and trienoic straight chain fatty acids, and, furthermore, we were able to distinguish among geometrical (*cis*- and *trans*-) and positional isomers of the olefinic acids.

EXPERIMENTAL PROCEDURES

All the fatty acids and the derivatization reagents were products of Applied Science Laboratories, Inc., (State College, PA). High purity methanol (Mallinckrodt Chrom AR, St. Louis, MO) and freshly-distilled water were used to prepare the eluant solvent systems.

Samples of the *p*-bromophenacyl esters were prepared by modification of the "Alkylation Method C" described by Durst, Grushka, et al. (6). In the modified procedure, anhydrous potassium carbonate was employed in place of anhydrous potassium hydrogen carbonate, and ground glass-jointed glassware was used in place of Reacti-Vials. The crown ether catalyst was dicyclohexyl-18-crown-6 (dicyclohexyl-1,4,7, 10,13,16-hexaoxacyclooctadecane). The derivatives were formed by boiling the reactants (acetonitrile solution) under reflux, with vigorous stirring, for 45 min. The solvent was then evaporated from the cooled reaction mixture. The residue was taken up in chloroform, and the resulting suspension was filtered to remove precipitated potassium bromide. The chloroform filtrate was diluted to an appropriate volume with additional chloroform. Aliquots of this solution were injected into the chromatograph.

Analyses were performed with a Du Pont model 820 liquid chromatograph (Instrument Products Division, E. I. Du Pont de Nemours and Co., Wilmington, DE). The model 820 is equipped with both UV (254 nm) and refractive index detectors. The former was employed in this study. The column was a 25 cm x 5 mm inside diameter stainless steel column packed with Applied Science Laboratories Inc. 5 μ particle size HI-EFF Micropart C_{18} phase bonded support. Specific HPLC conditions are given in the figures captions (Figs. 1 & 2).

RESULTS AND DISCUSSION

The crown ether-catalyzed reaction of potassium carboxylate and α,*p*-dibromoacetophenone developed by Durst, Grushka et al. (6,14) proved to be very effective for the rapid preparation of fatty acid *p*-bromophenacyl esters. Furthermore, the catalyst and excess alkylating

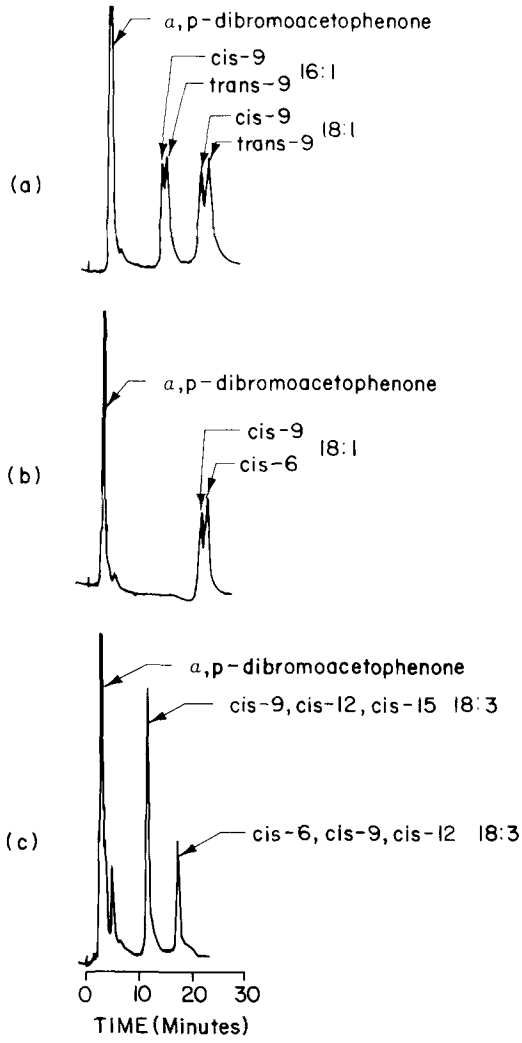

FIG. 1. Separation of the *p*-bromophenacyl esters of (A) palmitoleic (*cis*-9 16:1), palmitelaidic (*trans*-9 16:1), oleic (*cis*-9 18:1), and elaidic (*trans*-9 18:1) acids; (B) oleic (*cis*-9 18:1) and petroselinic (*cis*-6 18:1) acids; and (C) linolenic (*cis*-9, *cis*-12, *cis*-15 18:3) and γ-linolenic (*cis*-6, *cis*-9, *cis*-12 18:3) acids. Eluant: methanol/water (90:10, v/v). Flow rate: 2.0 ml/min at 1500 psig. Temperature: 25 C. Sample size: ca. 1.0 μg of each ester.

agent did not interfere with subsequent analyses: the crown ether did not absorb UV radiation at 254 nm, and α,*p*-dibromoacetophenone was eluted before any of the *p*-bromophenacyl esters. Side reactions appeared to be minimal.

The chromatograms illustrate the ability of the C_{18} reverse phase to separate compounds of slightly different polarities, such as geometrical and positional isomers of unsaturated fatty acids. In terms of resolution and analysis time, the separations are somewhat more efficient

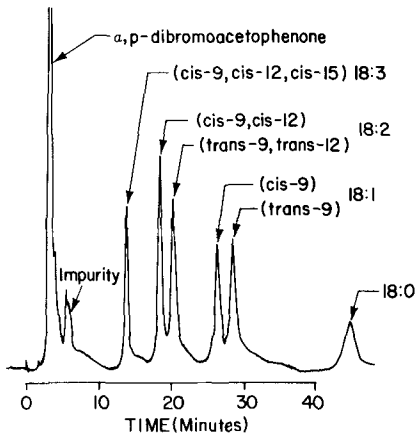

FIG. 2. Separation of the *p*-bromophenacyl esters of linolenic (*cis*-9, *cis*-12, *cis*-15 18:3), linoleic (*cis*-9, *cis*-12 18:2), linoleaidic (*trans*-9, *trans*-12 18:2), oleic (*cis*-9 18:1), elaidic (*trans*-9 18:1), and stearic (18:0) acids. Eluant: methanol/water (90:10, v/v). Flow rate: 1.5 ml/min at 1000 psig. Temperature: 25 C. Sample size: ca. 1.0 μg of each ester.

than those reported by Durst, Grushka, et al. (6), who employed a C_9 reverse phase column packing. Borch (12), using a C_{18} packing and the gradient elution technique, has effected separation of all the acids shown, as the phenacyl esters, with excellent resolution. Again, however, a longer analysis time was required.

The separations achieved can be best explained in terms of the relative intensity of the interaction of the *p*-bromophenacyl esters and the reverse phase. The more closely the ester and the reverse phase resemble each other structurally, the more intense is the interaction. The reverse phase is essentially of a nonpolar paraffinic nature, and in the case of a fatty acid *p*-bromophenacyl ester, that part of the molecule which most closely resembles the reverse phase is the nonpolar hydrocarbon "tail" (R in R-COOR′). The intensity of the interaction appears to be reduced by incorporating in the ester certain functional groups which increase its polarity. The most commonly encountered of such functional groups is the carbon-carbon double bond. The effect of thus increasing the polarity of the ester is illustrated in Figure 2. The stearic acid ester, which contains no carbon-carbon double bonds and thus structurally most resembles the reverse phase, interacts most strongly with the reverse phase and is eluted last. The linolenic acid ester, which contains three carbon-carbon double bonds and thus least resembles the reverse phase in structure, interacts weakly with the reverse phase and is eluted first. The monoenoic and dienoic

esters are eluted at intermediate times. This phenomenon—the more rapid elution of the more highly unsaturated acids—has also been observed with the free acids (4) and with the corresponding methyl (1-4), 2-napthacyl (8), and phenacyl (12) esters.

Small changes in the geometry and position of the carbon-carbon double bond also affect the polarity of the ester and, consequently, the interaction of the ester and the reverse phase. Among unsaturated esters differing only in the geometry of the double bond, the more polar *cis*-isomers are eluted more rapidly (Figures 1A and 2). Alteration of the position of the double bond has a similar effect: movement of the double bond towards the polar end of the molecule (–COOR′ in R-COOR′) reduces the polarity of the hydrocarbon "tail" (R- in R-COOR′), making it more paraffinic in nature. More intense interaction of the ester and the reverse phase is then observed (Figures 1B and 1C).

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Diester Waxes Containing 2-Hydroxy Fatty Acids from the Uropygial Gland Secretion of the White Stork (*Ciconia ciconia*)

ABSTRACT

The uropygial gland of the white stork secrets mono- and diester waxes as well as triglycerides, all of which contain unbranched medium chain fatty acids. n-Decanol and n-dodecanol have been the only alcohols detected in both types of waxes. The diester waxes contain 2-hydroxy fatty acids.

INTRODUCTION

Investigations of the chemical composition of uropygial gland secretions have been useful to the chemotaxonomy of birds (1). From the obviously heterogenous order *Ciconiiformes*, involving herons, storks, ibises, and unusual birds like the hammerhead, the shoebill and the boat-billed heron, the preen waxes of only two species, both of them herons, *Ardea cinerea* and *Nycticorax nycticorax* (2,3), have been investigated. This paper presents the analysis of the uropygial gland secretion of the white stork (*Ciconia ciconia*) and compares its composition with those of other ciconiiform species.

MATERIAL AND METHODS

The uropygial gland was excised from an adult animal immediately after death (♂, Schleswig-Holstein). Extraction, purification, and chromatographic separation procedures were performed as published previously (4) and yielded monoester waxes (91.5 mg = 81.9% of total lipid material), diester waxes (8.6 mg = 7.6%) and triglycerides (11.8 mg = 10.5%). Monoester waxes and triglycerides were esterified with 5% methanolic HCl, and the methanolysis products of the wax esters were separated into methyl esters and alcohols. The

esters are eluted at intermediate times. This phenomenon—the more rapid elution of the more highly unsaturated acids—has also been observed with the free acids (4) and with the corresponding methyl (1-4), 2-napthacyl (8), and phenacyl (12) esters.

Small changes in the geometry and position of the carbon-carbon double bond also affect the polarity of the ester and, consequently, the interaction of the ester and the reverse phase. Among unsaturated esters differing only in the geometry of the double bond, the more polar *cis*-isomers are eluted more rapidly (Figures 1A and 2). Alteration of the position of the double bond has a similar effect: movement of the double bond towards the polar end of the molecule (–COOR′ in R-COOR′) reduces the polarity of the hydrocarbon "tail" (R- in R-COOR′), making it more paraffinic in nature. More intense interaction of the ester and the reverse phase is then observed (Figures 1B and 1C).

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ABSTRACT

The uropygial gland of the white stork secrets mono- and diester waxes as well as triglycerides, all of which contain unbranched medium chain fatty acids. n-Decanol and n-dodecanol have been the only alcohols detected in both types of waxes. The diester waxes contain 2-hydroxy fatty acids.

INTRODUCTION

Investigations of the chemical composition of uropygial gland secretions have been useful to the chemotaxonomy of birds (1). From the obviously heterogenous order *Ciconiiformes*, involving herons, storks, ibises, and unusual birds like the hammerhead, the shoebill and the boat-billed heron, the preen waxes of only two species, both of them herons, *Ardea cinerea* and *Nycticorax nycticorax* (2,3), have been investigated. This paper presents the analysis of the uropygial gland secretion of the white stork (*Ciconia ciconia*) and compares its composition with those of other ciconiiform species.

MATERIAL AND METHODS

The uropygial gland was excised from an adult animal immediately after death (♂, Schleswig-Holstein). Extraction, purification, and chromatographic separation procedures were performed as published previously (4) and yielded monoester waxes (91.5 mg = 81.9% of total lipid material), diester waxes (8.6 mg = 7.6%) and triglycerides (11.8 mg = 10.5%). Monoester waxes and triglycerides were esterified with 5% methanolic HCl, and the methanolysis products of the wax esters were separated into methyl esters and alcohols. The

TABLE I

Composition of the Lipids from the Uropygial Gland Secretion of the White Stork (*Ciconia ciconia*)

Fatty acids (%)		Alcohols (%)		Hydroxy acids (%)	
		Monoester Waxes			
n-C_{10}	41.3	n-C_{10}	92.6		
n-C_{12}	54.5	n-C_{12}	7.4		
n-C_{14}	4.2				
		Diester Waxes			
n-C_{10}	80.3	n-C_{10}	77.9	2-OH-C_{10}	22.3
n-C_{12}	18.2	n-C_{12}	22.1	2-OH-C_{12}	70.2
n-C_{14}	1.5			2-OH-C_{14}	7.5
		Triglycerides			
n-C_{10}	35.4				
n-C_{12}	32.2				
n-C_{14}	10.5				
n-C_{16}	14.3				
n-C_{18}	2.7				
n-$C_{18:1}$	4.9				

alcohols were oxidized with CrO_3/acetic acid in cyclohexane to the corresponding acids which subsequently were esterified (4). Diester waxes were saponified with 1 N methanolic KOH. The acids of this fraction were esterified, and the alcohols oxidized as above. All fractions were identified by combined gas-liquid chromatography/mass spectrometry (GLC/MS) (10 m glass column with 3% OV 101 on Gas Chrom Q, mass spectrometer: Varian-MAT 111 (GNOM), 80 eV). The preen wax composition of a second specimen was qualitatively identical and quantitatively very similar.

RESULTS

The uropygial gland secretion of the white stork contains monoester waxes (81.9%), diester waxes (7.6%), and triglycerides (10.5%), the compositions of which are listed in Table I. The mass spectra recorded from the esters of the hydroxy acids were identical with those published in the literature (5), showing large $[M-59]^+$ and $[M-32]^+$ fragments due to elimination of $COOCH_3$ and CH_3OH respectively. Ions $[M-45]^+$ and $[M-50]^+$ were less abundant.

DISCUSSION

The white stork is the only bird investigated so far whose preen wax possesses considerable amounts of diester waxes containing 2-hydroxy fatty acids. 3-Hydroxy fatty acids have been detected earlier in *Columba palumbus* (6,7) and in *Lanius collurio* (Jacob, unpublished results).

From the chemotaxonomical viewpoint when compared with other ciconiiform species, *Ciconia ciconia* is closely related to *Ardea cinerea* (2), by possessing predominantly unbranched waxes and triglycerides with fatty acids of about the same chain length, although no diester waxes have been detected in *Ardea*. *Nycticorax nycticorax* (Night heron), however, is characterized by waxes containing secondary alcohols and, therefore seems to be more distant from *Ciconia*. The heterogeneity of the order *Ciconiiformes* is further demonstrated by the fact that ibises (Jacob, unpublished results) as well as hammerheads (Jacob, unpublished results) possess preen wax compositions which are different from *Ciconia* and *Nycticorax*. In the preen waxes of the ibis *Threskiornis aethiopica,* 2-methyl branched acids predominate and in those of *Theristicus caudatus* 2- and 4-ethyl branched acids predominate, whereas 4-methyl branched acids are the main constituents of the uropygial gland secretion of the hammerhead (Jacob, unpublished results).

Furhter preen wax analyses may elucidate the relationships within this order and to other orders.

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Blood Sampling Techniques for Studying Rapidly Turning over Metabolic Fuels in Mice[1]

ABSTRACT

Experiments were carried out in control and Ehrlich ascites carcinomatous mice to determine whether orbital venous sinus blood could be used to reflect blood in the systemic circulation (decapitation blood) in the case of a rapidly turning over metabolic fuel such as free fatty acids. The early time course of intravenously injected, labeled free fatty acids was measured using [9,10-^{3}H] palmitic acid and [1-^{14}C] linoleate complexed to mouse serum. No significant differences between decapitation and orbital sinus blood were found at early times in either group of mice. The orbital sinus clearly contains blood that is not stagnant and is replaced so rapidly that it is suitable for studying very rapidly turning over, circulating metabolites.

INTRODUCTION

Serious technical problems are commonly encountered by investigators who are interested in quantitative, dynamic aspects of circulating metabolic fuels in mice. These investigators usually recognize that they can minimize costs of radioactive tracers, radiation exposure, number of mice, duration of the study, and variance of the data by obtaining serial blood samples from individual mice. However, it is also difficult or impossible to obtain sufficient blood for analyses using peripheral blood vessels without resorting to invasive procedures that involve anesthesia and probable perturbation of metabolic rates. Although serial heart punctures are feasible and relatively easy to perform, it is difficult to imagine this procedure not producing a marked adrenal response, alteration of tissue cyclic nucleotide levels, and variation in flux of metabolic fuels, especially in a small animal.

An alternative to heart puncture was introduced by Riley (1) and later applied by Rerup and Lundquist to study circulating glucose levels in mice (2). This technique took advantage of the fact that the orbital blood sinus of unanesthetized mice could be sampled rapidly, repetitively, and, according to the authors (2), with little traumatic effect. However, there is some controversy with respect to this point (3). A number of investigators, ourselves included, have subsequently reported metabolic studies in mice using this technique. In the case of [^{14}C] glucose turnover in mice, we have found that the initial mixing and subsequent replacement of tracer glucose after intravenous injection is the same in animals killed at various times by decapitation (mixed, arterial blood) as in animals from which serial orbital blood samples were collected (4,5). However, these studies were carried out at separate times and do not establish that the serial orbital sinus sampling technique would be valid in the study of a metabolic fuel, such as free fatty acids (FFA), which responds more rapidly than glucose to changes in adrenergic hormones.

We are currently studying the flux of essential and nonessential FFA in normal and cancerous mice. In order to check the validity of the orbital sinus sampling technique, we have carried out simultaneous studies of orbital blood and blood from decapitated animals using an essential and a nonessential fatty acid (FA) in both normal and in tumor-bearing (Ehrlich ascites carcinoma) mice. Results of these studies indicate that the venous blood in the orbital sinus is in such rapid equilibrium

[1]Supported in part by VA Medical Research and NIH, USPHS Grant No. CA 15813.

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Blood Sampling Techniques for Studying Rapidly Turning over Metabolic Fuels in Mice[1]

ABSTRACT

Experiments were carried out in control and Ehrlich ascites carcinomatous mice to determine whether orbital venous sinus blood could be used to reflect blood in the systemic circulation (decapitation blood) in the case of a rapidly turning over metabolic fuel such as free fatty acids. The early time course of intravenously injected, labeled free fatty acids was measured using [9,10-^{3}H] palmitic acid and [1-^{14}C] linoleate complexed to mouse serum. No significant differences between decapitation and orbital sinus blood were found at early times in either group of mice. The orbital sinus clearly contains blood that is not stagnant and is replaced so rapidly that it is suitable for studying very rapidly turning over, circulating metabolites.

INTRODUCTION

Serious technical problems are commonly encountered by investigators who are interested in quantitative, dynamic aspects of circulating metabolic fuels in mice. These investigators usually recognize that they can minimize costs of radioactive tracers, radiation exposure, number of mice, duration of the study, and variance of the data by obtaining serial blood samples from individual mice. However, it is also difficult or impossible to obtain sufficient blood for analyses using peripheral blood vessels without resorting to invasive procedures that involve anesthesia and probable perturbation of metabolic rates. Although serial heart punctures are feasible and relatively easy to perform, it is difficult to imagine this procedure not producing a marked adrenal response, alteration of tissue cyclic nucleotide levels, and variation in flux of metabolic fuels, especially in a small animal.

An alternative to heart puncture was introduced by Riley (1) and later applied by Rerup and Lundquist to study circulating glucose levels in mice (2). This technique took advantage of the fact that the orbital blood sinus of unanesthetized mice could be sampled rapidly, repetitively, and, according to the authors (2), with little traumatic effect. However, there is some controversy with respect to this point (3). A number of investigators, ourselves included, have subsequently reported metabolic studies in mice using this technique. In the case of [^{14}C] glucose turnover in mice, we have found that the initial mixing and subsequent replacement of tracer glucose after intravenous injection is the same in animals killed at various times by decapitation (mixed, arterial blood) as in animals from which serial orbital blood samples were collected (4,5). However, these studies were carried out at separate times and do not establish that the serial orbital sinus sampling technique would be valid in the study of a metabolic fuel, such as free fatty acids (FFA), which responds more rapidly than glucose to changes in adrenergic hormones.

We are currently studying the flux of essential and nonessential FFA in normal and cancerous mice. In order to check the validity of the orbital sinus sampling technique, we have carried out simultaneous studies of orbital blood and blood from decapitated animals using an essential and a nonessential fatty acid (FA) in both normal and in tumor-bearing (Ehrlich ascites carcinoma) mice. Results of these studies indicate that the venous blood in the orbital sinus is in such rapid equilibrium

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with the circulation that it reflects accurately the turnover of FFA ($t_{1/2}$<60 sec) in the arterial circulation.

MATERIALS AND METHODS

Swiss-Webster mice were used (6). Details of tumor transplantation (6), complexing of radioactive FA to mouse serum albumin, and intravenous tracer injection have been previously described (7). [9,10-^{3}H]Palmitic acid and [1-^{14}C]linoleic acid (Dhom Products, Ltd., North Hollywood, CA, and Applied Science Labs., State College, PA, respectively) were obtained commercially and were purified, when necessary. The volume of mouse serum injected was 50 µl; it contained a maximum of 4 x 10^5 cpm ^{14}C and 1.5 x 10^6 cpm ^{3}H and ca. 1.5-20 nmol of added, labeled fatty acid.

Animals were decapitated immediately after orbital sampling or, in some cases, after serial sampling of orbital blood (ca. 50-100 µl blood/sample). Blood was collected in either case in heparinized capillaries which were stored on ice, after which plasma was separated by centrifugation. Radioactivity in plasma during the first 2 min after intravenous injection was assumed to reside entirely in FFA (8). Plasma aliquots (10 µl) were counted directly using a liquid scintillation counter. Plasma volume was found to be approximately 5.0% of body wt in both tumorous and control mice (manuscript in preparation).

RESULTS AND DISCUSSION

As shown in Figure 1, there was no significance difference, at any of the early times studied, between the plasma FFA "specific activities" (Sp.A., expressed as % dose/ml plasma) in orbital venous sinus blood and in blood from decapitated animals. The Sp.A. tended to be slightly higher in orbital blood than in blood from decapitated mice at ca. 10 sec; this was true for both nonessential (Fig. 1A) and essential FA tracer (Fig. 1B), and for tumorous as well as control mice.

The data lend strong support to the view that the orbital venous sinus contains an extremely rapidly turning over pool of blood that closely reflects arterial blood derived from the heart. Even using a tracer which turns over so fast that 70% is removed from the circulation in 60 sec (Figs. 1A and 1B), the mixing of heart blood with blood in this opthalmic capillary bed was rapid enough to provide almost identical values for fatty acid Sp.A. at even the earliest time points.

Our data do not provide any information either for or against the contention of Rerup and Lundquist (2) that repetitive sampling by this technique is nonstressful under their conditions. We have observed that after removing 200 µl plasma by serial sampling of orbital blood, the hematocrit tends to fall. It seems likely that the unanesthetized mouse is excited under these conditions and that FFA transport rates may be increased. However, this method, especially in the hands of a skilled technician,

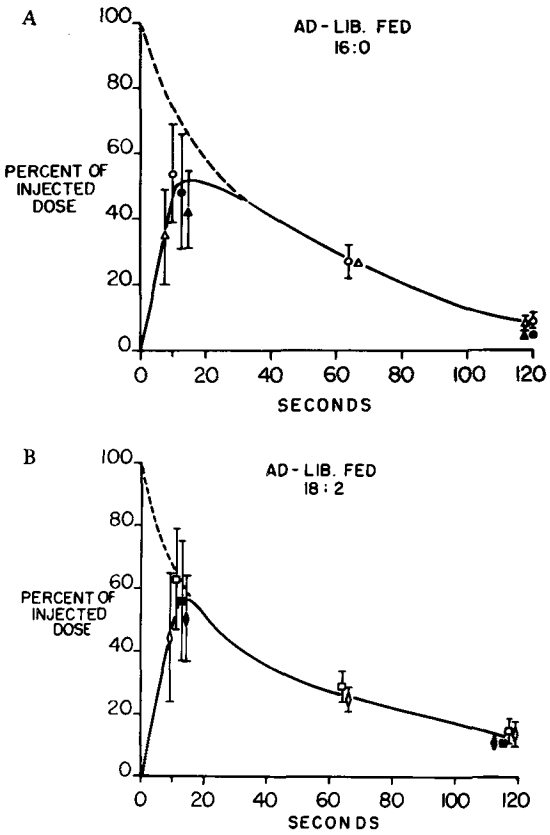

FIG. 1. Comparison of radioactivity in free fatty acids in blood plasma obtained by orbital venous sinus blood sampling and by decapitation in normal and Ehrlich ascites carcinomatous mice. A, after intravenous injection of [9,10-^{3}H] palmitic acid complexed to mouse serum (albumin); B, after intravenous injection of [1-^{14}C] linoleate, similarly complexed. Symbols: ●, ■ control, orbital sinus; ○, □, tumor, orbital sinus; ▲, ◆, control, decapitation; △, ◇, tumor, decapitation. Values are means ± S.D. (vertical bars), unless ± S.D. ≤ the size of the symbol. N = 5-10/point, orbital sinus; 3-6/point, blood from decapitated mice. Overlapping points have been slightly displaced for clarity. The broken line indicates the conventional assumption that mixing of an intravenously injected tracer is complete at zero time. Theoretically, this is not strictly correct; at zero time, 100% of the dose is injected into a tail vein and 0% of the dose is present in the heart and orbital sinus blood, as shown here.

can provide representative samples of blood within seconds, and the blood sample obtained is clearly not derived from a stagnant pool. Further analysis of the data shown in Figures 1A and 1B is the subject of a separate report (manuscript in preparation).

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LETTER TO THE EDITOR

Concerning the Extraction of Lipids from Yeast

Sir: In a recent paper in this journal [M.T. Sobus and C.E. Holmlund, Lipids, 11:341 (1976)], several methods for the extraction of lipids from yeast cells were compared. The report shows, however, that the discrepancies observed may be due to a modification of our procedure [T. Itoh and H. Kaneko, Yukagaku, 23:350 (1974)]. An essential principle of our extraction process is a combination of efficient cell disruption with a BRAUN cell homogenizer (complete disruption of yeast cells is accomplished within a few minutes [G. Schatz, H.S. Penefsky and E. Racker, J. Biol. Chem., 242:2552 (1967)]), and effective lipid extraction with chloroform–methanol. Sobus and Holmlund, however, used an ultrasonic cell homogenizer in place of the BRAUN cell homogenizer. In our hands, yeast cells were poorly disrupted by ultrasonic cell homogenization. In the experiment described in our paper, a five gram sample of wet cells was suspended in 20 ml of a chloroform-methanol mixture (1:2, v/v) together with 40 ml of glass beads (0.40–0.50 mm diameter), and the mixture was homogenized for 4 min at 4000 cpm in a mechanical cell homogenizer (BRAUN). The suspension of disrupted cells was filtered through a glass filter (4G). The residual cell debris was suspended in 50 ml of a chloroform-methanol mixture (2:1, v/v), stirred with a magnetic stirrer for 30 min at room temperature under an atmosphere of nitrogen, and then filtered. This stirring and filtering process was repeated three times. In addition, we checked the completeness of the lipid extraction by two methods: a portion of the lipid extracted cell debris (Residue-2) was further extracted twice with chloroform-methanol-conc. hydrochloric acid (120:60:1, v/v); another portion of the cell debris (Residue-2) was hydrolyzed in 6N-hydrochloric acid and the reaction mixture was extracted with petroleum ether. Neither of the combined lipid fractions exceeded 2% of the total amount of cellular lipid.

We take this as evidence that our lipid extraction procedure is highly suitable for the analysis of yeast lipids.

We wish to thank Dr. Sobus et al. for their informative comparison of statistical methodology.

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Relationships between Base-Exchange Reaction and the Microsomal Phospholipid Pool in the Rat Brain In Vitro

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ABSTRACT

The calcium-stimulated incorporation of ethanolamine, choline and L-serine into rat brain microsomal phospholipids has been investigated. The membranes were prelabeled in vitro in their choline or serine phosphoglycerides by base-exchange and then chasing experiments were done by displacing the lipid-bound base by ethanolamine, choline, or L-serine labeled with a different isotope. The results indicate that membrane phosphatidylcholine is presumably a substrate for the exchange with all the three bases, whereas phosphatidylserine exchanges only with ethanolamine and L-serine but not with choline. A small phospholipid pool (3-7% of the total available pool) is active in the calcium-dependent exchange with choline, ethanolamine, and L-serine. When the microsomal membranes are prelabeled in vitro in their phosphatidylcholine moiety through the cytidine-dependent pathway and then chasing experiments are performed with the three nitrogenous bases, as above, the small phospholipid pool is hardly detectable. In view of these and other results (Gaiti et al., FEBS Letters 49:361 1975), it is suggested that at least two different pools of phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine might exist in rat brain microsomes.

INTRODUCTION

In recent years, experimental evidence has indicated that labeled free ethanolamine, choline, and L-serine are incorporated in vitro into rat brain microsomal phospholipids by a Ca^{2+}-dependent base-exchange mechanism (1-6). The enzymic system is located exclusively in the microsomal fraction (2,3) and might operate in vivo (7-10).

The base-exchange enzymic system is specific for the polar head-groups of phospholipids and is thought to represent an exchange of nitrogenous bases with pre-existing endogenous membrane phospholipids (2-6,11). Kinetic data and differences found among the time-dependency curves of the various incubated bases, and of K_m and V_{max} values on varying the Ca^{2+} concentration and pH of the incubation medium, strongly support the view that more than one enzyme is involved in the exchange reactions (5,11), as proposed also for the liver system (12-13).

Although considerable rates of exchange of ethanolamine, choline, and L-serine take place in vitro in brain microsomes (1-6), little information has been given about the type of phospholipid substrates (14,15) that participate in the exchange reaction at the nerve membrane level. Recent data of Gaiti et al. (6), who have investigated the incorporation of ethanolamine by base-exchange, have indicated, by confirming previous results (2,13,16), that only a small pool of membrane phospholipid is active in the exchange reaction with the free bases. Different degrees of displacement of microsomal lipid-bound ethanolamine were obtained (6), in fact, by the exchange in vitro with free bases (ethanolamine, choline, L-serine), depending on the mechanism of prelabeling the PtdEtn-containing microsomes, thus pointing to the existence of at least two biochemically different pools of PtdEtn in brain microsomes.

It was decided, therefore, to extend these studies by examining the type and degree of exchange that take place in vitro at the level of brain microsomes between endogenous phospholipid and choline, ethanolamine, or L-serine, after the microsomal membranes were prelabeled in vitro in their PtdCho or PtdSer molecules. These studies, which were carried out in the past with microsomal membranes prelabeled in their PtdEtn (6), have been performed now by incubating tritiated choline or L-serine in a base-exchange system and by chasing successively the membrane-bound radioactivity of PtdCho or PtdSer with [^{14}C] labeled ethanolamine, L-serine, or choline, at proper time intervals. The present work attempts to establish by this procedure the presence of phospholipid pools at the microsomal level.

EXPERIMENTAL PROCEDURES

Microsomes

Brain microsomes were prepared from male

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Sprague-Dawley rats (110-130 g) and purified as described elsewhere (2,4). They were suspended by hand homogenization in 0.32 M sucrose containing 2 mM dithiothreitol to give a concentration of 8-10 mg protein/ml. The purity of the microsomal fraction was checked by published procedures (2), and the microsomes used immediately.

Prelabeling of Microsomes

Three distinct types of experiments were performed in order to prepare microsomes prelabeled at the level of the nitrogenous base moiety of their phospholipids.

Prelabeling by base-exchange: In this case (Experiment A), the purified brain microsomes (1-2 mg of protein) were incubated (0.5 ml of final volume) in a medium containing 2.5 mM $CaCl_2$, 40 mM HEPES buffer at pH 8.1, 0.32 M sucrose containing 2 mM dithiothreitol, and 3.3 mM [*Me*-^{3}H]choline [Specific activity (S.A.) of 1.62 nCi/nmol] or 1.95 mM [3-^{3}H] L-serine (S.A. of 2.22 nCi/nmol). After 15 min of incubation at 37 C during shaking (80 strokes/min), the incubation mixture was diluted 15-fold by adding a cold solution of 0.32 M sucrose containing 2 mM β-mercaptoethanol, and the microsomes washed and pelleted, as reported previously (6), in order to carry out the subsequent incubation (see the section, Incubation of Prelabeled Microsomes). Less than 0.5 nCi/mg protein was left in the form of residual radioactivity by the procedure adopted (6), as nonincorporated lipid-[*Me*-^{3}H] choline or [3-^{3}H] L-serine in the microsomal pellet (determined by scintillation on a last microsomal washing), and the obtained value was always considered in the final calculations for each experiment. Protein recovery was almost complete after this procedure.

Prelabeling by cytidine-depending mechanism (de novo synthesis): In the second case (Experiment B), 1-2 mg of microsomal protein (0.5 ml of final volume) was incubated for 40 min at 40 C with 1.18 mM labeled CDP-Cho (CMP-[1,2-^{14}C] phosphorylcholine), at a S.A. of 0.84 nCi/nmol, as described elsewhere (17). After incubation, the mixture was freed from any unreacted labeled CDP-Cho, as reported above.

Prelabeling in vivo: In this case (Experiment C), rats (110-130 g body wt) were injected intracerebrally (18) at 3 mm of depth with 2.31 μCi of [*Me*-^{3}H]choline (S.A. of 500 μCi/μmol), and microsomes prepared after 270 min from treatment, as described in the text. The subsequent incubation of the microsomal pellet was carried out, as reported in Incubation of Prelabeled Microsomes.

Incubation of Prelabeled Microsomes

After the prelabeling of the microsomes by the mechanisms reported under Experiments A, B, or C, the reisolated particles were incubated under standard incubation conditions (final volume of 0.5 ml), as follows: brain microsomes (1-2 mg protein), 2.5 mM $CaCl_2$, 40 mM HEPES buffer at pH 8.1, 0.32 M sucrose containing 2 mM dithiothreitol and either labeled [1,2-^{14}C]ethanolamine (1.98 mM, S.A. of 0.11 nCi/nmol), [1,2-^{14}C]choline (4.5 mM, S.A. of 0.22 nCi/nmol), or L-[3-^{14}C]serine (1.98 mM, S.A. of 0.17 nCi/nmol), in order to study the displacement of the lipid-bound base due to the exchange with a base labeled with a different isotope. In the case of incubation with bases of brain microsomes prelabeled in vitro with [^{14}C]labeled-CDP-Cho (see Experiment B of previous section), the microsomal pellet was incubated in the same conditions as reported above but with each of the following tritiated bases, in place of the [^{14}C]-labeled compounds: [1,2-^{3}H]ethanolamine (1.98 mM, S.A. of 4.59 nCi/nmol), [*Me*-^{3}H]choline (4.5 mM, S.A. of 2.2 nCi/nmol), or L-[3-^{3}H]serine (1.98 mM, S.A. of 4.4 nCi/nmol). In all the series of incubations, experiments were also performed in the absence of added base to study the displacement due to water alone.

After 3, 6, and 9 min of incubation, the samples were inactivated, as explained later. Samples were also treated soon after the addition of microsomes (zero time), in order to calculate the experimental data.

Assay for the Base-Exchange Reaction

The incubations of the prelabeled microsomes were terminated by adding 9 vol of chloroform:methanol (2:1 v/v), followed by a brief agitation. After one more extraction and filtration, the extracts were freed from contaminants, purified as reported elsewhere (2), and then taken to dryness in a stream of nitrogen. Fractionation of intact labeled lipid classes (ethanolamine phosphoglycerides, choline phosphoglycerides, and serine phosphoglycerides), their identification and treatment on thin layer chromatography (TLC) plates were carried out, as reported elsewhere (5). Counting of [^{14}C] and [^{3}H] in the same sample was carried out by scraping off the labeled lipids from the TLC plates into counting vials with the addition of 0.5 ml of diethyl ether:methanol:chloroform (10:10:1 v/v/v) and 10 ml of liquid scintillation fluid containing 33% Triton X-100 (v/v). Recoveries of about 95% of radioactivity were obtained by this method. Double isotope counting was carried out in a Packard Tri-Carb liquid scintillation spectrometer Model 3330,

by the method of the external standard for quenching correction. With various preliminary experiments, it has been checked that counting reliability and significance of the obtained values were always noticeably satisfactory for the dual labeling studies carried out in these series of experiments. Standard deviations for values of each set of similar experiments were less than 5-10%.

Other Determinations

Marker-enzyme assays were done as described elsewhere (2). Protein was determined according to Lowry et al. (19) with crystalline bovine serum albumin as a standard. Phospholipid P content of each TLC spot was estimated in quadruplicate, as described elsewhere (2,4).

Calculation of Experimental Data

The degree of the displacement of a lipid-bound base moiety (labeled with a first isotope) of prelabeled microsomal membranes, due to the addition of bases labeled with a second different isotope (see "Incubation of Prelabeled Microsomes" under "Experimental Procedures"), was determined (6) as the loss of radioactivity of the first isotope from the isolated phospholipid or occasionally also as the release of the base labeled with the first isotope into the aqueous phase. The incorporation of the base through base-exchange was followed under the same conditions in which the displacement of the bases was normally examined, by estimating the increase of radioactivity content of the second isotope in the isolated phospholipid.

Experimental data have always been corrected for the release due to water alone of the lipid-bound base, by performing incubation experiments with the prelabeled microsomes in the absence of added base. This release was always negligible, for the time considered, when microsomal PtdSer was used as the substrate for the displacement reactions, whereas in the whole series of experiments carried out with labeled microsomal PtdCho this release was of the order of ca. 0.05-0.12 nCi/mg protein, depending on time. Correction was also made for the small and always constant amount (less than 0.5 nCi/mg protein) of nonincorporated base which was left in the microsomal pellet after the washing procedure following the preincubation experiments (see "Prelabeling by Base-Exchange").

Data of the present work either will be reported as nCi/mg microsomal fraction/time considered, or are converted into nmol/mg protein/time considered.

Materials

[1,2-^{3}H]ethanolamine, [*Me*-^{3}H]choline, and [3-^{3}H] L-serine were purchased from the Radiochemical Center (Amersham, England), (CMP-[1,2-^{14}C]phosphorylcholine) (labeled CDP-Cho) was obtained from ICN (Cleveland, OH) and [1,2-^{14}C]ethanolamine, [1,2-^{14}C]choline, and L-[3-^{14}C]serine from New England Nuclear Corp. (Frankfurt, West Germany). Purified lipids, as reference standards, were obtained from Pierce Chemical Co. (Rockford, IL). Before use, all unlabeled and/or labeled substrates were tested by TLC on cellulose or silica gel layers for their chemical purity and/or their isotope content and radiochemical purity. Trace contaminants have never been found by the use of radiochromatoscanner. Organic solvents were all freshly distilled over calcium chloride and stored under nitrogen. Scintillation chemicals were products of Packard S.A. (Zürich, Switzerland).

RESULTS

Prelabeling of Microsomes

Analyses performed either by hydrolytic procedures (2,4) or by direct TLC (5) on the total lipid extract after prelabeling of microsomes by Experiments A, B, or C, have indicated that the choline and L-serine moieties incorporated into the brain microsomal lipid were associated only with the Cho-PG and Ser-PG, respectively. This finding confirms previous results (2-5). The same was true when the microsomal membranes were prelabeled in vitro with radioactive CDP-Cho. It was also shown, by following published procedures (2), that the radioactivity of Cho-PG and Ser-PG was entirely localized in the base-component of each radioactive lipid. In all the subsequent part of the work, Cho-PG, Etn-PG and Ser-PG will be referred as to PtdCho (phosphatidylcholine), PtdEtn (phosphatidylethanolamine) and PtdSer (phosphatidylserine), respectively.

Incubation of Prelabeled Microsomes

The type of procedure reported in the present contribution allows a demonstration of the displacement of the lipid-bound base moiety of prelabeled membranes due to the addition of free bases of similar or different structure but labeled with a different isotope. By following this procedure and by performing chase experiments at given intervals, it has been possible to estimate, through base-exchange, the degree of displacement (and simultaneously the degree of incorporation of the displacing base) of the choline or serine moieties of brain microsomal PtdCho or PtdSer, which had been synthesized previously by different mechanisms (see Experiments A, B, or C, under "Experimental Procedures").

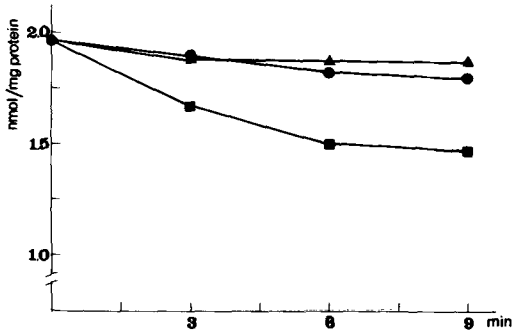

FIG. 1. Displacement of [*Me*-^{3}H]choline from rat brain microsomal labeled PtdCho by the addition of [^{14}C]labeled ethanolamine (■—■—■), L-serine (●—●—●), and choline (▲—▲—▲) in the reincubation medium. See the text for experimental procedures. The loss of radioactivity of microsomal PtdCho, expressed as nmol/mg protein, is plotted against time or reincubation (min). Estimations done in quadruplicate.

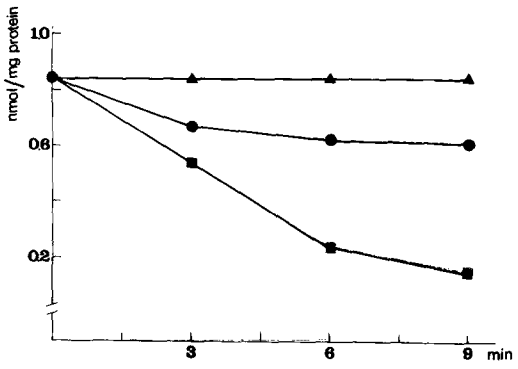

FIG. 2. Displacement of [3-^{3}H]serine from rat brain microsomal labeled PtdSer by the addition of [^{14}C]labeled ethanolamine (■—■—■), L-serine (●—●—●), and choline (▲—▲—▲) in the reincubation medium. For other details, see Figure 1. Estimations done in quadruplicate.

Before referring to the various results, it must be mentioned that control experiments carried out by omitting the displacing bases in the incubation mixture have frequently shown, with few exceptions (see "Experimental Procedures"), that there was no decrease in vitro of lipid radioactivity during the chase period (0-9 min). The decrease of lipid radioactivity which will be reported later on and which takes place during the chase period cannot depend on degradation of phospholipids due to water alone and leading to production of water soluble products. These findings confirm previous results (6,13).

Displacement of Microsomal Lipid-Choline and Lipid-Serine Prelabeled by Base-Exchange (Experiment A)

Figure 1 shows that ethanolamine, L-serine, and choline all displace the choline moiety of microsomal PtdCho, synthesized by base-exchange, though at different rates. In separate experiments (not shown in the figure), it was observed that the released tritiated choline was almost quantitatively estimated in the aqueous phase. The data of Figure 1, as well as those of the forthcoming figures and tables, have all been corrected for the release of lipid radioactivity due to water alone (see "Experimental Procedures"). Figure 1 shows that 25%, 8.6%, and 5.2% of tritiated choline in the labeled PtdCho were displaced after 9 min of reincubation in the exchange medium, with [^{14}C] labeled-ethanolamine, L-serine, and choline, respectively. Therefore, PtdCho can act as a membrane-bound substrate for the incorporation of ethanolamine and possibly also of L-serine and choline, the degree of displacement by these two last compounds being more hypothetical. By estimating the degree of incorporation of the three [^{14}C]labeled bases during the displacement reaction (see "Experimental Procedures"), only 4.65, 1.94, and 0.44 nmol/mg microsomal protein/9 min of ethanolamine, L-serine, and choline, respectively, were found to be incorporated, and these data, together with the results on percentages of displacement (Fig. 1), indicate that the total available pool of PtdCho for all types of exchange was ca. 18-20 nmol (8 nmol in the exchange of choline), which is only a small amount (3-6%) of the total PtdCho (ca. 300 nmol) present in 1 mg of brain microsomal protein (20,21). The value of this pool size is strikingly similar to that already found (6) for exchanging PtdEtn molecules (5-6%). It must be mentioned that the base concentration used in all the displacement reactions (and therefore in all the reincorporation experiments) was saturating, namely well above the K_m values, at the pH and Ca^{2+} concentration used, which are 8×10^{-5}M, 4×10^{-4}M, and 5.88×10^{-4}M for ethanolamine, L-serine, and choline, respectively (5).

Figure 2 shows that after 9 min of incubation, ethanolamine and L-serine are able to displace by 82% and 27%, respectively, microsomal lipid-serine, synthesized by base-exchange, being contemporarily reincorporated during the displacement reaction, at the same time intervals, for 4.44 and 1.47 nmol/mg microsomal protein. This result indicates again, as reported also in Figure 1, that the total pool of PtdSer available for the exchange with both ethanolamine and L-serine was small, ca.

TABLE I

Displacement of Rat Brain Phosphatidylcholine Synthesized in Vitro by the CDP-Choline-Mediated Pathway[a]

Addition	nmol of lipid-bound choline displaced at the indicated min of reincubation[b]		
	3	6	9
Ethanolamine	0.22	0.20	0.20
L-Serine	0.32	0.34	0.32
Choline	0.08	0.09	0.10

[a]Experiments were carried out by incubating the brain microsomes with [^{14}C]labeled CDP-Cho (1.17 mM, S.A. of 0.84 nCi/nmol) for 40 min, as reported in Experiment B of preincubations (see "Experimental Procedures"), and by reincubating the particles under conditions of base-exchange, as reported under "Experimental Procedures," in the presence of either [1,2-^{3}H]ethanolamine, L-[3-^{3}H]serine, or [Me-^{3}H]choline. Experiments were done in quadruplicate.

[b]Nmol of prelabeled PtdCho at zero time: 5.33 per mg of protein (estimations in quadruplicate). Correction of data has been made for the release of radioactivity by water alone (see "Experimental Procedures"). Data reported in nmol/mg of microsomal protein.

TABLE II

Displacement of Rat Brain Phosphatidylcholine Synthesized from Administered Choline in Vivo[a]

Addition	nCi of lipid-bound choline displaced at the indicated min of reincubation[b]		
	3	6	9
Ethanolamine	0.14	0.29	0.51
L-Serine	0	0	0
Choline	0.23	0.36	0.41

[a]Experiments were performed by injecting intracerebrally (18) rats (110-130 g) with 2.31 μCi of [Me-^{3}H]choline (S.A. of 500 μCi/μmol) and by preparing microsomes, as described in the text after 270 min from the administration. Prelabeled microsomes were reincubated as reported in the text, and in quadruplicate.

[b]NCi of prelabeled PtdCho at zero time: 3.44/mg of protein (estimations in quadruplicate). Correction of data has been made for the release of radioactivity by water alone (see "Experimental Procedures"). Data reported in nCi/mg of microsomal protein.

5.4 nmol, i.e., only 7% of the total PtdSer (80 nmol) present in 1 mg of microsomal protein (22). These data agree well with those reported for the "active" pools of PtdEtn (6) and PtdCho (present work). Figure 2 indicates, in addition, that choline is not able to displace labeled L-serine from the phospholipid, and therefore that this free base cannot exchange with membrane-bound PtdSer, at least in the indicated experimental conditions. In all the experiments depicted in this section, saturating concentrations of bases were used (5).

Displacement of Microsomal Lipid-Choline Prelabeled by Cytidine-Depending Mechanisms (Experiment B)

Experiments have been carried out, in light of previous results, by performing the displacement reaction between free nitrogenous bases and microsomal membranes which had been prelabeled in their PtdCho moiety through the CDP-choline-mediated pathway (part of the de novo synthesis mechanism), in place of the base-exchange reaction (see "Experimental Procedures"). Table I shows that after 9 min of incubation, L-serine and ethanolamine displace the choline moiety from PtdCho by only 6% and 4%, respectively, and that choline displaces by only 2%. The degree of displacement does not change already after 3 min of reincubation. These data differ essentially from those reported in Figure 1, which reported the percentages of displacement rates of lipid-choline prelabeled by base-exchange, and which were 25%, 8.6%, and 5.2% for ethanolamine, L-serine, and choline, respectively.

Displacement of Microsomal Lipid-Choline Prelabeled in Vivo (Experiment C).

Experiments have been performed by reincubating for the displacement reaction brain microsomes which had been prelabeled in vivo with choline (18) in their PtdCho molecules and then isolated after 270 min from administration. Table II shows that, after 9 min of reincubation, ethanolamine and choline displace by 15% and 12%, respectively, the choline moiety of microsomal PtdCho synthesized by the in vivo administration of tritiated choline,

whereas L-serine is without effect. These data are different from those of Figure 1 and Table I, where the displacement reaction was examined on microsomal PtdCho synthesized, respectively, by base-exchange and by de novo synthesis and seem to reflect an intermediate situation. By estimating the degrees of incorporation of the [^{14}C]labeled choline and ethanolamine (see "Experimental Procedures") during the displacement reaction reported in Table II, only 3.26 and 5.76 nmol/mg microsomal protein/9 min were found to be incorporated, and these data, together with the results on percentages of displacement (Table II), indicate that the total available pool of PtdCho for the exchanges of choline and ethanolamine is ca. 26-37 nmol, which is only 9-12% of the total available PtdCho pool (300 nmol) present in 1 mg of brain microsomal protein (20,21).

DISCUSSION

The results of the present work show that both PtdCho and PtdSer act as membrane-bound substrates for the Ca^{2+}-stimulated base-exchange reaction in rat brain microsomes. In previous work (6) it was shown that PtdEtn acts similarly. Ethanolamine, and probably L-serine and choline, exchange with membrane-bound PtdCho (Fig. 1) and PtdEtn (6), while PtdSer exchanges only with ethanolamine and L-serine and apparently not with choline (Fig. 2). This last result contrasts with Bjerve's data (13), which indicated that in liver microsomes choline exchanged with PtdSer but not with PtdEtn. Probably differences in competitions between bases of different structures for the same enzyme or enzyme site are at play in explaining these discrepancies. The other displacement reactions between membrane-bound labeled lipid and free bases, reported in this work, agree with those reported for the liver system (6).

PtdCho can thus be converted to PtdEtn and probably to PtdSer in brain microsomes, and PtdSer only to PtdEtn; moreover, PtdEtn can be converted both to PtdCho and PtdSer (6). The method of using differently labeled bases has enabled us to perform simultaneously in the same experiment the displacement reaction by a free base and the incorporation of the displacing base into the microsomal lipid. This method, among probably others, has allowed us to observe that the total available pool of PtdCho for the exchange is only 3-6% of the total PtdCho molecules present in the microsomal membranes, and similarly that of PtdSer only 7% of the total. These results, which confirm previous findings (6), about the "active" PtdEtn pool (5-6% of the total), are probably explained by an inhomogenous labeling of the phospholipid pool with the consequence that only a small pool participates in the exchange mechanism at the membrane level (2,6,13).

Table I indicates that poor values of displacement of prelabeled PtdCho are given by ethanolamine (4%), L-serine (6%), and choline (2%), when the radioactivity of the lipid synthesized in vitro through cytidine-dependent mechanisms (which is part of the de novo synthesis) is chased by added bases, and this contrary to the findings of Figure 1, which showed much higher degrees of displacement. This finding again indicates that only a small part of the total PtdCho of brain microsomes, of high specific activity, is available to the exchange enzyme, and that when the PtdCho molecules are more homogenously labeled by the Kennedy's pathway, in place of the exchange reaction, the high degree of base displacement due to the exchange enzyme is no longer observed. This finding was even more evident when PtdEtn synthesized by the de novo pathway was examined (6).

When the chasing experiments were done on the microsomal PtdCho synthesized in vivo by choline administration, the data indicated that the displacement rates were rather high (Table II) at intermediate values between those obtained on membrane PtdCho synthesized in vitro by base-exchange and "net synthesis," respectively. To what extent this result could mean that the PtdCho of brain microsomes must have been labeled in vivo at least partially by base-exchange is not known, although previous indications (7-10) do not exclude this possibility.

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Lecithin Inhibits Fatty Acid and Bile Salt Absorption from Rat Small Intestine In Vivo[1]

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ABSTRACT

During digestion of a fatty meal, long chain free fatty acids (FFA) and lecithin are among the lipids solubilized in intestinal contents as mixed micelles with bile salts. We hypothesized that if lecithin were not hydrolyzed, the mixed micelles would be abnormal, and absorption of FFA and bile salts would be depressed. To test this hypothesis, isolated segments of rat small intestine were infused in vivo with micellar solutions of 2 mMolar linoleic acid and 10 mMolar taurocholate to which was added 3 mMolar 1-palmitoyl, 2-oleoyl lecithin (a common lecithin in bile and food), or 1-palmitoyl lysolecithin (the hydrolytic product of lecithin). Absorption of FFA and bile salt was measured under steady state conditions using a single-pass technique. Lecithin depressed the rate of FFA absorption by 40% ($p<0.025$) in jejunal and ileal segments whereas lysolecithin was associated with normal rates of FFA absorption. Lecithin also reduced taurocholate absorption from the ileum by 30% ($p<0.05$). These data support the idea that lecithin may depress FFA and bile salt absorption from the small intestine in pancreatic insufficiency.

INTRODUCTION

During the digestion of a fatty meal, pancreatic lipase cleaves fatty acids from dietary triglyceride, while pancreatic phospholipase hydrolyzes biliary and dietary lecithins to lysolecithins. The free fatty acids (FFA) are transported as soluble multimolecular aggregates with bile salts (mixed micelles) through luminal water to the absorptive cell surface; the lysolecithins are dispersed readily in water and absorbed (1-6).

Might an impairment in lecithin hydrolysis affect FFA absorption? Rampone (7,8), and Rodgers and O'Connor (9) reported that lecithin could depress uptake of FFA and cholesterol by rat jejunum in vitro. Lecithins form mixed micelles with bile salts and FFA. Were lecithins to stay unhydrolyzed, mixed micelles might remain abnormally large, diffusing less rapidly through luminal water. We speculated that, in pancreatic insufficiency, lecithins might retard the absorption of FFA as well as of bile salts.

To evaluate our speculation, we measured the rate of absorption of a long chain fatty acid (linoleate) and a bile salt (taurocholate) from isolated rat small intestine in vivo in the presence of a commonly occurring lecithin or its hydrolytic product. Water absorption was measured simultaneously because inhibition of water transport might be responsible for depression of solute absorption. Lipids were infused in concentrations which are similar to those in postcibal duodenojejunal contents of patients with pancreatic insufficiency (10).

METHODS

Materials

Palmitic, oleic, and linoleic acids were obtained from Nu-Chek Prep Inc., Elysian, MN.

Taurocholate was synthesized and crystallized. The final product contained, according to thin layer chromatography (TLC), a trace of cholic acid but no taurine. 1-Palmitoyl glycerophosphoryl choline (lysolecithin) and 1-palmitoyl, 2-oleoyl glycerophosphoryl choline (lecithin) were prepared from egg glycerophosphoryl choline and were purified on columns of silicic acid according to Wells and Hanahan (11). Ratios of phosphorus to weight of sample (mmoles/mg) varied by less than 5% from theoretical values for both synthetic phospholipids.

[1-^{14}C] linoleic acid, and [24-^{14}C] taurocholic acid (Amersham/Searle, Arlington Heights, IL) were purified by TLC and autoradiography. The purified fatty acid behaved as authentic linoleate on gas liquid chromatography.

[1,2-^{3}H] polyethylene glycol (New England Nuclear Corp., Boston, MA) was measured in a toluene-based phosphor (12) in a Beckman liquid scintillation counter; [^{14}C]-toluene and [^{3}H]-toluene were used as internal standards.

Mixtures for infusing intestinal segments consisted of NaCl, 118 mM; $NaHCO_3$, 10 mM; KCl, 6 mM; $CaCl_2$ 0.2 mM; sodium taurocholate, 10 mM; sodium linoleate, 2 mM; [^{3}H]-polyethylene glycol, 0.5 mM; [^{14}C]-linoleate, or [^{14}C]-taurocholate; gas phase, 5% CO_2 in O_2; pH 7.1-7.2.

[1]The following trivial names are used: lecithin (1,2-diacyl-*sn*-glycero-3-phosphorylcholine); lysolecithin (1-acyl-*sn*-glycero-3-phosphorylcholine).

TABLE I

Absorption of Water and Fatty Acids from Segments of Rat Intestine in vivo[a]

Segment	Addition	Water absorbed (mg/min/g)	Linoleate absorbed (nmoles/min/g)
Jejunum	None	79 ± 15 (9)	471 ± 24 (9)
	Lecithin, 3 mM	93 ± 20 (7)	290 ± 53 (7)[b]
	Lysolecithin, 3 mM	93 ± 18 (8)	552 ± 60 (8)
Ileum	None	69 ± 14 (13)	494 ± 45 (13)
	Lecithin, 3 mM	82 ± 11 ((7)	321 ± 47 (7)[b]
	Lysolecithin, 3 mM	92 ± 16 (7)	572 ± 58 (7)

[a]Infusions contained 2 mM [^{14}C] linoleate in buffered bile salt. All infusions were optically clear. The figures are means ± SE (number of experiments).

[b]Significantly different from control, $p<0.025$.

Animals

We used male Sprague-Dawley rats which were fasted overnight. The single-pass technique for measuring water absorption in vivo from isolated segments (13) was modified slightly because we had to ensure that steady-state conditions existed when rates of absorption of fatty acid or bile salt were being determined.

A segment of jejunum or ileum was infused at 0.5 ml/min for 15 min (washout), then for 30 min (equilibration), then for a final test period of 30 min when six 5-min samples of intestinal effluent were collected. Intestinal output of water (13) and ^{14}C in each of the six 5-min periods was calculated, and the regression line between time and cumulative output of [^{14}C]-linoleate or of [^{14}C]-taurocholate was determined by the method of least squares. About 10% of the experiments were rejected because the coefficient of variation of the slope of this regression line was greater than 2.5%; the mean ± 2 SD of the coefficient of variation of the slopes of 17 regression lines relating time and cumulative delivery of [^{14}C]-linoleate by the Gilson peristaltic pump was 0.9 ± 1.6%.

RESULTS

Lecithin depressed the rate of absorption of FFA by 38% in jejunum, and by 35% in ileum (Table I). Rates of FFA absorption were normal when lecithin was replaced by lysolecithin. Neither phospholipid significantly altered water absorption from control values (Table I).

We could not detect absorption of [^{14}C] taurocholate from jejunal segments. Nine ileal segments, however, absorbed 2.9 ± 0.3 (SE) μmoles of taurocholate/min/g, and 68 ± 17 mg of water/min/g. Lecithin, 3 mM, depressed taurocholate absorption to 1.9 ± 0.3 in seven experiments ($t = 2.29$; $p<0.05$) without altering water absorption. Lysolecithin, 3 mM, did not depress taurocholate absorption by the ileum (3.1 ± 0.4 μmmoles/min/g, four experiments).

DISCUSSION

These experiments indicate that lecithin retarded the absorption of micellar FFA from infused segments of rat jejunum and ileum in vivo, whereas its hydrolytic product, lysolecithin, did not depress FFA absorption (Table I). Lecithin also depressed bile salt absorption from rat ileum.

How lecithins depress FFA absorption from mixed micelles is unknown, although the phenomenon has been documented by experiments in vitro in two other laboratories (8,9). Lecithins increase the size of bile salt micelles (6). Large micelles would be expected to diffuse more slowly through luminal water and to retard delivery of FFA to the absorptive cell surface. Another possibility is that persistence of lecithin-bile salt micelles at the absorptive cell surface may alter FFA partitioning. Absorption of FFA could be reduced if FFA preferred the aqueous environment of mixed micelles rather than the lipid membrane of intestinal absorptive cells.

Our results suggest aspects of lipid malabsorption in pancreatic insufficiency other than simple derangement of triglyceride absorption. Dietary and biliary lecithins would be poorly absorbed in this disease (14); they may retard jejunal and ileal absorption of micellar FFA which is present in low concentrations (10). In the ileum, lecithin could depress absorption of bile salts. Such a decreased retrieval of bile salts might explain the increased losses of fecal bile salts which are described in pancreatic insufficiency (15). An increased delivery of bile salts and FFA into the colon may impair colonic absorption of water (16,17), thereby contributing to the increased fecal water in patients who have pancreatogenous steatorrhea (18).

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Comparative Effect of Glucagon, Dibutyryl Cyclic AMP, and Epinephrine on the Desaturation and Elongation of Linoleic Acid by Rat Liver Microsomes

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ABSTRACT

The effect of glucagon, dibutyryl cyclic adenosine 3',5'-monophosphate, and epinephrine on the biosynthesis of polyunsaturated fatty acids of the linoleic acid family was studied. The incubations were performed with rat liver microsomes and labeled linoleic acid under desaturating and elongating conditions. Under desaturating conditions, linoleic acid was converted to γ-linolenic acid, whereas under elongating conditions it was converted to 20:2ω6. Glucagon, dibutyryl cyclic AMP, and epinephrine decreased the oxidative desaturation of linoleic acid to γ-linolenic acid while the elongating reaction was not modified in the experimental conditions tested. Consequently, the results support the hypothesis that the oxidative desaturation of linoleic acid to γ-linolenic acid is the main controllable step in the biosynthesis of polyunsaturated fatty acids of the linoleic acid family in the microsomes.

INTRODUCTION

Enough evidence has been collected during the last few years to show that unsaturated fatty acids are both elongated and desaturated by rat liver microsomes (1-3). In a previous report, it was demonstrated that the first step in the biosynthesis of polyunsaturated fatty acids involves a Δ6 desaturation reaction (2) and that this reaction is modified by nutritional factors. Fasting produces a decrease on the desaturation activity of linoleic acid to γ-linolenic but refeeding enhances the activity of the enzyme (4,5). A high protein diet evokes a significant activation in liver Δ6-desaturation activity (4,6,7), whereas glucose inhibits the enzyme (7,8). It has also been demonstrated that this enzyme is under hormonal control. Alloxan diabetic rats showed a decrease in Δ6-desaturation activity that was restored by parenteral injection of insulin (9,10). The administration of glucagon or dibutyryl cyclic AMP abolished the increase of the Δ6-desaturase activity elicited by refeeding (5). Similarly, epinephrine produced a significant decrease in the linoleic and α-linolenic acid desaturation activity (11). However, little is known about the factors that regulate chain elongation enzymes that are present in microsomes. Torrengo and Brenner (12) have shown that a decrease of environmental temperature produces an increase in oleic, linoleic, and α-linolenic acid desaturation and elongation activity of fish microsomes. Sprecher has shown that elongation of octadeca-6,9,12-trienoic acid was depressed by fasting (13). Nevertheless, dietary protein produced no changes in the elongation of linoleic acid (14). Desaturation and lengthening of linoleic acid is presumably controlled by quite different mechanisms. The present study was undertaken in an attempt to clarify whether the microsomal chain elongation of linoleic acid is under the influence of glucagon, dibutyryl cyclic AMP, or epinephrine.

MATERIAL AND METHODS

Chemicals

1-^{14}C-linoleic acid (56.2 mCi/mmole, 99% radiochemical purity) was purchased from New England Nuclear Corp. (Boston, MA). N^6-2'-O-Dibutyryl 3',5' cyclic AMP (sodium salt). glucagon and theophylline were purchased from Sigma Chemical Company (St. Louis, MO). Epinephrine was provided by Biol. Lab. Argentina.

Animals

Adult female Wistar rats weighing 200-250 g and maintained on standard Purina chow were used.

Treatment of Animals

The rats were divided into groups of three animals each and two experiments were done. In the first experiment, all rats were fasted for 48 hr and then refed a fat-free diet for 48 hr. The distribution of calories in the diet was 30% protein and 70% carbohydrate. From the begin-

[1]The authors are members of the Carrera del Investigador Científico of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

ning of the refeeding period, the different groups were administered glucagon, dibutyryl cyclic AMP, and/or theophylline. Water was given ad libitum. A group of rats maintained on the fat-free diet without treatment and another group of rats fasted for 48 hr were used as controls. Glucagon was administered at a dose of 200 μg/8 hr/100 g of body weight. Dibutyryl cyclic AMP was administered at a dose of 5 mg/8 hr/100 g of body wieght. Theophylline was given at a dose of 2 mg/8 hr/100 g of body weight. Control groups received saline solution every 8 hr. All the drugs were injected intraperitoneally.

In the second experiment, all rats were fasted for 24 hr and then refed with Purina chow for 1 hr. Water was given ad libitum. Three hours after the end of the refeeding period the rats were injected subcutaneously with epinephrine at a dose of 1 mg/kg body weight and killed 12 hr after the injection. Therefore, from the refeeding up to the time of killing, the animals were fasted again (15 hr). The rats used as controls were treated identically except for the substitution of 0.9% saline injection in place of epinephrine. In both experiments, the administration of the drugs did not produce changes in body weight compared to the controls.

Isolation of Microsomes

The rats were killed by decapitation without anesthesia. Livers were rapidly excised and placed immediately in ice cold homogenizing medium (15). After homogenization, the microsomes were separated by differential centrifugation at 100,000 x g by the procedure described prebiously (15).

Incubation Procedure for Oxidative Desaturation

The desaturation of linoleic acid to γ-linolenic acid by liver microsomes was measured by estimation of the percent conversion of 100 nmoles of linoleic acid to γ-linolenic acid. Three nmoles of labeled acid and 97 nmoles of unlabeled acid were incubated with 5 mg microsomal protein with the necessary cofactors at 35 C for 20 min according to the procedure described in a previous paper (5).

Incubation Procedure for Chain Elongation

The elongation of linoleic acid by the liver microsomal preparation was measured by estimating the conversion of 1-^{14}C-linoleic acid to eicosa-11,14-dienoic acid. The basic incubation medium contained 10 μmoles ATP, 1.25 μmoles NADH, 0.1 μmole CoA, 5 μmoles $MgCl_2$, 2.25 μmoles glutathione, 62.5 μmoles NaF, 0.5 μmole nicotinamide, 62.5 μmoles phosphate buffer (pH 7), 1.5 μmoles KCN, and 0.2 μmoles malonyl CoA in a total volume of 1.5 ml of 0.15 M KCl and 0.25 M sucrose solution. Five nmoles labeled linoleic acid and 135 nmoles unlabeled acid were incubated with 5 mg microsomal protein in tubes fitted with rubber serum stoppers. All the above components except the microsomes were added to the incubation tubes. The tubes were gassed by inserting two hypodermic needles through the rubber stoppers and allowing nitrogen to flow through the tubes for 10 min. The incubations were initiated by injecting the microsomes into the tubes with a Hamilton syringe. All incubations were carried out at 35 C for 10 min in a metabolic shaker.

Analysis of the Fatty Acids

Incubations were stopped by addition of 2 ml of 10% KOH in methanol. The fatty acids were recovered by saponification of the incubation mixture. The acids were esterified with 3 M HCl in methanol (3 hr at 68 C). The distribution of the radioactivity between linoleic acid and γ-linolenic acid or the elongation product was determined by gas liquid radiochromatography in an apparatus equipped with a Packard proportional counter by the procedure described previously (15). The labeled methyl esters were identified by equivalent chain length determination and comparison with authentic standards.

RESULTS AND DISCUSSION

The ability of rat liver microsomes to desaturate and elongate linoleic acid has been well demonstrated. The study of the biosynthesis of polyunsaturated fatty acids from linoleic acid led to the recognition of two metabolic pathways: the desaturating and the elongating routes. The first one begins with a Δ6-desaturation of linoleic acid to γ-linolenic acid and is followed by an active elongation to 20:3ω6 (Δ8-11-14), a further desaturation to arachidonic acid (1,16), a new elongation to 22:4ω6 and a weakly active desaturation to 22:5ω6 (2,3). 18:2 (Δ9-12) → 18:3 (Δ6-9-12) → 20:3 (Δ8-11-14) → 20:4 (Δ5-8-11-14) → 22:4 (Δ7-10-13-16) → 22:5 (Δ4-7-10-13-16). The elongation route is initiated by the elongation of linoleic acid to 20:3ω6 and is followed by a desaturation to 20:3ω6 (Δ5-11-14). 18:2 (Δ9-12) → 20:2 (Δ11-14) → 20:3 (Δ5-11-14). This route apparently ends with this acid since it is not converted to arachidonic acid (17,18). However, 20:2ω6 may be elongated to 22:2ω6 (3). The existence of similar metabolic routes has been shown for α-linolenic acid by Alaniz et

TABLE I

Effect of Glucagon and Dibutyryl Cyclic AMP on Chain Lengthening and Oxidative Desaturation of 1-^{14}C- Linoleic Acid

Condition	Treatment	nmoles of product formed in the incubation: Desaturation[a] (6-9-12) 18:3	nmoles of product formed in the incubation: Elongation[b] (11-14) 20:2
Fasted	none	12.3[c] ± 0.9	2.4 ± 0.4
Refed	none	21.3 ± 1.7	6.0 ± 0.6
Refed	+ glucagon + theophylline	5.8 ± 2.3	5.6 ± 0.8
Refed	+ dibutyryl cyclic AMP + theophylline	8.0 ± 1.5	4.3 ± 0.7
Refed	+ theophylline	26.1 ± 3.0	5.7 ± 1.4

[a]100 nmoles of linoleic acid were incubated with 5 mg microsomal protein at 35 C for 20 min with the cofactors detailed in material and methods.

[b]140 nmoles of linoleic acid were incubated with 5 mg microsomal protein at 35 C for 10 min with the cofactors detailed in material and methods.

[c]Means of three animals, each analyzed in duplicate ± SEM.

al. (19) in hepatoma tissue culture (HTC) cells. The importance of the desaturating route is obvious since it synthesizes the higher polyunsaturated acids, arachidonic, 22:4ω6 and 22:5ω6. The reason for the existance of the second route that synthesizes 20:2ω6 is not clear since the work of Sprecher (18) shows that it does not operate as an alternative way of arachidonic acid synthesis in rat liver microsomes. However, an increase of 20:2ω6 has been found in certain lipid fractions, specially cholesteryl esters of human serum, in cases of atherosclerotic lessions in patients with ischaemic heart disease (20).

As is shown in Tables I and II, linoleic acid is elongated by rat liver microsomes to eicosa-11,14-dienoic acid when incubated under strictly elongating conditions. In 10 min incubation, the only product of elongation found was 20:2ω6.

The same microsomes are also able to desaturate linoleic acid to γ-linolenic acid under desaturating conditions. However, the reactions are regulated by different mechanisms. From the data reported by Sprecher (13) and those obtained in the present experiment, it is evident that fasting produces a decrease in the elongation of linoleic acid to eicosa-11,14-dienoic acid, and refeeding reactivates the reaction (Table I). Similar results were obtained when Δ6-desaturation activity was investigated (Table I) (4,5). However, as can be seen in this experiment (Table II) and was demonstrated previously (7), 15 hr of fasting did not modify significantly Δ6 desaturation activity. However, both reactions are affected differently when the animals are treated with glucagon, dibutyryl cyclic AMP (Table I) or epinephrine (Table II).

TABLE II

Effect of Epinephrine on Chain Lengthening and Oxidative Desaturation of 1-^{14}C-Linoleic Acid

Condition	nmoles of product formed in the incubation: Desaturation (6-9-12) 18:3	nmoles of product formed in the incubation: Elongation (11-14) 20:2
Control	22.3[a] ± 2.6	12.5 ± 2.7
Epinephrine	11.1 ± 2.8	12.2 ± 0.7

[a]Means of three animals analysed each in duplicate ± SEM. Experimental conditions as in Table I.

Administration of glucagon or dibutyryl cyclic AMP abolished the reactivation of linoleic acid desaturation evoked by refeeding fasted animals (Table I) corroborating a previous experiment (5). However, the same treatment did not significantly alter the reactivation of linoleic acid elongation. Theophylline injection alone did not modify either the desaturation or the elongation reactions. Similarly, linoleic acid conversion to γ-linolenic acid was significantly depressed by epinephrine administration (Table II) as has also been shown previously (11) while the elongation reaction was not changed by the same treatment Therefore, these results show that, whereas the Δ6-desaturation of linoleic acid is a reaction that is modified by hormones, the microsomal elongation enzyme is insensitive to the same stimulus under the conditions of our experiment.

Experiments with HTC culture cells incubated with α-linolenic acid have provided evidence that dibutyryl cyclic AMP in vivo produces an increase in the elongating pathway with a simultaneous decrease in the desatu-

rating route (19). The difference between these results is undoubtedly a consequence of the different experimental conditions of both assays. In experiments shown in Tables I and II, only the microsomes of treated rats are incubated with the labeled acid in excess of malonyl CoA and reduced cofactors. In consequence, it demonstrates that the enzymatic activity of the elongating system of the microsomes is not altered by the two glycogenolytic hormones tested and dibutyryl cyclic AMP. In the experiment of Alaniz et al. (19), HTC cells were incubated with dibutyryl cyclic AMP. Therefore, the whole living cell including mitochondria, microsomes, and supernatant may contribute to the increase of the elongation. Glucagon produces glycogenolysis and increases the level of acetyl CoA in liver (21) through an enhancement of intracellular levels of cyclic AMP. Therefore, we might suspect that an increase of malonyl CoA could be produced in the cell experiment by carboxylation of acetyl CoA and that this increase could enhance the elongation. However, dibutyryl cyclic AMP inhibits acetyl CoA carboxylase (22). Hence, in the cell culture experiment, it is possible to suspect that the increase of the elongation of linoleic acid to 20:3ω6 was not evoked by the microsomal elongating complex that requires malonyl-CoA but by the mitochondrial acetyl-CoA dependent enzymes. This last system was discovered by Wakil (23). Moreover, this hypothesis is supported by Seubert and Podack's experiments (24) in which it is shown that linoleic and α-linolenic acids are physiological substrates of mitochondrial chain elongation in liver. In addition, microsomal enzymes prefer fatty acid structures of the type of γ-linolenic acid in which a double bond occurs in the Δ6 position, whereas the mitochondrial system elongates preferentially linoleic and α-linolenic acids.

However, an alternative mechanism can be proposed to explain the increase of the elongation route in the cell when the desaturation pathway decreases. Since both enzymes use the same substrate, it is possible that a decrease of the Δ6-desaturation reaction may evoke an increase of the elongation pathway by reduction of the competing desaturating enzyme (19). Notwithstanding, incubation of labeled acid with liver microsomes of diabetic and non-diabetic animals under simultaneous elongation and desaturation conditions has shown that, in spite of a decreased desaturation, there was no increase of 18:2 elongation (25). Therefore, the contribution of the mitochondrial enzymes to the elongation of linoleic acid in the whole cell is apparently the most acceptable explanation for the result found in the experiment with HTC cells. Furthermore, the insensitivity of the elongation to the hormones investigated in the present experiment could be attributed to the absence of the mitochondrial elongating system under our experimental conditions. However, the proposal requires further investigation.

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Lipid Composition of 30 Species of Yeast[1]

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ABSTRACT

The detailed composition of cellular lipid of more than 23 species of yeast has been determined quantitatively by thin-chrography on quartz rods, a method previously used for estimating cellular lipids of seven species of yeast. That data was fortified by neutral and phospholipid quantitations on 30 species of yeast cells. Most of the test organisms contained 7-15% total lipid and 3-6% total phospholipid per dry cell weight, except for the extremely high accumulation of triglycerides in two species of *Lipomyces*. Qualitatively, 30 species of yeast cells contained similar neutral lipid constituents (triglyceride, sterol ester, free fatty acid, and free sterol) and polar lipid components (phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, cardiolipin, and ceramide monohexoside) without minor constituents. Based on the quantitative composition of neutral lipids, the 30 species of yeast were divided into two groups, the triglyceride predominant group and the sterol derivative group. These groupings were fairly well overlapped from the standpoint of the distribution characteristics of fatty acid. The relative polar lipid compositions also grossly resembled each other. Only one exception of polar lipid composition in yeast cells was found in *Rhodotorula rubra* species which contained phosphatidyl ethanolamine as the most abundant phospholipid. Fatty acid distribution patterns in yeast cells consistently coincided with other reports concerning fatty acid composition of yeast cells. Correlation of lipid composition and classification of yeasts are suggested and discussed.

INTRODUCTION

Despite considerable interest in the production of fat yeast, comparatively few detailed investigations concerning the nature of yeast lipids have appeared in the literature. Especially there is a lack of quantitative data on the distribution of the lipids in the cells and on the lipid productivity of many species of yeast. Previously a simple method for estimating the cellular lipids of yeast was established in this laboratory (1). YM-agar plate culture was used for the production of yeast cells. YM-medium is the most generally used for the preservation and maintenance of yeast strains. Moreover, this culturing method is less laborious and gives the average value about various growth stages of cells.

The principal purpose in this series of investigations is to acquire basic information about lipid components in as many yeast species as possible. Work with the pyrophosphatidic acid of *Cryptococcus neoformans* in this laboratory (2,3) also suggested that there are significant relationships between yeast taxonomy and distribution of pyrophosphatidic acid in yeast species (4).

This paper reports the lipid compositions and contents of 23 species of yeast cells and compares them with previously published compositions of seven species.

EXPERIMENTAL PROCEDURES

Yeast Strains

In addition to the seven species of yeast in a preceding report (1), the following 23 species were used in this study.

Ascosporogenous yeast: *Saccharomyces rosei* W-70, *Saccharomyces carlsbergensis* BH1-3, *Saccharomyces rouxii* MS1-3, *Kluyveromyces polysporus* EC12-4, *Debaryomyces hansenii* MY-45, *Debaryomyces nilssonii* Z-9-6, *Pichia membranaefaciens* IV-5-1, *Pichia farinosa* WH3-1, *Hansenula anomala* WH16-2, *Lipomyces lipoferus* 0673, *Saccharomycodes ludwigii* Shin 3-5.

Ballistospologenous yeast: *Sporobolomyces salmonicolor* WF-174.

Asporogenous yeast: *Cryptococcus laurentii* Z-6-5, *Torulopsis colliculosa* J-5, *Torulopsis candida* MYA-3, *Candida Krusei* WF-16, *Candida toropicalis* Shin 1-3, *Candida mycoderma* WF-8, *Candida pulcherrima* 33C, *Trigonopsis variabilis* S-3-9, *Trichosporon cutaneum* KC4-3, *Rhodotorula glutinis* H3-9-1, *Rhodotorula rubra* AY-2, *Rhodotorula rubra* Np. 2-17-4B. All cultures were obtained from the

[1]A part of this investigation has been reported at the 14th conference of the Japan Oil Chemists' Society, Nagoya, Japan, October 1975.

TABLE I

The Amount and Composition of the Lipids in 30 Species of Yeast[a]

Species	TL (% of DC)	PL (% of DC)	PL (% of TL)	Lipid productivity[b] (mg)
Ascosporogenous yeast				
Schiz. pombe	9.1 ± 0.1[c]	4.0 ± 0.5[c]	42.7 ± 4.2[c]	10.0
Sacch. cerevisiae	7.2 ± 0.3[c]	3.7 ± 0.4[c]	52.0 ± 5.0[c]	18.5
Sacch. rosei	8.5 ± 0.3	4.8 ± 0.0	56.8 ± 1.8	21.1
Sacch. carlsbergensis	7.1 ± 0.2	4.4 ± 0.1	61.7 ± 0.7	13.2
Sacch. rouxii	8.1 ± 0.2	4.1 ± 0.1	49.9 ± 1.1	17.3
K. polysporus	10.7 ± 0.4	5.5 ± 0.3	51.6 ± 1.3	10.9
Schw. occidetalis	9.0 ± 0.6[c]	4.8 ± 0.2[c]	53.4 ± 2.2[c]	14.1
Deb. hansenii	7.0 ± 0.4	4.2 ± 0.1	59.5 ± 3.2	16.0
Deb. nilssonii	6.7 ± 0.3	3.9 ± 0.2	57.9 ± 0.3	19.5
P. membranaefaciens	11.3 ± 0.4	5.0 ± 0.2	43.6 ± 2.9	29.2
P. farinosa	8.7 ± 0.1	4.4 ± 0.1	50.3 ± 1.9	31.4
H. anomala	8.6 ± 0.1	4.6 ± 0.1	52.6 ± 1.3	23.8
L. starkeyi	32.3 ± 2.3[c]	5.3 ± 1.8[c]	15.5 ± 4.9[c]	51.7
L. lipoferus	25.8 ± 1.5	3.8 ± 0.3	14.4 ± 0.1	78.0
S'codes. ludwigii	9.6 ± 0.1	4.7 ± 0.2	48.0 ± 1.4	12.6
Ballistospologenous yeast				
Sp. salmonicolor	13.4 ± 0.3	4.0 ± 0.1	29.8 ± 1.3	39.8
Asporogenous yeast				
Cr. neoformans	9.8 ± 0.9[c]	6.0 ± 0.9[c]	63.1 ± 8.3[c]	17.2
Cr. laurentii	11.5 ± 0.2	3.1 ± 0.2	26.7 ± 1.4	46.6
T. colliculosa	8.9 ± 0.2	5.1 ± 0.1	57.8 ± 0.3	21.2
T. candida	8.9 ± 0.2	2.9 ± 0.1	32.2 ± 0.5	29.0
C. utilis	10.6 ± 1.8[c]	4.0 ± 0.3[c]	37.8 ± 5.5[c]	23.9
C. krusei	7.3 ± 0.1	4.1 ± 0.1	55.5 ± 0.4	23.5
C. tropicalis	6.3 ± 0.0	3.4 ± 0.0	53.2 ± 0.1	19.6
C. mycoderma	11.5 ± 0.0	5.0 ± 0.1	43.1 ± 0.3	27.0
C. pulcherrima	7.1 ± 0.1	4.8 ± 0.2	67.7 ± 2.1	15.2
Kl. apiculata	6.6 ± 0.2[c]	2.4 ± 0.4[c]	37.3 ± 3.8[c]	4.6
Trig. variabilis	8.7 ± 0.4	5.5 ± 0.3	63.6 ± 4.2	20.7
Tr. cutaneum	13.3 ± 0.9	5.3 ± 0.3	39.5 ± 0.6	33.3
Rh. glutinis	11.2 ± 1.3	4.6 ± 0.0	41.3 ± 4.8	30.7
Rh. rubra AY-2	14.8 ± 0.3	3.9 ± 1.3	25.4 ± 2.5	40.9
Rh. rubra Np. 2-17-4B	18.6 ± 0.1	3.0 ± 0.3	16.5 ± 1.2	60.0

[a]Abbreviations: DC, dry cell weight; TL, total amount of lipid; PL, total amount of phospholipid. The figures in this table are the mean values ± SD of 5-10 repeated analyses of lipid samples. The yeast cultures were repeated 2-4 times.

[b]Lipid productivity (mg) is expressed as the total amount of cellular lipid which has been obtained from cells grown on a unit of cultural medium (40 ml YM-agar medium, 12 cm diameter petri dish). These data have about ± 10 percent error for mean values.

[c]These data had been reported in a previous report (1).

culture collection maintained at the Faculty of Engineering, Yamanashi University.

Cell production was substantially the same as described previously (1). The YM-culture medium contained per liter: 10 g glucose, 3 g yeast extract, 3 g malt extract, 10 g peptone, and 20 g agar. Yeast cells freshly grown on the slope of the agar medium for 48 hr at 30 C were inoculated on the surfaces of agar plates. After cells had grown to cover the surfaces of the plates, they were harvested.

Total Cellular Lipid Preparation

The total lipid of the yeast was obtained from fresh cells according to the previous paper (1). A 5 g sample of the wet cells was suspended in 20 ml of a chloroform:methanol mixture (1:2 v/v) together with 40 ml of glass beads (0.40-0.50 mm diameter) and the mixture was homogenized for 4 min at 4000 cpm in a cell homogenizer (Braun Model MSK). The disrupted cell suspension was filtered through a glass filter (4G). The residual cell debris were stirred on the magnetic stirrer with 50 ml of a chloroform:methanol mixture (2:1 v/v) for 30 min at a room temperature under an atmosphere of nitrogen and filtered. This stirring and filtering process was repeated three more times. The combined chloroform-methanol filtrate was washed with a 0.9% NaCl solution to remove the nonlipid contaminants according to Folch's method (5).

Quantitative Analysis

The membrane filter (Sartorius MF-100) was

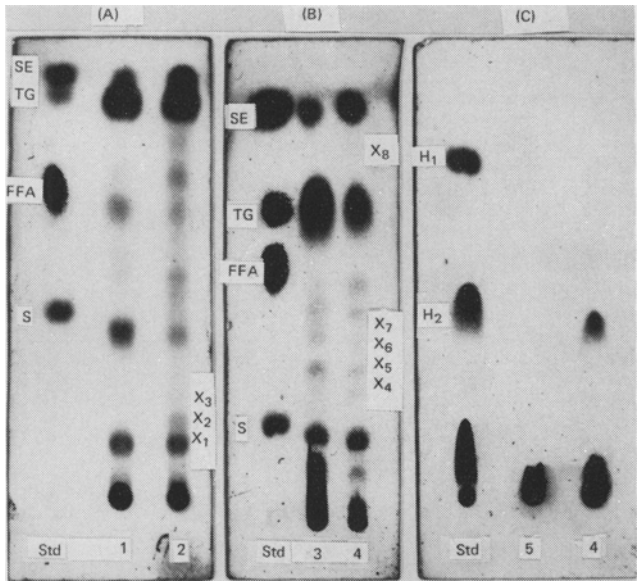

FIG. 1. Thin layer chromatograms of neutral lipids of five yeast species. Plate: silica gel sintered plate. Developing solvent systems: (A) petroleum ether:ethyl ether:gracial acetic acid (80:30:1, v/v); (B) petroleum ether:ethyl ether:gracial acetic acid (90:10:1 v/v); (C) *n*-hexane. Detection: charring at 140 C for 20 min after spraying conc. sulfuric acid. Abbreviations: Std, standard sample; H_1, saturated hydrocarbon (squalane); H_2, unsaturated hydrocarbon (squalene); SE, sterol ester (cholesterol palmitate); TG, triglyceride (tripalmitin); FFA, free fatty acid (oleic acid); S, sterol (cholesterol); X_1-X_8, (unidentified lipids). Samples: 1, *C. krusei*; 2, *T. colliculosa*; 3, *H. anomala*; 4, *Deb. nilssonii*; 5, *P. membranaefaciens*.

used for the determination of the amount of dry cells. The total amount of lipids was estimated gravimetrically. The phosphorus content in a total lipid was determined by the method of King (6). The total amount of phospholipids was calculated by multiplying the phosphorus value by 25.

Thin Layer Chromatography (TLC)

Lipids were chromatographed on silica gel sintered plates (Yamato Kagaku, Tokyo, Japan) and silica gel precoated plates (Merck, Darmstadt, West Germany). Solvent systems and detecting reagents were as described previously (7). Relative neutral lipid composition was determined by thinchrography, a quantitative TLC using a new apparatus which was equipped with silica gel sintered rods (0.9 x 150 mm) and a hydrogen flame ionization detector (Iatroscan TH-10, Iatron, Tokyo). The solvent system was petroleum ether:ethyl ether:acetic acid (90:10:1 v/v) (8). Relative phospholipid composition was determined with quantitative two-dimensional TLC combined with phosphorus estimation (9,10). Developed chromatograms were stained by iodine vapor, and detected spots were scraped from thin layer plates and digested in 0.6 ml $HClO_4$ at 180 C for 2 hr. After addition of 0.2 ml of 5% ammonium molybdate and 0.2 ml of reducing reagent, the total volume was adjusted to 5 ml with distilled water. The reaction mixtures were heated for 7 min in boiling water and centrifuged at 3000 rpm for 10 min. The optical density of the supernatant at 830 nm was estimated.

Fatty Acid Composition

Fatty acid methyl esters were prepared by the HCl-methanol method (11). Gas liquid chromatography was carried out by the use of Hitachi K-53 and 063 gas chromatographs. The column packings used were 10% diethyleneglycolsuccinate (170 C) and 3% SE-30 (70-250 C, 5 C/min) on acid washed Gas Chrom W (60-80 mesh). The flow rate of the N_2 carrier gas was 30 ml/min at 170 C. The injection port and the detector were operated at 320 C. Each peak was identified by comparing the retention time with those of the authentic methyl esters of even numbered straight chain saturated acids (C_{12}-C_{18}), palmitoleic acid, oleic acid, and linoleic acid (Nippon Oil & Fat). All of the samples were chromatographed through the polar and nonpolar columns. Fatty acid composition was expressed as percentage of peak area.

Chemicals

The organic solvents were of reagent grade and freshly distilled before use. Other organic

TABLE II

Neutral Lipid Composition of 30 Yeast Species[a]

Species	S (%)	FFA (%)	TG (%)	SE (%)	Unidentified NL[b]
Ascosporogenous yeast					
Schiz. pombe	4.2 ± 0.6	4.0 ± 0.9	54.4 ± 5.0	28.9 ± 1.7	2
Sacch. cerevisiae	3.5 ± 0.6	2.5 ± 1.6	29.9 ± 6.3	52.9 ± 5.2	2
Sacch. rosei	6.6 ± 0.9	T[c]	14.4 ± 1.4	76.4 ± 2.0	5
Sacch. carlsbergensis	11.1 ± 1.6	10.7 ± 0.4	19.2 ± 0.7	57.1 ± 1.7	5
Sacch. rouxii	8.9 ± 1.5	3.0 ± 1.2	47.0 ± 2.5	38.1 ± 3.6	5
K. polysporus	6.7 ± 0.3	2.8 ± 1.0	26.0 ± 1.7	54.8 ± 5.2	6
Schw. occidentalis	3.0 ± 1.5	7.2 ± 0.1	76.0 ± 5.5	4.9 ± 2.7	3
Deb. hansenii	13.8 ± 1.7	10.0 ± 1.8	65.8 ± 5.2	1.8 ± 0.8	4
Deb. nilssonii	21.2 ± 2.0	2.1 ± 0.5	41.2 ± 1.0	34.4 ± 3.0	5
P. membranaefaciens	3.8 ± 0.9	4.4 ± 1.3	70.4 ± 6.7	6.5 ± 0.6	4
P. farinosa	25.9 ± 2.5	3.5 ± 1.3	59.6 ± 4.3	4.5 ± 1.0	4
H. anomala	11.9 ± 4.0	T	74.0 ± 1.1	7.6 ± 4.0	5
L. starkeyi	2.4 ± 0.7	3.2 ± 0.8	93.4 ± 1.8	T	5
L. lipoferus	1.8 ± 0.2	7.6 ± 0.6	85.4 ± 0.6	T	4
S'codes. ludwigii	3.9 ± 1.9	3.8 ± 1.0	36.1 ± 5.7	46.1 ± 4.7	5
Ballistospologenous yeast					
Sp. salmonicolor	7.2 ± 1.9	15.9 ± 1.3	66.0 ± 6.2	5.0 ± 1.0	2
Asporogenous yeast					
Cr. neoformans[d]	3.6 ± 0.1	1.2 ± 0.2	90.7 ± 0.3	3.1 ± 0.1	4
Cr. laurentii	6.3 ± 1.5	67.2 ± 1.3	21.3 ± 1.5	2.5 ± 0.8	3
T. colliculosa	6.7 ± 1.5	1.7 ± 0.9	39.0 ± 1.6	46.6 ± 2.8	8
T. candida	3.1 ± 0.9	1.1 ± 0.4	92.4 ± 2.3	1.0 ± 0.2	5
C. utilis	3.1 ± 1.4	2.2 ± 0.6	87.6 ± 3.4	2.3 ± 3.0	3
C. krusei	19.4 ± 2.0	4.5 ± 1.0	71.1 ± 1.1	4.9 ± 2.3	5
C. tropicalis	18.4 ± 0.8	4.8 ± 0.8	69.7 ± 1.7	7.0 ± 0.8	5
C. mycoderma	6.5 ± 1.1	1.6 ± 0.2	70.9 ± 2.5	1.3 ± 0.2	4
C. pulcherrima	30.3 ± 2.5	T	62.5 ± 2.9	2.0 ± 0.7	4
Kl. apiculata	7.1 ± 1.7	29.3 ± 3.4	51.2 ± 3.8	4.8 ± 1.4	3
Trig. variabilis	27.7 ± 1.6	14.3 ± 1.2	32.0 ± 2.8	6.9 ± 0.6	4
Tr. cutaneum	1.8 ± 0.6	T	93.4 ± 2.1	2.5 ± 1.4	4
Rh. glutinis	6.7 ± 1.2	7.0 ± 1.4	79.7 ± 3.7	4.5 ± 1.0	3
Rh. rubra AY-2	3.6 ± 0.3	8.0 ± 0.8	84.8 ± 1.7	1.7 ± 0.3	3
Rh. rubra Np. 2-17-4B	1.3 ± 0.1	6.6 ± 0.3	90.9 ± 0.3	0.8 ± 0.1	3

[a]The figures are obtained from the same experiments as in Table I. Abbreviations: S, sterol; FFA, free fatty acid; TG, triglyceride; SE, sterol ester; NL, neutral lipid.

[b]The number of unidentified lipids detected on the area between the origin and sterol ester on the silica gel thin layer chromatograms (Fig. 1).

[c]T<1%.

[d]That was obtained from liquid cultured cells (10).

and inorganic reagents, of analytical grade or of the highest quality commercially available, were used without further purification.

RESULTS AND DISCUSSION

Lipid Productivity and Lipid Content

The lipid productivity by yeast is expressed as the total amount of lipid obtained from cells grown on a unit of cultural medium. The total amount of cellular lipid per dry cell weight, the total amount of cellular phospholipid per dry cell weight, and the cellular lipid production for 30 species of yeast are summarized in Table I. The cells belonging to ascosporogenous yeasts contained relatively constant levels of total lipid (about 7-11%), but two strains of yeast of the so-called fat yeast, *L. starkeyi* and *L. lipoferus*, contained 32% and 26%, respectively. Lipid abundance in asporogenous yeasts ranged from 7 to 18%. A similar range of total phospholipids in ascosporogenous yeasts was found. Table I also shows that the high production of lipid by *L. starkeyi* and *L. lipoferus* was due largely to the deposit of neutral lipids.

Neutral Lipid Composition

Typical chromatograms for cellular neutral lipids of yeast on silica gel plate are shown in Figure 1. The neutral lipid composition of 30 species of yeast cells is presented in Table II. Each of them was found to contain at least four kinds of neutral lipids: triglyceride, sterol ester, free sterol, and free fatty acid. For many

TABLE III

Phospholipid Composition of 30 Yeast Species[a]

Species	PC	PE	PI	PS	CL	PA	PG	LPC	pyro-PA	A[b]	D[b]	G[b]	Unidentified[c] polar lipids
Ascosporogenous													
Schiz. pombe	50.6 ± 0.4	13.1 ± 0.5	13.8 ± 0.7	12.3 ± 0.4	5.5 ± 0.9	T[d]	-[e]	-	-	-	-	-	
Sacch. cerevisiae	42.0 ± 1.5	25.3 ± 1.1	15.8 ± 0.4	7.0 ± 0.6	9.4 ± 0.6	T	-	-	-	-	-	-	
Sacch. rosei	39.2 ± 0.6	21.8 ± 0.2	18.3 ± 0.4	7.3 ± 0.6	9.2 ± 0.4	1.0 ± 0.5	-	-	-	3.2 ± 0.2	-	-	E
Sacch. carlsbergensis	34.1 ± 0.8	25.4 ± 0.4	16.4 ± 0.4	8.5 ± 0.5	8.3 ± 0.2	1.1 ± 0.1	-	T	-	5.2 ± 0.4	-	-	E
Sacch. rouxii	44.2 ± 1.7	16.6 ± 0.6	15.4 ± 0.7	8.5 ± 1.0	7.6 ± 0.8	1.7 ± 0.3	-	T	-	5.5 ± 0.1	-	T	E
K. polysporus	42.3 ± 2.6	14.8 ± 0.1	18.0 ± 1.3	7.4 ± 1.0	5.3 ± 0.7	1.6 ± 0.4	-	-	-	8.0 ± 0.8	-	T	E
Schw. occidentalis	48.2 ± 3.0	16.3 ± 0.3	10.1 ± 1.0	11.9 ± 0.1	7.4 ± 0.6	T	4.1 ± 1.8	1.2 ± 1.1	-	-	-	-	
Deb. hansenii	41.5 ± 1.5	15.6 ± 0.3	9.1 ± 0.6	11.8 ± 0.3	8.7 ± 0.8	2.9 ± 0.4	3.5 ± 0.2	T	-	5.1 ± 0.5	T	-	E
Deb. nilssonii	33.2 ± 0.3	20.1 ± 0.3	17.7 ± 0.8	11.5 ± 1.1	7.4 ± 1.1	1.3 ± 0.6	-	-	-	7.5 ± 0.3	-	1.2 ± 0.3	E, F
P. membranaefaciens	37.7 ± 1.0	17.1 ± 0.4	16.1 ± 0.7	6.9 ± 0.8	6.6 ± 0.2	1.3 ± 0.6	-	-	-	13.1 ± 1.7	-	1.1 ± 0.1	B, E, F
P. farinosa	25.3 ± 0.7	17.6 ± 0.3	16.5 ± 0.8	12.6 ± 0.3	6.0 ± 0.4	3.5 ± 0.3	-	-	-	17.8 ± 0.2	-	T	E, F
H. anomala	39.8 ± 1.8	15.8 ± 0.2	11.0 ± 0.6	13.2 ± 0.5	8.6 ± 0.7	T	-	-	-	7.8 ± 0.9	-	-	E, F
L. starkeyi	43.1 ± 0.6	25.4 ± 0.5	11.7 ± 0.8	10.9 ± 0.5	5.1 ± 0.1	1.3 ± 0.4	-	1.9 ± 0.6	-	-	-	-	
L. lipoferus	37.7 ± 0.7	19.9 ± 0.4	15.8 ± 0.5	9.4 ± 0.9	3.2 ± 0.6	2.2 ± 1.5	-	-	-	10.8 ± 2.4	T	-	E
S'codes. ludwigii	46.7 ± 1.5	13.7 ± 1.4	7.5 ± 0.7	18.6 ± 0.6	8.1 ± 1.0	T	-	-	-	4.6 ± 1.0	-	-	E
Ballistospologenous yeast													
Sp. salmonicolor	28.9 ± 0.7	30.4 ± 1.4	11.5 ± 0.8	12.6 ± 0.6	7.5 ± 0.2	2.7 ± 0.5	-	-	T	6.5 ± 0.6	-	-	B, C, E, F
Asporogenous yeast													
Cr. neoformans[f]	48.5 ± 1.0	27.5 ± 0.5	7.2 ± 0.5	7.8 ± 0.5	3.5 ± 0.5	T	T	2.5 ± 0.5	T	T	T	-	
Cr. laurentii	30.0 ± 1.0	13.1 ± 0.4	10.9 ± 1.0	4.2 ± 0.4	7.9 ± 0.2	10.8 ± 0.5	-	-	1.1 ± 0.3	18.7 ± 0.6	3.2 ± 1.1	-	C, E, F
T. colliculosa	34.4 ± 0.2	27.4 ± 0.3	17.1 ± 0.4	5.9 ± 0.4	9.4 ± 0.3	1.2 ± 0.6	T	T	-	2.7 ± 0.4	T	-	B, E
T. candida	28.5 ± 0.5	16.1 ± 0.2	16.1 ± 0.2	13.5 ± 0.8	7.9 ± 0.4	4.3 ± 1.1	3.8 ± 0.5	-	-	8.6 ± 0.9	-	1.3 ± 0.9	E, F
C. utilis	31.7 ± 0.6	27.3 ± 0.4	12.7 ± 0.4	12.1 ± 0.5	8.0 ± 0.2	2.7 ± 0.3	2.1 ± 0.9	T	-	2.6 ± 0.1	-	-	
C. krusei	33.4 ± 0.4	17.1 ± 0.2	19.9 ± 0.8	9.0 ± 0.5	6.1 ± 0.1	2.0 ± 0.3	T	T	-	11.0 ± 0.2	-	T	E
C. tropicalis	30.9 ± 0.6	19.2 ± 0.4	14.9 ± 1.1	13.9 ± 1.0	8.3 ± 0.2	3.3 ± 1.0	1.2 ± 0.2	-	-	7.3 ± 0.6	T	T	
C. mycoderma	35.2 ± 0.2	17.3 ± 0.9	16.7 ± 0.8	6.6 ± 0.5	7.6 ± 0.3	1.5 ± 0.8	1.4 ± 0.4	T	-	11.3 ± 0.7	-	1.6 ± 0.4	E, F
C. pulcherrima	41.3 ± 0.5	17.5 ± 0.4	13.0 ± 0.9	10.9 ± 0.7	6.2 ± 0.3	T	T	T	-	9.2 ± 0.8	-	-	F
Kl. apiculata	30.6 ± 2.7	16.5 ± 0.5	8.2 ± 1.4	10.6 ± 0.4	24.6 ± 0.6	T	-	5.7 ± 0.9	-	-	3.6 ± 0.8	-	
Trig. variabilis	41.1 ± 0.7	17.8 ± 0.1	11.5 ± 0.4	9.9 ± 0.4	6.6 ± 0.1	1.6 ± 0.4	-	-	-	10.5 ± 0.8	-	1.0 ± 0.5	B, E, F
Tr. cutaneum	28.9 ± 0.4	15.1 ± 0.5	10.6 ± 0.2	11.6 ± 0.2	5.8 ± 0.6	6.6 ± 0.1	-	1.8 ± 0.3	1.6 ± 0.3	13.4 ± 0.4	-	-	B, E, F
Rh. glutinis	40.2 ± 1.5	28.7 ± 2.6	11.9 ± 1.7	4.7 ± 1.7	7.6 ± 0.4	2.8 ± 0.5	-	-	T	-	3.8 ± 0.2	-	E
Rh. rubra AY-2	15.6 ± 1.2	28.7 ± 2.6	7.1 ± 0.5	16.4 ± 0.9	11.8 ± 0.3	6.4 ± 0.7	-	-	2.4 ± 1.0	9.7 ± 1.4	-	T	B, C, E, F
Rh. rubra Np. 2-17-4B	6.5 ± 0.3	29.8 ± 0.3	7.4 ± 0.6	15.7 ± 0.2	13.8 ± 0.4	13.4 ± 0.1	-	-	2.8 ± 0.3	10.7 ± 0.5	-	-	B, C, E, F

[a]The figures are obtained from the same experiments as in Table I. Abbreviations: PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; PS, phosphatidyl serine; CL, cardiolipin; PA, phosphatidic acid; PG, phosphatidyl glycerol; LPC, lysophosphatidyl choline; pyro-PA, pyro phosphatidic acid.

[b]These lipids are unidentified phospholipids. Their chromatographic characteristics are described in Figure 2 and in the text.

[c]Those unidentified lipids were negative to the molybdenum blue reagent (5).

[d]T<1%.

[e]-, not detected;

[f]That was obtained from liquid cultured cells (10).

TABLE IV

Fatty Acid Composition of 23 Species of Yeast[a]

Species	C_{10}	C_{12}	C_{14}	C_{15}	$C_{16:0}$	$C_{16:1}$	C_{17}	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	C_{20}	C_{22}	C_{24}
Ascosporogenous yeast														
Sacch. rosei	T[b]	1.7 ± 0.2	3.7 ± 0.2	T	14.7 ± 0.9	47.5 ± 3.5	-[c]	1.0 ± 0.3	33.3 ± 2.5	-	-	-	-	-
Sacch. carlsbergensis	-	T	2.8 ± 0.2	T	6.2 ± 0.2	50.8 ± 0.9	-	1.2 ± 0.3	37.6 ± 0.3	-	-	-	-	-
Sacch. rouxii	-	T	T	-	13.6 ± 0.2	16.4 ± 0.6	T	3.4 ± 0.1	31.6 ± 0.2	34.4 ± 0.2	-	-	-	-
K. polysporus	T	2.8 ± 0.6	11.0 ± 0.0	T	14.6 ± 0.4	51.3 ± 3.7	-	1.3 ± 0.3	21.0 ± 1.5	-	-	-	-	-
Deb. hansenii	-	-	T	T	18.9 ± 1.0	1.0 ± 0.1	2.0 ± 0.0	10.5 ± 0.7	43.7 ± 1.3	18.7 ± 0.6	4.7 ± 0.1	2.8	T	T
Deb. nilssonii	T	T	1.3 ± 0.1	T	10.7 ± 0.1	33.1 ± 0.4	T	2.5 ± 0.1	35.3 ± 0.3	17.0 ± 0.1	-	-	-	-
P. membranaefaciens	-	-	T	T	12.3 ± 1.0	14.8 ± 1.7	T	T	40.9 ± 0.3	23.6 ± 1.5	7.7 ± 0.9	T	-	-
P. farinosa	-	-	T	T	25.5 ± 1.5	1.5 ± 0.1	T	1.3 ± 1.1	23.9 ± 0.1	46.4 ± 1.7	-	T	T	T
H. anomala	-	-	T	T	17.2 ± 2.0	1.3 ± 0.1	T	3.8 ± 0.1	34.7 ± 0.9	35.6 ± 0.9	7.5 ± 0.2	-	-	-
L. lipoferus	-	-	T	T	37.3 ± 1.0	4.3 ± 0.3	-	6.7 ± 0.3	48.3 ± 1.3	3.2 ± 0.3	-	T	T	T
S'codes. ludwigii	T	T	1.0 ± 0.5	-	13.4 ± 1.1	3.0 ± 0.7	-	T	80.2 ± 2.6	-	-	T	-	-
Ballistospologenous yeast														
Sp. salmonicolor	-	-	T	T	24.0 ± 4.7	T	T	6.1 ± 2.7	35.9 ± 2.1	36.3 ± 9.1	3.4 ± 1.6	-	-	-
Asporogenous yeast														
Cr. laurentii	-	-	T	T	17.2 ± 0.2	T	T	8.5 ± 0.4	37.5 ± 0.1	36.5 ± 0.5	-	T	T	T
T. colliculosa	T	1.2 ± 0.1	3.4 ± 0.2	-	13.9 ± 0.3	46.5 ± 1.0	-	T	34.5 ± 1.0	-	-	-	-	-
T. candida	-	-	T	T	27.9 ± 1.4	3.7 ± 0.3	1.8 ± 0.0	9.0 ± 0.1	42.5 ± 2.0	11.9 ± 0.3	2.7 ± 0.5	-	-	T
C. krusei	-	T	T	T	15.0 ± 0.2	6.2 ± 0.2	T	1.1 ± 0.1	48.2 ± 2.0	14.9 ± 0.6	13.7 ± 1.4	T	T	-
C. tropicalis	-	T	T	1.2 ± 0.0	21.8 ± 0.2	5.4 ± 0.2	2.6 ± 0.1	9.4 ± 0.6	28.6 ± 0.5	26.2 ± 0.2	4.4 ± 0.3	-	-	T
C. mycoderma	-	-	T	T	14.1 ± 0.6	17.1 ± 0.8	2.4 ± 0.1	2.3 ± 0.2	41.1 ± 1.4	18.6 ± 1.0	4.7 ± 0.7	T	-	-
C. pulcherrima	-	-	1.4 ± 0.1	T	21.0 ± 0.1	7.4 ± 0.6	T	1.5 ± 0.2	41.4 ± 2.0	25.6 ± 0.6	1.4 ± 0.3	-	-	-
Trig. variabilis	-	-	T	T	19.0 ± 0.5	34.4 ± 1.6	1.5 ± 0.1	T	22.9 ± 1.8	21.5 ± 1.6	-	-	-	-
Trich. cutaneum	-	-	T	T	24.0 ± 4.7	T	T	6.1 ± 2.7	35.9 ± 2.1	36.3 ± 9.1	3.4 ± 1.6	-	-	-
Rh. glutinis	-	T	T	T	12.3 ± 1.0	T	T	1.9 ± 0.6	31.6 ± 2.2	53.1 ± 1.9	-	T	-	T
Rh. rubra AY-2	-	-	T	T	22.2 ± 1.6	T	T	4.2 ± 0.8	61.0 ± 0.7	10.2 ± 0.8	2.7 ± 0.4	-	-	-
Rh. rubra Np. 2-17-4B	-	-	2.0 ± 0.2	-	23.8 ± 3.7	T	-	2.3 ± 0.1	56.9 ± 0.1	11.5 ± 1.8	3.2 ± 1.3	-	-	-

[a]The figures are the average percentages of relative peak area ± SD obtained from the same experiments as in Table I.

[b]T<1%.

[c]-, not detected.

species, triglyceride made up the major component (50-90% of the total amount of neutral lipid), whereas sterol ester, free sterol, and free fatty acid constituted 2-5%, 3-7%, and trace to 5%, respectively. All of the strains of *Saccharomyces, K. polysporous,* and *T. colliculosa* tested did not fit the above generalization. They contained sterol derivatives as a main neutral lipid (50-80%). *Cr. laurentii* and *Kl. apiculata* contained abnormally high levels of free fatty acids. These results suggest that extensive lipolytic activities (lipase and/or phospholipase) had been functioning in the course of cell growth.

Most of the test organisms except *Pichia, Lipomyces, C. mycoderma,* and *Kl. apiculata* contained hydrocarbon as the minor constituents of neutral lipids. Additionally, the following minor components were detected commonly in most of the test strains: X_1 and X_3 were located on silica gel thin layer chromatogram between the original point and the free sterol; X_4-X_7 were situated between the free sterol and the free fatty acid (Fig. 1). One or two of those spots were stained positively with sterol reagents. Further fractionation of sterols and sterol derivatives will be reported elsewhere.

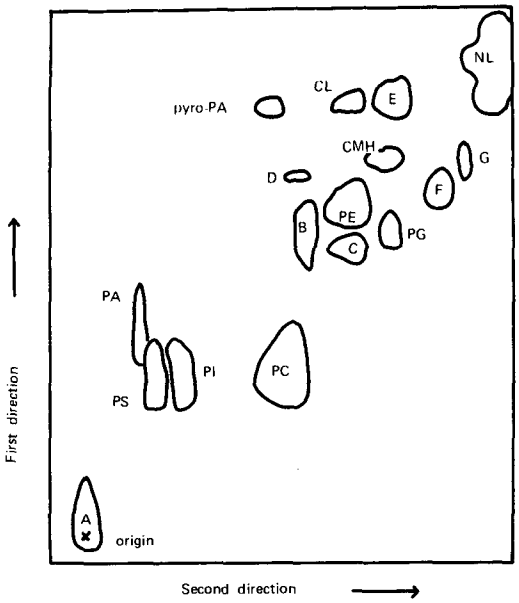

FIG. 2. A typical illustration of a thin layer chromatogram of the polar lipids of yeast. Plate: silica gel sintered plate. Developing solvent systems: first direction, chloroform:methanol:water (65:25:4 v/v); second direction, chloroform:methanol:conc. ammonium hydroxide (65:35:5 v/v). Abbreviations: A, B, C, D, E, F, and G, unidentified lipids; the others, see footnotes to Table III. A, D, and G were phosphorus positive spots. Spot A was also positive for anthrone reagent. Spot C was colored yellow with ninhydrin reagent. B, E, and F were detected with sulfuric acid-charring.

Polar Lipid Compositions

A typical illustration of the two dimensional thin layer chromatogram for yeast cellular lipids is presented in Figure 2. Relative quantitative phospholipid composition is listed in Table III. Most of the yeast species examined in this study possessed similar polar lipid patterns. Phosphatidyl choline constituted the most abundant component, and phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, cardiolipin, phosphatidic acid, and ceramide monohexoside were present. These polar lipid distribution patterns in the yeast cells agreed consistently with those patterns reported by Letters (12). The only exceptions observed were in two strains of *Rh. rubra* which contained phosphatidyl ethanolamine as the most abundant phospholipid in the cells. The specific distribution of pyrophosphatidic acid in six species of yeast was reported previously and was discussed in relation to yeast taxonomy. Phosphatidyl glycerol was also detected in some species. Other unusual distributions of phospholipids were recognized. An unidentified glycophospholipid (A), which remained almost at the starting point on silica gel thin layer chromatograms for several kinds of developing systems, was about 20% per total phospholipid in *Pichia* and *Cr. laurentii*. It is considered likely to be a derivative of the mannosylinositol phosphorylceramide complex or of inositol phosphorylceramides (13). Cardiolipin was contained in relatively large amounts in *Kl. apiculata*. Phosphatidic acid deposits in *Cr. laurentii* and *Rh. rubra* Np. 2-17-4B suggest intense phospholipase activity. From its chromatographic behavior, IR spectrum, and staining behavior, we propose that spot H probably is the *N*-methyl derivative of phosphatidyl ethanolamine (14), and that spot I may be lyso-*bis*-phosphatidic acid (15,16).

Fatty Acid Composition

Relative fatty acid composition of 23 species of yeasts is given in Table IV. Fatty acid distribution patterns in yeast cells closely resembled each other and consisted of analogous series of saturated and unsaturated even numbered aliphatic acids ranging in chain length from C_{10} to C_{24}. Their fatty acid patterns also overlap with others (17). The amounts of C_{16} plus C_{18} acids accounted for over 95% of the total fatty acid in all species again. The species tested in this study were arbitrarily divided into two groups based on fatty acid constitution: one in

which more than 50% of the lipids had chain lengths less than 16 carbons, and one in which more than 50% of the lipids had carbon chain lengths greater than 18 carbons. The three strains of *Saccharomyces, K. polysporus* and *Kl. apiculata* were placed in the first group. All of the remaining 25 species were included in the second group. All of the species of *Saccharomyces, K. polysporous* contained sterol derivatives as the main neutral lipid (50-80%). This property closely coincided with the remarkable distribution pattern of fatty acids in these groups.

Generally speaking, with a few exceptions, there were no significant differences in lipid pattern (neutral lipid, phospholipid, and fatty acid compositions) of yeasts grouped according to plant, animal, or human sources. Furthermore, these data aid in selection of the yeast species needed for raw material for preparation of certain lipids or lipid related material. For example, *Cr. neoformans* and *Rh. rubra* are suitable strains for the preparation of pyrophosphatidic acid (2).

Subsequent studies of additional new species of yeast are in progress to clarify the relationship between lipid composition and taxonomy or the phylogenetic evolution of yeast.

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Metabolism of Chenodeoxycholic Acid in Hamsters

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ABSTRACT

The study on the metabolism after oral administration of chenodeoxycholic acid-24-^{14}C was performed by analysis of radioactivity that had appeared in bile and feces of male hamsters. The radioactive bile acids were analyzed by thin layer chromatography and identified by the isotope dilution method. In the bile of the hamsters with bile fistula, radioactivity was originated from unchanged chenodeoxycholic acid for the most part, and 7-ketolithocholic acid, lithocholic acid, and β-muricholic acid for the remainder. In the feces lithocholic acid, dehydrolithocholic acid, isolithocholic acid, and unchanged form were identified. After the multiple dosing of chenodeoxycholic acid-24-^{14}C for 6 days, β-muricholic acid was also identified in the feces.

INTRODUCTION

Chenodeoxycholic acid (CDCA), the primary bile acid, has been widely explored as a therapeutic agent for gallstone dissolution because this bile acid decreased the saturation of cholesterol in bile, but cholic acid, the other primary bile acid, did not (1). The metabolism of CDCA has already been studied in the rat (2,3), mouse (4), pig (5), rabbit (6), chicken (7) and man (8,9). However, there has been no report concerning the metabolism of CDCA in the hamster which has been recently used as an experimental animal model for the study of pathogenesis of cholelithiasis (10,11). In this report, the metabolism of CDCA in Golden hamsters was investigated to determine metabolites of CDCA in bile and feces.

EXPERIMENTAL PROCEDURES

Chenodeoxycholic acid-24-^{14}C (^{14}C-CDCA; New England Nuclear Corp., Boston, MA; Specific activity, 54 mCi/mmole) was administered orally (1 mg/kg) to male Golden hamsters (6-9 wk of age) as 0.05% solution of 1% $NaHCO_3$. The bile was collected for 24 hr after the administration of ^{14}C-CDCA in three hamsters with bile fistula. The biliary radioactive bile acids were extracted according to the method of Mahowald et al. (2). The conjugated bile acids were hydrolyzed with 8% NaOH at 80 C for 27 hr in screw-capped tubes. In this method, the conjugated bile acids were completely hydrolyzed and artifacts were not observed. Feces were collected for 7 days after a single administration of ^{14}C-CDCA in three hamsters without bile fistula, and for 12 days in the animals given six equal doses (1 mg/kg) once a day. The feces were dried in vacuo for 24 hr and pulverized. After extracting radioactive bile acids twice by refluxing 0.2 g of the feces in 8% NaOH-ethanol (1:8) for 2 hr, ethanol in the filtrate was evaporated. After the extraction the fecal residue contained radioactivity less than 1% of the total. Before washing out neutral lipids from the fecal extract with petroleum ether, the fecal extract was hydrolyzed at 80 C for 27 hr in screw-capped tubes. The solution was acidified with conc.-HCl and

TABLE I

Biliary Exeretion of Radioactivity 24 Hours after Single Oral Administration of ^{14}C-CDCA

Hamster No.	I	II	III
Recovery in bile (% of dose)	76.5	82.1	75.0
Fraction[a]	% of total radioactivity		
Glyco-CDCA	10.9	6.2	6.4
Tauro-CDCA	78.9	81.1	80.0
Sum	89.9	87.3	86.4

[a]Solvent system: n-butanol/acetic acid/water (10:1:1).

TABLE II

Nature of Radioactivity Recovered in Bile and Feces after Single Oral Administration of ^{14}C-CDCA

Compounds[b]	% of total	
	Bile	Feces
Dehydrolithocholic acid	N.D.[a]	2.1-3.7
Isolithocholic acid	N.D.	5.2-8.9
Lithocholic acid	0.5-0.9	53.1-68.2
7-Ketolithocholic acid	8.0-10.0	Trace
CDCA	75.9-85.2	3.3-7.1
Unknown fr.-2	1.1-5.2	2.3-3.8
Unknown fr.-1	N.D.	N.D.

[a]N.D. = Not detected

[b]Solvent system: benzene/dioxane/acetic acid (75:20:2.0)

TABLE III

Composition Percentage of Radioactivity Recovered in Feces from Day 6 to Day 7 after the Oral Administration of 6 Doses of Equal Dose (1 mg/kg) of ^{14}C-CDCA Once a Day

Compounds[a]	Hamsters A	Hamsters B
Dehydrolithocholic acid	3.0	4.1
Isolithocholic acid	9.7	6.1
Lithocholic acid	29.2	35.2
7-Ketolithocholic acid	2.2	2.3
CDCA	7.3	12.8
Unknown fr.-2	3.4	6.7
Unknown fr.-1	21.5	13.7

[a]Solvent system: benzene/dioxane/acetic acid (75:20:2.0)

TABLE IV

Identification of β-Muricholic Acid in Feces[a]

Recryst. No.	Specific activity (x 10^2 dpm/mg)
1	7.04
2	6.04
3	4.47
4	4.50
5	4.54

[a]β-Muricholic acid (45.1 mg) was added to 6.08 x 10^4 dpm (13.5 x 10^2 dpm/mg) and crystallized from aqueous methanol.

the radioactive fecal bile acids were extracted with ether.

The biliary and fecal bile acids were analyzed by partition column chromatography (1) or thin layer chromatography (TLC) using Silica Gel 60 plates (E. Merck Co., Darmstadt, Germany). The identification of radioactive bile acids was performed by isotope dilution method. CDCA (Weddel Pharmaceuticals, London, England), lithocholic acid (Tokyo Kasei, Tokyo, Japan), isolithocholic acid (13), dehydrolithocholid (3-keto-5β-cholanoic) acid (13), 7-ketolithocholic acid (14), and β-muricholic acid (14) were used as standard samples for TLC and isotope dilution methods.

The radioactivity was determined by a liquid scintillation counter (Aloka, Model LSC-652, Nihon Musen, Tokyo, Japan).

RESULTS AND DISCUSSION

Table I shows the radioactivity recovered in the bile for 24 hr after a single oral administration of ^{14}C-CDCA in the hamsters with bile fistula, and the percentages of the fractions corresponding to taurochenodeoxycholate and glycochenodeoxycholate. As shown in Table I,

TABLE V

Identification of β-Muricholic Acid in Bile[a]

Recryst. No.	Specific activity (x 10^2 dpm/mg)
1	3.53
2	2.56
3	2.09
4	1.77
5	1.57
6	1.83

[a]β-Muricholic acid (28.6 mg) was added to 3.26 x 10^4 dpm (11.4 x 10^2 dpm/mg) and crystallized from aqueous methanol.

most of the biliary radioactivity was detected in the fractions of taurine- and glycine- conjugate of CDCA. The hydrolyzates of biliary and fecal bile acids were analyzed by TLC. The percentage composition of CDCA and its metabolites are shown in Table II. The biliary radioactivity was composed mainly of the unchanged ^{14}C-CDCA. This result with hamsters showed a tendency similar to that with rats (2,3), pigs (5), chickens (7) and man (8).

The main metabolite of CDCA in the hamster bile was 7-ketolithocholic acid, which has been identified as a metabolite of CDCA in the rat bile (3), the urine of surgically jaundiced mouse (4), and the duodenal fluid of rabbit (6). Lithocholic acid and unknown fr.-2 were observed as minor metabolites in the bile. The unknown fr.-2 corresponded to dihydroxy-bile acid or hyodeoxycholic acid, but not to ursodeoxycholic acid. Fr.-2 was also confirmed to be different from hyodeoxycholic acid by the isotope dilution method.

In the feces obtained from the hamsters for a week after the administration of ^{14}C-CDCA, 85-90% of the radioactivity was recovered. Lithocholic acid, isolithocholic acid, and dehydrolithocholic acid were identified as fecal metabolites of CDCA (Table II). 7-Ketolithocholic acid, CDCA, and unknown fr.-2 were also detected in the feces. Lithocholic acid has been reported as the main fecal metabolite of CDCA in rabbits (6) and man (9), as in hamsters. It is, therefore, suggested that most of CDCA administered is transformed to metabolites less polar than CDCA and excreted in feces.

Table III shows the percentage composition of radioactivity recovered in feces from day 6 to day 7 after the termination of multiple dosing of ^{14}C-CDCA. The percentage composition of lithocholic acid in the feces of multiple dosing (Table III) was lower than that in a single dosing (Table II). On the other hand, the percentages of unknown fr.-1 corresponding to

trihydroxy-bile acids on the TLC and unknown fr.-2 were larger in comparison with those in a single dosing. The unknown fr.-1 was further analyzed by TLC with the solvent system of chloroform/methanol/acetic acid (80:12:3) (15). A small portion of the radioactivity was detected in the fraction of β-muricholic acid and identified as β-muricholic acid by the isotope dilution method (Table IV).

A similar analysis was performed on the bile of hamsters with bile fistula (Table V). These results indicate that the small portion of CDCA administered is transformed to β-muricholic acid in hamsters, which has been known as a metabolite of CDCA in the mouse (4) and the rat (2,3). However, α-muricholic acid, a metabolite of CDCA in the mouse (4) and the rat (2,3), was not detected in the present experiment.

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Effect of Acute Ethanol Ingestion on Fat Absorption[1]

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ABSTRACT

A test meal (300 mg casein, 600 mg sucrose, 100 mg corn oil, tracer dose of 9.10^3H oleic acid) was given to fasting adult rats with intestinal lymph fistulas. One group received an acute oral dose of ethanol (3.2 g/kg body weight) simultaneously with the test meal. Controls received 2.5 ml of water instead of ethanol. Ingestion of ethanol temporarily delayed the removal of lipid radioactivity from the stomachs. More than 25% of radioactivity fed remained 8 hr after feeding whereas with control rats less than 10% of lipid radioactivity fed remained 6 hr after feeding. In controls and ethanol-treated rats, the amounts of exogenous lipids in the intestinal lumen and mucosa were low and similar enough. Quantities of endogenous and exogenous lipids found in the lymph collected during 24 hr after feeding were similar in the two groups, but the fat absorption peak was found after 6 hr in alcoholic rats and before 6 hr in controls. This delay was probably due to the retention of lipids in the stomach. More of the exogenous lipid was always transported by small particles moving in the region of α_1 globulins in cellulose acetate electrophoresis than by larger particles remaining at the origin. This proportion was enhanced in the ethanol-treated animals. The larger fat particles were richer in endogenous fatty acids in alcohol-treated rats than in controls.

INTRODUCTION

This study was undertaken to investigate the effects of acute ethanol administration on the lymph lipid output and the lymph fat particle size after lipid feeding. Previously (1,2) we have shown that cellulose acetate electrophoresis of mesenteric lymph obtained from rats fed a diet containing ^{14}C or ^{3}H fatty acids exhibited two labeled bands. The first (M_0) remained at the origin whereas the second ($M_{\alpha 1}$) moved in the region of α_1-globulins (see Fig. 1). Since these two kinds of lipoproteins can also be separated by density gradient zonal centrifugation (2) and investigated by electron microscopy (2,3), we have observed that after feeding corn oil, the particles of band M_0 have a larger diameter ($0.1\ \mu m \leqslant d$; mean value $0.15\ \mu m$) than those of band $M_{\alpha 1}$ (mean value $0.05\ \mu m$).

Ethanol has been shown to delay the intestinal absorption of triolein or oleic acid (4) as well as the absorption of various water-soluble nutrients (5-7) and even the transit of inert substances (8). Ethanol has also been reported to increase both intestinal synthesis and output of triglycerides (9,10) into the lymph in fasting rats (10), but there is little information concerning the effects of ethanol ingestion on the transport of dietary lipids into the lymph (11,12). Although there was an increased lymph flow and lipid and protein output, mainly in the first hour after ethanol administration (intragastric or intraduodenal route), no hyperlipemia occurred under these conditions. These findings seem curious, because it is well known that ingestion of ethanol with a high fat meal enhances the usual postprandial lipemia in man (13-15) and in animals (16,17). Moreover, a gastric retention of fats and proteins has been observed after ethanol ingestion (4) which might explain the prolonged postprandial lipemia.

In the present investigation, in addition to the experiments on lymph, we determined the gastrointestinal transit time of lipids after

[1]These experiments are a part of a thesis of "Doctorat d'Etat" (Speciality: Biology) n° C.N.R.S. A0 11.965 presented in Dijon on February 6, 1976.

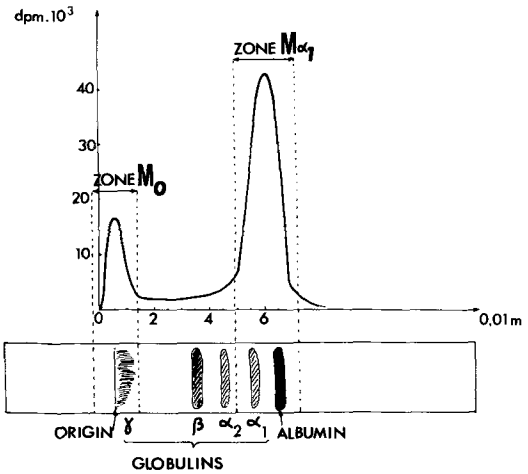

FIG. 1. Radiogram and cellulose acetate electrophoresis pattern of lymph of a rat fed corn oil labeled with 0.10 mCi 18:1 9-10^{3}H (staining amidoschwarz).

simultaneous ethanol ingestion.

MATERIAL AND PROCEDURES

Adult male or female Wistar rats, 200-300 g body weight, fasted overnight were used in these experiments. They were maintained on a commercial diet (UAR-Villemoisson, France) containing 17% protein and 4% lipid.

Collection of Lymph

Cannulation of the main mesenteric lymph duct was performed (18). Sixteen hours later, the animals received the test-meal. Lymph was collected during 24 hr at 2 or 4 hr intervals. Operated animals received a solution of 0.7% NaCl 0.2% KCl prior to and 8 hr after the meal. To study the effects of ethanol on fat gastrointestinal transit time, animals were killed 1.5, 3, 6, and 8 hr after the test meal. No liquid was given in this case.

Composition of the Test Meal

The test meal given by intubation contained 100 mg corn oil with 9, 10^3H oleic acid (0.10 mCi-10 Ci/mM) (CEA Saclay, France), 300 mg casein, 600 mg sucrose, 2.5 ml water (group C) or a solution of 55% ethanol (3.2 g per kg body weight) (group E). Examination of ^{3}H oleic acid by thin layer chromatography (TLC) and gas liquid chromatography (GLC) indicated a radiopurity greater than 98%.

ANALYTICAL METHODS

Cellulose acetate electrophoresis of lymph and measurement of radioactivity have been previously described (1,2). After electrophoresis of lymph, 5 mm strips were cut to separate the two radioactive zones and radioactivity and lipid mass of these strips measured. The lipid mass was determined after direct saponification of each radioactive zone (M_0 and $M_{\alpha 1}$) with a 5% KOH-MeOH solution in a small closed vessel at 100 C. Fatty acids were analyzed as methyl esters after the addition of a known amount of heptadecanoic acid as internal standard. The lipids from the other biological samples (total lymph, stomach contents, small intestine contents and mucosa, caecum and whole large intestine) were extracted with 15 volumes dimethoxymethane-methanol (4:1, v/v) (2). General lipid analyses were performed by previously described methods (1,2).

RESULTS

Proportions of Lipid Radioactivity Recovered in the *Different Parts of the* Gastrointestinal Tract (Table I)

Ingestion of ethanol led to a marked reten-

TABLE I

Proportions of Lipid Radioactivity (Percentages of Administered Dose) Recovered in the Gastrointestinal Tract in Groups C (Control Rats) and E (Ethanol-treated Rats) 1.5, 3.6, and 8 hr after Feeding[a]

	C (control rats)				E (ethanol-treated rats)				
	Time (hr)								
	1.5 (3)[b]	3 (6)	6 (6)	8 (3)	1.5 (6)	3 (6)	6 (9)	8 (6)
Stomach[c]	26.3 ± 1.9	23.9 ± 2	9.6 ± 1	2.7 ± 0.7	38 ± 3	35.7 ± 2.7	33.2 ± 3.6	28.8 ± 4.8
Intestinal[d]								
Lumen	1.4	0.9	0.9	0.7	2	0.25	1	0.35
Mucosa	4.5	8 (7 - 9.5)	3 (2.7 - 3.4)	5	3.8 (3.4 - 4.2)	1.7 (1.3 - 2.1)	2.1 (1.7 - 2.4)	4.4 (3.9 - 4.9)

[a]Test meal: 300 mg casein, 600 mg sucrose, 100 mg corn oil with added oleic acid 9-10^3H (microdose), 2.5 ml of solution of 55% ethanol (3.2 g/kg body weight (group E) or 2.5 ml of water (group C).

[b]( ): number of rats.

[c]Analyses were carried out on each stomach: the values represent the mean ± SE.

[d]Analyses were carried out on three combined samples: the values represent either one sample or the mean of two or three samples with the extreme values according to the number of rats involved.

TABLE II

Effects of Acute Administration of Ethanol on Intestinal Lymph Lipid Output

	C (control rats)			E (ethanol-treated rats)		
	$\frac{\text{dpm}^3\text{H lipid lymph (24 hr)}}{\text{dpm}^3\text{H fed}} \times 100$					
	52.3 ± 0.8			52 ± 0.7		
	ml of lymph in 24 hr					
	12.7 ± 0.4			13.7 ± 1.1		
	mg of lymph fatty acids in 24 hr					
	118.6 ± 18.6			118.6 ± 9.5		
Time after feeding (hr)	A[a]	B[b]	C[c]	A	B	C
0.2	13.8 ± 1.5[d]	17.2 ± 1.8	15.3 ± 5	11.8 ± 4.1	22.3 ± 1.7	9.9 ± 1.5
2-4	29.3 ± 0.5	28.4 ± 1.1	20.8 ± 1.8			
4-6	25.4 ± 2.3	25.8 ± 6	28.0 ± 2.2			
6-8	31.5 ± 1.5	28.6 ± 3.3	16.5 ± 1.7	49.1 ± 4	31.8 ± 5.4	19.1 ± 2.1
8-12			10.0 ± 1			
12-24			10.0 ± 1	39.1 ± 7.9	46.4 ± 5.2	13.9 ± 2.1

[a]A: distribution of lipid radioactivity (in %) between the collections of lymph during 0-24 hr periods after feeding.

[b]B: distribution of lipid mass (expressed as total fatty acids) (in %) between the collections of lymph during 0-24 hr periods after feeding.

[c]C: Percentages of lipid radioactivity found in zone Mo by comparison with the total radioactivity present in zone M_0 + zone $M_{\alpha 1}$ in some collections of lymph during 0-24 hr periods after feeding.

[d]The values represent the mean ± SE of 5 rats.

tion of the radioactivity in the stomach, since, at each time, the proportions of ^3H were always higher in group E than in the control. The values decreased in both groups with time, but much more rapidly in group C. Six hours after feeding, the amounts of radioactive lipids in stomach contents in group C were 1/3 those in group E. The amounts of radioactivity recovered in the intestinal lumen and mucosa were always low and very similar, despite high individual variation. In the caecum and large intestine, the amounts of labeled lipids were very low (1-3% of ingested ^3H material) in all the groups examined.

Studies on the Intestinal Lymph after Ethanol Feeding (Table II)

In all animals, lymph ^3H lipid output was similar during the 24 hr period of collection and the flow of lymph and the output of total fatty acids of lymph during 0-24 hr after feeding were very similar in the two groups. Fifty-two percent of the ^3H lipids which were fed was recovered. The lymph flow output was higher in group E than in group C during the first hour after feeding (x 1.5 to 2) but the lipid concentration of this sample was very low.

The distribution of lymph lipid radioactivity and mass in the aliquots of lymph collected during 0-24 hr periods was different in the two groups. The peak output was found in samples collected between 6 and 12 hr after feeding in group E and between 0 and 6 hr after feeding in group C.

In all animals, the proportion of radioactivity in zone M_0 was always lower than that in zone $M_{\alpha 1}$. On the other hand, the percentage of radioactivity of M_0 compared to the total radioactivity in the two zones ($M_0 + M_{\alpha 1}$) was the highest at the peak output (Table II). Moreover, percentages of radioactivity of zone M_0 were often lower in rats fed ethanol than in control group.

The proportion of lipid mass and radioactivity of zone M_0 determined at the peak output compared to the total lipid mass and radioactivity of the two zones ($M_0 + M_{\alpha 1}$) (Fig. 2) exhibited some differences in the two groups of animals. The data relative to the proportions of radioactivity of M_0 were higher than those relative to the proportions of mass of M_0 in group C whereas the opposite was seen in group E.

DISCUSSION

Results indicate that a large dose of ethanol administered simultaneously with corn oil delayed the absorption of the fat since the peak of absorption in lymph was observed later than in the controls. About 68% and 12% of the

radioactivity were recovered in control and alcoholic rats, respectively, in the first 6 hr of lymph collection. It is apparent that this delay is due chiefly to the retention of fat in the stomach because, even if the amounts of radioactivity of intraluminal and mucosal lipids were significantly different in the two groups, no special retention in the intestine was observed in group E as compared to group C. A small deficit of radioactivity recovered in the gastrointestinal tract and lymph in comparison with administered radioactivity was observed and is probably due to lipid transport by other small chyliferous vessels. Our data indicating the impairment of gastric emptying after ethanol feeding are in agreement with the previous observations of Barboriak and Meade (4), but the reaction that we found was less pronounced than in their experiments perhaps because the amounts of fat and ethanol given our rats were lower than those administered by these authors.

The delay of fat absorption observed in the present investigation was not due to an inhibition of pancreatic lipase by ethanol, since the proportion of free fatty acids to total lipids of the lumen contents was similar in the two groups. This conclusion is consistent with the reports of Barboriak and Meade (4), Capitaine et al. (19), and Belleville and Clement (20); the last group observed an increase in pancreatic lipase activity 2 hr after ingestion of ethanol, but this activity returned to normal after 8 hr. Barboriak and Meade (4), on the other hand, found no important effect of ethanol on intestinal lipase activity.

Ethanol feeding caused no change of lymph flow in 24 hr. We confirmed the observations of Baraona et al. (11) concerning the increase of lymph output during the first hour after ethanol feeding, but the lymph sample was poor in lipids in our experiments and in this respect our results were different from those described by these authors.

No reduction of exogenous fat absorption was seen in alcohol-treated rats by comparison with controls in 24 hr. Of administered radioactivity, 52% was recovered in lymph in the two groups. These data (concerning lymph volume and radioactivity) were lower than usual (15 to 30 ml and more than 60% of radioactivity recovered in 24 hr) but no liquid was given our rats for 8 hr after the meal intake, and it is well known that ingestion of water increases the lymph flow and lipid output.

Concerning the delay of lipid output after ethanol feeding, our results did not agree with those of Lieber et al. (11,12), but the administration of liquid diets (with or without ethanol) may have some effects on the gastric

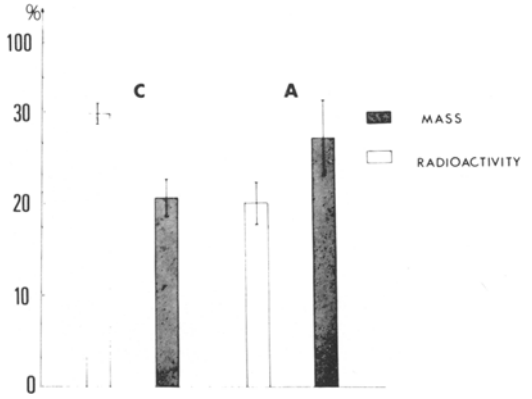

FIG. 2. Percentages of radioactivity and mass in zone M_0 found at the absorption peak by comparison with total radioactivity and mass in both zones M_0 and $M_{\alpha 1}$ in rats C and E (see text). Each value represents the mean ± SE of 5 rats. Note. The percentages of lipid mass of zone M_0 by comparison with the total mass ($M_0 + M_{\alpha 1}$) were determined in each lymph collection but as the mass of M_0 was low (in lymph collected before or after the absorption peak) the values obtained were unreliable and therefore only the peak values were reported in this figure where the mass of M_0 was high enough for accurate determination.

and intestinal lipid transit time. Three hours after the administration of food, all labeled dietary lipids were absorbed in their experiments; this observation is very different from the findings well established with solid diets. Direct relationships have been determined (21) between the amounts of lipids leaving the stomach and those being absorbed by the intestine; our data are in agreement with those observations.

Our results show that exogenous lipid transport in lymph is mainly effected by the smaller fat particles in the two groups as the proportions of radioactivity in zone M_0 (larger particles) were always lower than those found in zone $M_{\alpha 1}$. This finding confirms our previous results (2,3). Moreover, ethanol ingestion seemed to increase the exogenous fat transport in lymph as small fat particles since the proportions of radioactivity in zone M_0 were lower in group E than in group C. It should be noted that Ockner et al. (22) observed that the ingestion of ethanol enhanced the production of endogenous intestinal very low density lipoproteins (VLDL) (in the fasting state).

The highest proportions of radioactivity in zone M_0 (= larger particles) were always found at the absorption peak in both groups; this finding is supported by the work of Fraser et al. (23). However, the proportion of endogenous fatty acids in zone M_0 was higher in group E than in group C (as the proportion of mass was higher than the proportion of radioactivity

in group E by comparison with group C; see Fig. 2). The SA of fatty acids in zone M_0 was 1060 ± 114 dpm/mg in group E and 1380 ± 83 dpm/mg in group C. Palmitic, oleic, and stearic acids were the main endogenous fatty acids.

In this report we were not concerned with whether the electrophoretic moving particles in cellulose acetate electrophoresis (= zone $M_{\alpha 1}$) were small chylomicrons or VLDL because this has been discussed previously (1,2). However, these particles have the size of VLDL described by Jones and Ockner (24) even if they do not have the classic mobility on electrophoresis.

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Relative Autoxidative and Photolytic Stabilities of Tocols and Tocotrienols

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ABSTRACT

The relative stabilities of selected individual tocols and tocotrienols and of equimolar mixtures of either α- plus γ- or α- plus δ- tocopherols were determined in methyl myristate and methyl linoleate during autoxidation and photolysis. Solutions containing 0.05% of the appropriate tocopherol(s) or tocotrienols were subjected to UV light (254 nm) or to a flow of 4.3 ml/min of oxygen, both at 70 C. Tocopherols (T) and tocotrienols (T-3) were determined by gas chromatography without preliminary separation or purification. Under photolytic conditions, stabilities in increasing order in methyl myristate were γ-T-3 <α-T-3 <δ-T <α-T <γ-T <5,7-T <β-T and in methyl linoleate were α-T <α-T-3 ≤γ-T-3 ≤β-T ≤5,7-T <γ-T <δ-T. A solvent effect on the initial rate of photolysis was observed for 5-methyl substituted tocols but not for the tocols with an unsubstituted 5-position or for the tocotrienols. Under autoxidative conditions, stabilities in increasing order in methyl myristate were α-T=α-T-3 <β-T-3 <γ-T-3 <δ-T-3 <γ-T <δ-T=β-T and in methyl linoleate were α-T <α-T-3 <γ-T-3 <β-T <γ-T <δ-T. Tocopherols were much more stable during autoxidation in methyl myristate than they were in methyl linoleate. In mixtures, there was no significant protection of α-tocopherol by either γ- or δ-tocopherol under any of the conditions used. However, α-tocopherol was highly effective in protecting γ- and δ-tocopherols in methyl myristate during both photolysis and autoxidation and in methyl linoleate during photolysis. During autoxidation in methyl linoleate, α-tocopherol protection of γ- and δ- tocopherols after 24 hr was slight tough measurable.

INTRODUCTION

The tocopherols, in addition to their vitamin E function, are natural antioxidants in foods and are important in promoting the stability of vegetable oils. This antioxidant aspect of tocopherol chemistry has been studied extensively (1-5), especially with regard to the relative antioxidant activities of α-, γ-, and δ-tocopherols, the forms commonly found in vegetable oils. In contrast, relatively little information is available on the fate of the various tocopherols themselves during fat oxidation, even though this information could be important in evaluating possible changes in vitamin E content during cooking or storage. Of particular interest is the possibility that some tocopherol forms may exert a sparing action on others, acting as antioxidants for them. Since α-tocopherol, the form with the highest vitamin E potency, is frequently accompanied by γ- and δ-tocopherols, any sparing effects of these latter two forms on α-tocopherol would be advantageous.

Investigations of tocopherol concentration changes during fat oxidation have been hampered in the past by methodological difficulties. The analysis of tocopherol mixtures by chemical methods has presented problems of separation, and, in addition, the presence of hydroperoxides from unsaturated fatty acid oxidation has required further steps in already lengthy procedures. Tocopherol separations and analysis by gas liquid chromatography (GLC) have essentially eliminated these problems. By the GLC technique, specific tocopherols can be analyzed in the presence of fatty acid methyl esters, hydroperoxides, other oxidation products, and mixtures of tocopherols with no sample preparation other than the formation of the trimethylsilyl (TMS) ethers.

This paper reports a study of the effects of either autoxidation or photolysis on the stabilities of tocopherols in model systems. Individual tocopherols in methyl myristate and in methyl linoleate were exposed to oxygen or to UV light (254 nm) at 70 C. Also, mixtures of α tocopherol with either γ or δ tocopherol were tested under these same conditions.

EXPERIMENTAL PROCEDURES

Materials

α-, β-, γ-, and δ-Tocopherol (α-T, β-T, γ-T, and δ-T) (Eastman Kodak Co., Rochester, NY); 5,7-dimethyltocol (5,7-T) (Pierce Chemical Co., Rockford, IL); and α-tocotrienol (α-T-e) (Hoffmann-La Roche Ltd., Nutley, NJ), were used as

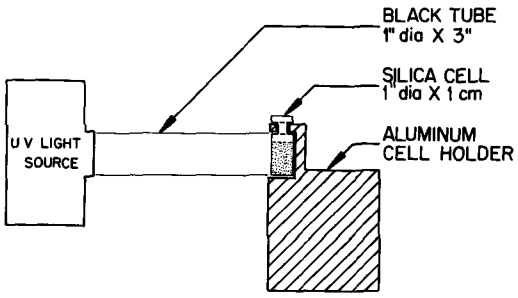

FIG. 1. Apparatus for photolysis.

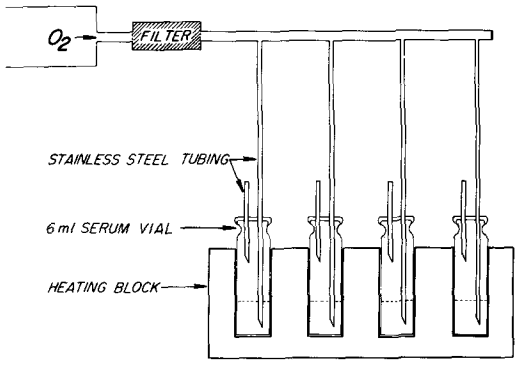

FIG. 2. Apparatus for autoxidation.

received. δ- and γ-Tocotrienols (δ-T-3 and γ-T-3) were extracted from natural latex (Uniroyal Chemical Co., Mishawaka, IN) with $CHCl_3$:MeOH (1:1); β-tocotrienol (β-T-3) was extracted from wheat bran with ethanol. Lipid extracts containing the tocotrienols were saponified and tocotrienols were then purified by repeated thin layer chromatography (TLC). Methyl myristate (Hormel Institute, Austin, MN) was used as received. Methyl linoleate (Analabs, Inc., North Haven, CT) was purified by passing 10 ml diluted 1:1 with distilled hexane through 10 g of hexane washed chromatographic grade silica gel (Analabs, Inc.) 100/110 mesh. Methyl linoleate was eluted with hexane, and solvent was removed with a stream of nitrogen.

Dotriacontane and hentriacontane (Analabs, Inc.) were used without further purification. Dotriacontane was used as the internal standard for all tocopherols and tocotrienols except δ-T-3, which had the same retention time during GLC on OV-17. For δ-T-3, hentriacontane was substituted as the internal standard.

Trimethylsilylating (TMS) reagent was prepared by mixing pyridine, hexamethyldisilazane, and trimethylchlorosilane, all distilled, in the ratio 10:9:6 v/v/v.

METHODS

One gram aliquots of a 0.05% solution of the appropriate tocopherol(s) or tocotrienol in methyl myristate or methyl linoleate plus the hydrocarbon internal standard were exposed at 70 C either to molecular oxygen in the dark or to 254 nm UV light under a nitrogen atmosphere. (Mixtures contained 0.05% of α-T plus 0.05% of either γ-T or δ-T).

Photolysis

The apparatus used for photolysis is shown in Figure 1. A circular silica spectrophotometer cell measuring 1 in. diameter by 1 cm light path was placed in an aluminum cell holder in a heating block maintained at 70 C. The UV light source (254 nm; Mineralight Model V41) was placed 3 inches from the cell and the whole system was put under a nitrogen atmosphere in a dry box. Ten-μl aliquots were withdrawn with a 10-μl syringe without disturbing the reaction.

Autoxidation

The apparatus used for autoxidation is shown in Figure 2. Filtered oxygen at a flow rate of 4.3 ml/min was distributed to individual samples from a stainless steel manifold. Serum vials (6 ml) containing the samples were sealed and placed in a heating block maintained at 70 C. A 6 in. length of 16-gauge stainless steel hypodermic needle tubing was inserted through the serum cap far enough to reach the bottom of the vial and was connected to the oxygen manifold. A 2 in. length of 16-gauge tubing was also inserted through the cap as an outlet, through which 10-μl aliquots were taken without interrupting the oxygen flow.

Gas Chromatography

Tocopherols were analyzed by using a F&M 810 gas liquid chromatograph equipped with a flame ionization detector and operated isothermally at 250 C. Columns were 15 ft by 0.125 in. OD glass coil packed with 100/120 mesh Gas Chrom Q (Applied Science Laboratories, Inc., State College, PA) coated with 2% OV-17. A 10-μl aliquot of the reaction mixture, taken as described previously, was immediately mixed with 10 μl of TMS reagent. After standing 5 min at room temperature, 1 μl of silylated mixture was injected onto the chromatographic column. Peaks were quantitated by reference to the hydrocarbon internal standard (dotriacontane or hentriacontane). This procedure did not require the removal of the methyl ester solvents, since they eluted in the solvent front. Hydroperoxides also caused no problems, at

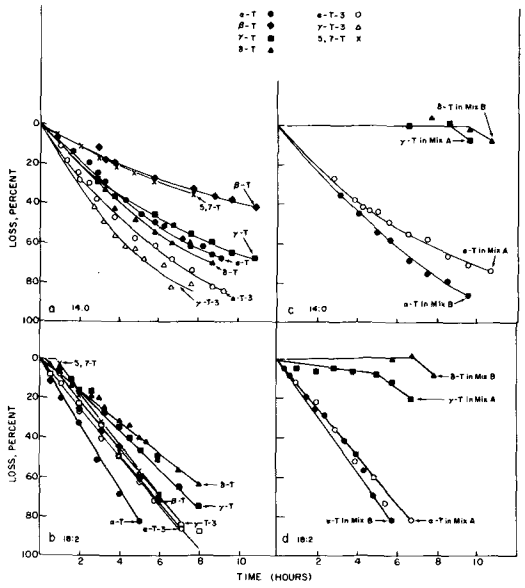

FIG. 3. Tocopherol losses during photolysis in methyl myristate (3a and 3c) and methyl linoleate (3b and 3d). The disappearance of individual tocopherols present singly in solution is shown in 3a and 3b. The disappearance of individual tocopherols from mixtures is shown in 3c and 3d. [Mix A=α-T+γ-T, Mix B=α-T+δ-T]

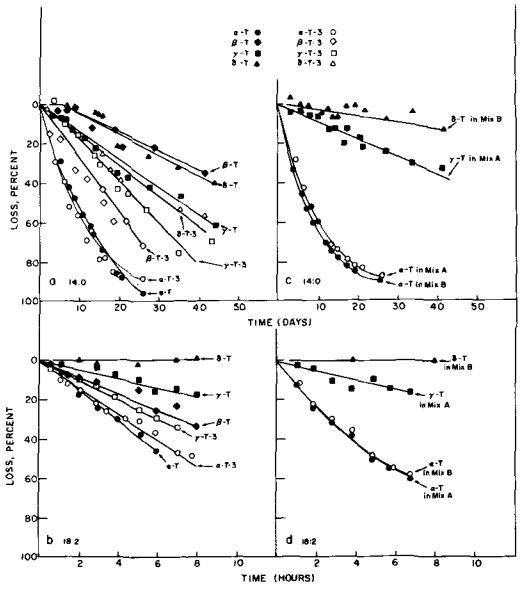

FIG. 4. Tocopherol losses during autoxidation in methyl myristate (4a and 4c) and methyl linoleate (4b and 4d). The disappearance of individual tocopherols present singly in solution is shown in 4a and 4b. The disappearance of individual tocopherols from mixtures is shown in 4c and 4d. [Mix A=α-T+γ-T, Mix B=α-T+δ-T]

least at the low peroxide value reached in these oxidations. The accuracy of the method was evaluated by comparing the total tocopherol content determined by GLC with that measured by fluorescence (6). The results obtained by these two methods were in good agreement.

Peroxide Determination

The AOAC method for determining peroxide values (7) modified to use 100 mg or less of sample. Sodium thiosulfate solution was prepared and standardized as described in the official method. The oil was weighed into a #3 hollow polyethylene stopper (Nalgene No. 6190) containing a micro stirring bar. $CHCl_3$-HAc (0.6 ml) was added and the mixture was stirred gently. Saturated aqueous KI (10 μl) was added with a 10-μl syringe and the mixture stirred for 1 min. Boiled distilled water (0.5 ml) was added and the mixture was titrated with sodium thiosulfate solution to the point just before disappearance of the yellow color. Starch solution was added and the titration continued until the disappearance of the blue color.

RESULTS AND DISCUSSION

Peroxide Values

When tocopherols were initially present in the sample, peroxide values (meq. peroxide/kg sample) at the termination of the reaction varied from 0 to 3. In the absence of tocopherols, methyl linoleate reached a peroxide value of 28 after oxygen was bubbled through for 8 hr and a peroxide value of 3 when exposed to UV light for 8 hr.

Photolysis. Effects of UV light in the Absence of Oxygen

Tocopherols exposed to UV light at 70 C were rapidly destroyed in both methyl myristate (Fig. 3a) and methyl linoleate (Fig. 3b). The reaction rates (Fig. 3b and Table I) for tocopherol destruction in methyl linoleate and the initial reaction rates (until ca. 20% loss) for tocopherol destruction in methyl myristate (Fig. 3a and Table I) were all zero order. From an inspection of the curves (Fig. 3a and 3b), tocopherol stabilities in methyl myristate increased in the order: γ-T-3 <α-T-3 <δ -T <α-T <γ-T <5,7-T <β-T; and the stabilities in methyl linoleate increased in the order: α-T <α-T-3 ≤γ-T-3 ≤β-T ≤5,7-T <γ-T <δ-T.

A comparison of the rates of loss in methyl myristate and methyl linoleate (Table I) shows that the rates were different for the two solvents for the tocols having a methyl group in the 5-position (α-T, β-T and 5,7-T) but were

TABLE I

Initial Rates of Tocopherol Disappearance in Methyl Myristate (14:0) and Methyl Linoleate (18:2) during Photolysis

Compound	Reaction rate 14:0	18:2
	%/hr	%/hr
α-T	8.7	16.6
α-T (+ γ-T)	9.5	12.0
α-T (+ δ-T)	10.7	13.2
α-T-3	11.0	12.1
β-T	4.6	12.3
γ-T	9.3	9.4
γ-T (+ α-T)	0	2.2
γ-T-3	14.1	12.3
δ-T	10.2	8.3
δ-T (+ α-T)	0	0
5,7-T	5.3	12.4

essentially the same in both solvents for the tocotrienols (α-T-3, γ-T-3) and for the tocols not methyl substituted in the 5-position (γ-T and δ-T). The rates of loss for α-T, β-T and 5,7-T in methyl linoleate were, in order, 1.9, 2.7, and 2.3 times the rates in methyl myristate.

When equimolar mixtures of α-T with either γ-T or δ-T were irradiated, there was a distinct sparing of γ-T and δ-T in both methyl myristate and methyl linoleate. For these mixtures in methyl myristate, there was little or no loss of either γ-T or δ-T (Fig. 3c). For these mixtures in methyl linoleate, there was no loss of δ-T until after 7 hr, and while there was some loss of γ-T this was considerably less then that in the absence of α-T (Fig. 3b and 3d). In contrast to the increased stability of γ-T and δ-T in mixtures with α-T, the rate of loss of α-T in these same mixtures was only slightly increased in methyl myristate and slightly decreased in methyl linoleate.

Autoxidation

Tocopherols dissolved in methyl myristate were oxidized only slowly by O_2 (Fig. 4a), an observation in agreement with the report by Swift et al. (8) that tocopherols in methyl laurate were resistant to attack by molecular oxygen. The disappearance of both α-T and α-T-3 followed first order kinetics, with a half-life for each form of 7.8 days. Reaction orders for the non-α forms could not be determined from these data. Stabilities in methyl myristate increased in the order: α-T=α-T-3 <β-T-3 <γ-T-3 <δT-3 <γ-T <δ-T=β-T.

After 8 hr autoxidation in methyl linoleate, there was no measurable loss of δ-T, and only α-T and α-T-3 were half destroyed (Fig. 4b). Relative stabilities in increasing order were α-T <α-T-3 <γ-T-3 <β-T <γ-T <δ-T. These relative stabilities for the tocopherols are similar to the relative antioxidant activities that have been reported. Lea and Ward (9) found that the antioxidant activities of the saturated tocopherols in methyl linoleate at 50 C and in lard esters at 90 C varied in the order α-T <β-T <γ-T <δ-T. In lard and in oleo oil at 75 C, Olcott and Emerson (1) found that antioxidant activities for the first three of these varied in the order: α-T <β-T <γ-T. Among the tocols, at least, stabilities and antioxidant activities in the protection of fats and oils vary in the same direction with the most stable δ-T being the best antioxidant. Our results agree with those of Chow and Draper (10) who reported the rates of loss during autoxidation of γ-T, γ-T-3, α-T and α-T-3 in corn oil and γ-T and δ-T in soybean oil. They found no significant differences between the rates of loss of the saturated and unsaturated tocopherol pairs (i.e., α-T vs. α-T-3 and γ-T vs. γ-T-3). In corn oil, they found that γ-T was more stable than α-T, and in soybean oil, δ-T was more stable than γ-T.

In mixtures during autoxidation, the influence of γ-T or δ-T on the disappearance of α-T in either methyl myristate (Fig. 4c) or methyl linoleate (Fig. 4d) was minor or nonexistent. The presence of α-T had a marked effect, however, in decreasing the rate of loss of both γ-T and δ-T in methyl myristate. During the first 8 hr of autoxidation in the methyl linoleate systems, no loss of δ-T and only 20% loss of γ-T was seen either with or without added α-T (Fig. 4b and 4d). After 24 hr, α tocopherol protection of both γ-T and δ-T was slight though measurable (values not shown).

These data, both autoxidative and photolytic, indicate that, at least in terms of antioxidant interactions among the tocopherols, α-T is a better antioxidant than either γ-T or δ-T. This is in contrast to the relative antioxidant activities discussed above for the protection of fats and oils, where both δ-T and γ-T have been reported (1,9) as being more effective than α-T as antioxidants.

The possibility of tocopherol reaction with minor contaminants initially present in the methyl linoleate was considered. α-T (0.05%) in methyl linoleate was treated with N_2 instead of O_2. No loss was seen after 8 hr, compared to a loss of more than 50% in the presence of O_2, indicating that O_2 was necessary to account for the loss measured. Since all forms were relatively stable to O_2 in methyl myristate, it may be inferred that O_2 reacts slowly with tocopherols and that the losses in methyl linoleate were due to reaction products of linoleate and

O_2. This might explain why the reaction rates (with respect to tocopherol concentration) in methyl myristate were first order while those in methyl linoleate were zero order.

Consideration was given to the possibility that tocopherol oxidation products may have caused errors in the GLC method. Knapp and Tappel (11) reported that in autoxidizing methyl linoleate at 37 C, α-T is oxidized to α-tocopheryl quinone. The separation factors for the TMS ethers of the quinones and their corresponding tocopherols on OV-17 are: α-T, 1.03; α-T-3, 1.00; β-T, 1.17; β-T-3, 1.17; γ-T, 1.31; and δ-T, 1.26. No tocopheryl quinone peaks were found in any chromatograms. α-T and α-T-3 would not be separated from their quinones, but the absence of quinone peaks from the other tocopherols and the agreement of the GLC and the fluorescence methods did not suggest quinone formation under the conditions used. Also, tocopherol dimers, another common reaction product that may have been produced, have been reported (12) to decompose in the GLC injection port to yield tocopherol monomers. Either additional tocopherol TMS ether or a peak for the free tocopherol would have appeared in the chromatogram. Since no free tocopherol peak was seen and since the fluorescence and GLC methods agreed, the dimer if formed did not decompose in the GC. In summary several observations can be made: (a) Under the conditions of autoxidation and photolysis in this study, α-T protected γ-T and δ-T but not vice versa; (b) during cooking or storage of vegetable oils and other products, α-T would be expected to decrease in concentration first; and (c) tocopherol stabilities during photolysis were solvent dependent under the conditions of this study for those tocols having a methyl substitution in the 5-position but not for tocotrienols or for tocols with an unsubstituted 5-position.

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The Occurrence and Distribution of Octadecapentaenoic Acid[1] in a Natural Plankton Population. A Possible Food Chain Index

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ABSTRACT

It is shown that marine dinoflagellates under natural conditions synthesize the unusual fatty acid octapentadecaenoic (18:5ω3). This acid is very likely a characteristic of certain groups of phytoplankters and is not an artifact from artificial culture conditions. Various species of herbivorous copepods as well as contemporary carnivorous chaetognaths living in the same environment present traces of this fatty acid. The decreasing quantity of 18:5ω3 on moving up the food chain, and its absence in certain species, makes it a possible ecological tracer.

INTRODUCTION

The idea that certain fatty acids of exotic structure could be better used as tracers in the marine food chain than could the major fatty acids which are common to most trophic levels (1) is not new (2) but has not received much attention, probably because the necessary fatty acid analyses were usually restricted in scope by experimental design or by apparatus limitations. The use of capillary column gas chromatography allows the detection of the less apparent minor components, some of which must be specific to the first trophic level and hopefully could be followed through higher levels. Octadecapentaenoic acid (18:5ω3) (See footnote 1) has been reported to be synthesized by various species of dinoflagellates (3), as well as by certain symbiotic algae (4), but to our knowledge has not been reported in any zooplankters. In the present study we present the distribution of the acid 18:5ω3 through the various levels of a neritic (coastal) planktonic food chain.

[1]Referred to in text as 18:5ω3, shorthand notation for chain length, number of double bonds, and position ultimate methylene-interrupted double bond relative to terminal methyl group.

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EXPERIMENTAL PROCEDURES

Samples of Lipid Recovery

Zooplankton and phytoplankton were captured separately during the late summer and fall of 1974 in Bedford Basin (Nova Scotia, Canada). The phytoplankton component (mainly dinoflagellates Ceratium), was collected with an 85 μm net by horizontal surface tows. After collection, the plankton was sieved through a 85 μm bolting cloth to remove any small zooplankton. Further microscopic examination revealed that 92% (on a volume basis) of the organisms in the sample were phytoplankton. Neritic copepods (*Acartia, Temora, Oithona* and *Eurytemora*) were captured at 5 meters depth with a #6 net (240 μm mesh) equipped with a double release mechanism. The plankton was first sieved through a 800 μm bolting cloth to remove any large carnivorous zooplankton (mainly chaetognaths) and then washed in a 240 μm bolting cloth to eliminate any phytoplankton. Phytoplankton and neritic samples were scraped into plastic bags and deep-frozen in dry ice. The two larger zooplankters were the crustacean *Calanus finmarchicus*, Stage V (copepoda), and the arrow worm *Sagitta elegans* (chaetognatha). They were collected with a coarse net (800 μm mesh) by an oblique tow from 60 meters to the surface. After capture they were transferred into plastic coolers, diluted with water from 60 meters, and returned immediately to the laboratory to be sorted into stages with a binocular microscope.

All samples were extracted for lipids as soon as possible after capture (usually a few hours, sometimes overnight) according to the method of Bligh and Dyer (5). The chloroform phase containing the lipids was recovered and stored under nitrogen at -20 C until further analysis. Aliquot fractions (100 mg) were evaporated to dryness and the phospholipids separated from other lipids on a 25 mm x 400 mm gel column (Dowex styrenedivinylbenzene copolymer beads, X_2, 200-400 mesh) eluted with benzene (6,7). The other lipid classes were separated by preparative scale thin layer chromatography (TLC) on Prekotes (Adsorbosil 5; Applied Science Labs, State College, PA) eluted by hexane:diethyl ether:acetic acid (85:15:1). Esters of fatty acids were obtained by refluxing

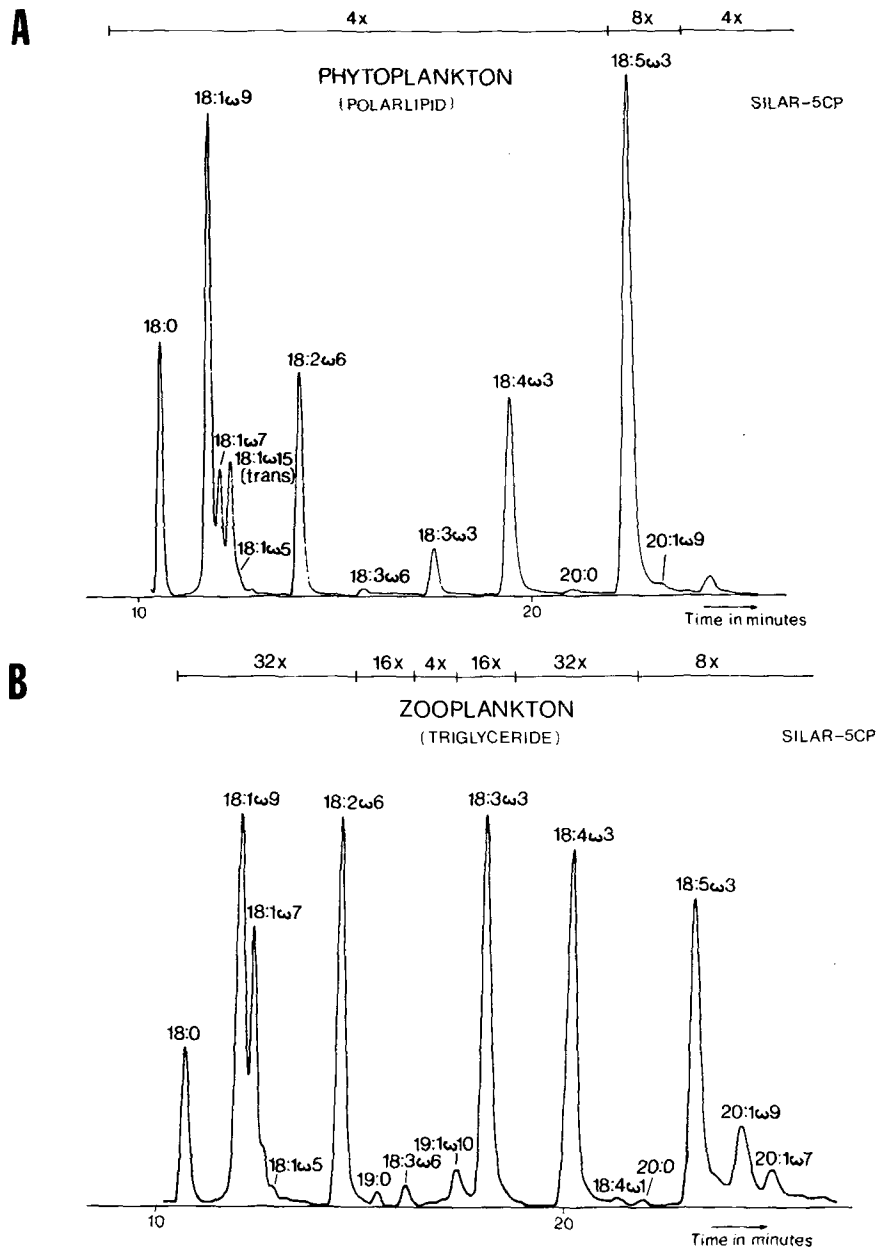

FIG. 1. Comparison of relative proportions of 18:5ω3, 18:4ω3, and other C_{18} fatty acids in the polar lipids of a phytoplankter (A, above) and in the triglycerides of a zooplankter (B, below). Note attenuation changes and reduction in proportion of 18:5ω3. Wall-coated open-tubular column with SILAR-5CP as the liquid phase.

lipid fractions with BF_3-methanol for 30 min or 1 hr. The transesterification product from wax esters was recovered whole and separated by TLC into methyl esters and free fatty alcohols. The latter were converted to acetate esters by refluxing with acetic anhydride for one hour.

Analyses of Sample

Gas liquid chromatography (GLC) analyses of all esters were carried out on 46 m length x 0.25 mm ID stainless steel open-tubular (capillary) columns coated with Silar 5-CP (Applied Science Labs) or Apiezon-L, in Perkin-Elmer series 900 apparatus equipped with flame ioni-

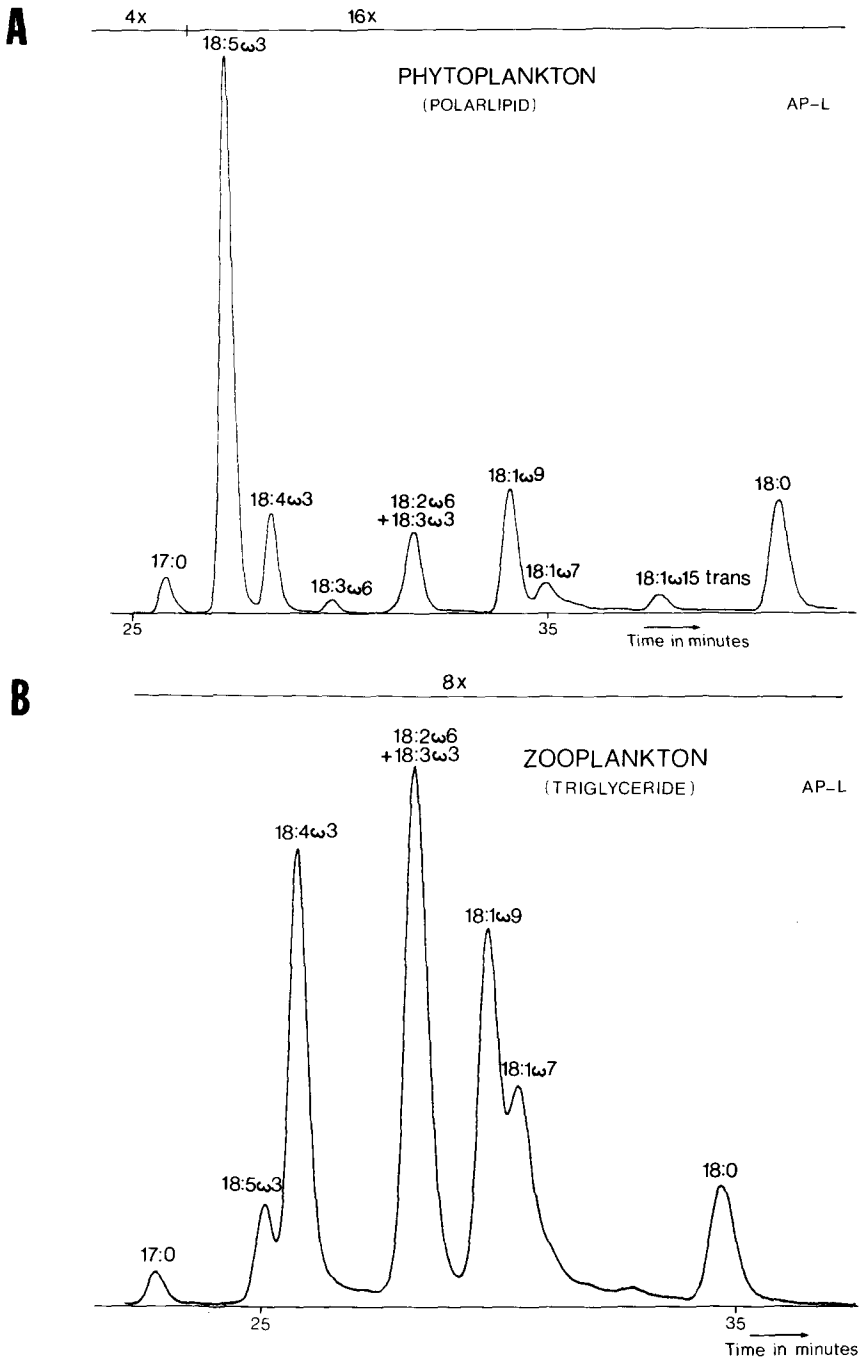

FIG. 2. Comparison of relative proportions of 18:5ω3, 18:4ω3, and other C_{18} fatty acids in the polar lipids of a phytoplankter (A, above) and in the triglycerides of a zooplankter (B, below). Note attenuation changes. Figure 2B may also be compared with Figure 1B as a demonstration of the identification of 18:5ω3. Wall-coated open-tubular column with Apiezon-L as the liquid phase. 2A and 2B were obtained with different Ap-L columns.

zation detectors. The columns were operated isothermally at 180 C (Silar 5-CP) and 190 C (Apiezon-L). Helium was used as carrier gas at 50 psig (Silar 5-CP) and 80 psig (Apiezon-L). Detector and injector temperatures were maintained at 250 C. In addition to the examination

TABLE I

Distribution (wt %) of 18:5ω3 in the Lipid Classes of Various Species of Plankton

Species	Trophic type	Lipid classes[a]			
		PL	TG	WE.FA.	WE.Alc.
Ceratium sp	-	8.55[b]	1.71	-	-
Acartia + Temora	herbivorous	0.25	1.79	0.12	ND[c]
Oithona + Eurytemora	herbivorous	0.15	1.96	0.13	ND
Sagitta elegans mature	carnivorous	ND	0.13	0.11	ND
Calanus finmarchicus Stage V	herbivorous	ND	ND	ND	ND

[a]PL = phospholipids, TG = triglycerides, WE.FA. = Wax esters Fatty acids, WE.Alc. = Wax esters Fatty alcohols.

[b]Includes glycolipids.

[c]ND = not detected.

of esters as recovered, parts of all ester samples were completely hydrogenated and the product examined quantitatively and qualitatively by GLC. The quantitative results are given to two decimal places to permit the inclusion of the minor components, but this does not imply this order of accuracy. Major components (>10%) should be accurate to ± 5%, moderate sized component (1 to 9%) to ± 10%, and minor components (< 1%) to up to ± 30%.

RESULTS AND DISCUSSION

Octadecapentaenoic acid has been fully identified for various species of dinoflagellates and its equivalent chain length calculated for various types of columns (3,8). Earlier Ackman et al. (9) had tentatively identified the same compound using structural element retention data. In both cases equivalent chain length (ECL) values for polar columns ranged from 20.02 to 21.98. Ackman et al. (9), using the polar phase SILAR 5-CP (170 C) and the nonpolar phase Apiezon-L (180 C), found ECL values of 20.13 and 17.18, respectively. Our corresponding ECL values were in the range 20.16-20.21 at 180 C and 17.12-17.21 at 190 C for various samples from this study run at various times on different columns.

The GLC behavior of the peak we identified as 18:5ω3 is presented for two samples (a phytoplankter, basically *Ceratium*, and a zooplankton mixture of *Acartia + Temora*) run on the polar phase (Silar 5-CP, Fig. 1A and 1B) and on the nonpolar phase (Apiezon-L, Fig. 2A and 2B). On the polar phase, the unusual peak runs between 20:0 and 20:1ω9, but it falls between 17:0 and 18:4ω3 on the nonpolar phase. Hydrogenation of the esters followed by quantitation of the chromatograms confirmed that we were dealing with a straight chain C_{18} fatty acid. These results and those obtained from the chromatographic behavior on $AgNO_3$-TLC confirm the octadecapentaenoic acid.

The transfer of organic matter through the marine food chain has conventionally been considered essentially in terms of total organic matter or of basic elemental components (carbon, nitrogen, phosphorus). Of the three major biochemical classes of material, only certain lipids have received further attention as constituents retaining identifiable structures. Most of the published studies on marine plankton have been concerned essentially with phylogenetic aspects of lipid and fatty acid composition of either the phytoplankton or zooplankton species (10-12). Nevertheless, some have dealt with lipid transfer through artificial experimental food chains (13-15), but relatively few with naturally occurring trophic structures (16-19).

The structure of the trophic food web in Bedford Basin is relatively simpler than in the open ocean. During the summer months, the surface waters are inhabited by small neritic copepods (20) which graze on phytoplankton and nonliving particulate matter (21). The main carnivore during that period of 1974 (20) was the chaetognath *Sagitta elegans* which migrated diurnally to feed on the surface copepods. The waters below the thermocline were also inhabited by various species of herbivorous copepods such as *Calanus finmarchicus, C. hyperboreus, Pseudocalanus minutus,* etc., whose position in the food chain at this time of the year is not clearly understood.

The distribution of the octadecapentaenoic acid in the various species of plankton (Table I) confirmed the identification and precisely outlined the structure of the food chain. The greatest amount of 18:5ω3 (8.5%) was found in the polar lipids of the phytoplankters, which agrees with the conclusions of Joseph (3) that this fatty acid is synthesized by dinoflagellates; but some was also present in the triglycerides (1.7%). Smaller amounts were found in two

samples of the neritic, herbivorous copepods and were located predominantly in the triglyceride fraction (1.7 and 1.9%). Even smaller amounts (0.1%) were found in *Sagitta*, equally distributed in the triglyceride and wax ester fatty acid fractions. According to Joseph (3), there is evidence that the 18:5ω3 is synthesized through the loss of an acetate unit from 20:5ω3 rather than through desaturation of 18:4ω3. The proportionately large amount of 20:5ω3 and 22:5ω3 in our sample of dinoflagellates certainly supports such a view. Once assimilated by the grazers, the 18:5ω3 is very likely degraded by the mitochondrial enzymes to yield Δ^3-*cis*-enoyl-CoA intermediates (22). Nevertheless, a portion of the 18:5ω3 ingested by the herbivores appears to be readily included during the synthesis of triglyceride, and also incorporated to a minor extent into the fatty acid moiety of the wax esters and the phospholipids. Similarly the carnivorous chaetognaths at the next higher step in the food chain accumulate some 18:5ω3 in their triglyceride and wax ester fractions. Although their phospholipid fraction represents more than 80% of the total lipids (11,23), it contains no 18:5ω3. Such a distribution agrees with the general concept that triglycerides as well as wax esters reflect the dietary fatty acids, whereas phospholipid reflect specific biosynthetic requirements (14,15,24). An interesting exception is the complete lack of 18:5ω3 in the lipids of the herbivorous copepod *C. finmarchicus* (Stage V). Since this species always remains under the thermocline throughout the summer, it can mean either that Stage V *Calanus* feed on particulate matter of different origin or that they do not feed at all during the summer months. A total catabolism seems unlikely, as 18:4ω3 and 20:5ω3 are normal components of such copepods (23,25).

Although *Ceratium* is not the species of dinoflagellate most readily assimilated by copepods, the dominance of dinoflagellates such as *Peridinium* (3), also rich in 18:5ω3, at the time of collection of the copepod samples maintains the proposed trophic relationship. From the results obtained, it is clear that the octadecapentaenoic acid is potentially very useful as a qualitative index since it is a recognizable percentage of the total fatty acid of primary consumers and its proportions decrease in the depot lipids of the secondary consumers. Nevertheless, it is likely that it disappears at upper levels of the food chain where other tracers such as nonmethylene-interrupted dienoic fatty acids become useful (26).

Whether or not the decrease of 18:5ω3 through the food chain, observed in the present study, is precise enough so that quantitative data on nutritional status can be obtained from study of its dynamic distribution, is related to two questions: (a) how widely is it distributed in the naturally occurring particulate matter, and (b) what is its fate in the metabolism of the various consumers feeding on that particulate matter or being fed on by higher predators. This second question is now the object of further investigation.

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Localization of a Marine Source of Odd Chain-Length Fatty Acids. I. The Amphipod *Pontoporeia femorata* (Kröyer)

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ABSTRACT

The amphipod *Pontoporeia femorata* (Kröyer) contains approximately equal amounts of odd chain length and even chain length fatty acids. Mature males of this species are released into the waters of Jeddore Harbour during winter months as the result of a regular reproductive cycle and become food for smelt *Osmerus mordax* moving into the harbor in preparation for spring spawning runs, thus accounting for the previously reported unusual fatty acid composition of these smelt. The exceptionally high levels of odd chain length fatty acids in *P. femorata* occur at all stages of maturity in both sexes of the animal and are found in *P. femorata* in other locations.

INTRODUCTION

The exceptional occurrence of high levels of odd chain length fatty acids (OCFA) in smelt *Osmerus mordax* taken from Jeddore Harbour, Nova Scotia, was first observed by Addison and Ackman (1). Further studies showed that OCFA maxima occurred on a seasonally and geographically limited basis (2). Attempts to relate these phenomena to the general fatty acid and hydrocarbon compositions of Jeddore harbour water proved unsuccessful (3). Consequently, a more specific source of OCFA in smelt was sought by a comparative study of dietary components in smelt from Jeddore Harbour and from the adjacent and superficially similar Musquodoboit Harbour where smelt retain low OCFA levels. An amphipod, *Pontoporeia femorata* (Kröyer), was found to occur only in Jeddore Harbour smelt stomachs and only during winter months. This crustacean contained as much as 50% OCFA, more than any previously reported plant, animal, or normal microbial source (4-7). This finding prompted a further investigation of lipids in relation to the life cycle of the amphipod. A mysid from the same location was also examined for lipids and fatty acids for comparative purposes.

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EXPERIMENTAL PROCEDURES

Smelt were gill-netted between the months of October and April 1971-1974 in both Jeddore Harbour and Musquodoboit Harbour, Nova Scotia. The fish were initially obtained from several locations in each harbor, but sampling stations were later reduced to one location near the head of each harbor. A few lots of smelt and bottom samples were also obtained from St. Margaret's Bay.

The weight, length, and sex of fish were recorded within 2 to 3 hr of landing. Stomachs were then immediately removed and graded as empty, partially full, or full. The presence of specific dietary components was noted. Stomach contents were washed into saline or saline-formalin solutions and pooled, then segregated into groups of recurring faunal components. Components were counted, weighed, tentatively identified, and transferred to vials containing 10% saline-formalin for storage and future reference.

Benthic samples were obtained manually by divers who drew a fine mesh net bag through the top of 10 cm of sediment. This material was transferred to containers above the water surface till 10-15 liters of wet sediment had been collected. Large particles of vegetation and gravel were removed by hand. The bottom samples were then passed through a series of graded wire-mesh sieves, allowing rapid removal of fine silt. The contents of each sieve were transferred with salt water to pans for manual collection of animals. Animals recovered in this way were either preserved in a manner analogous to that described above for smelt stomach contents or examined immediately for lipids. Isolation of eggs from gravid *P. femorata* in some spring bottom samples required micromanipulation under a magnification field of 50x.

Samples of *P. femorata* and other invertebrates were further examined to determine sex, degree of maturity, and morphological characteristics and, where circumstances warranted, were subjected to lipid extraction.

A mud sample collected from Jeddore Harbour in December was held in the running seawater facilities of the Halifax Laboratory until April. At that time *P. femorata* were recovered from the mud (referred to as live-held) for fatty acid examination.

TABLE I

Lipid Composition of Dietary *P. femorata* and *M. stenolepis* Obtained from Jeddore Harbour Smelt Stomachs, January 14, 1972

Dietary component as % of stomach contents	Total Lipid (wt. %)	Non-saponified	Triglycerides	Polar lipids	Fatty acids
P. femorata (49%)	3.0	2.9	71.6	15.9	10.3
M. stenolepis (34%)	1.5	11.2	38.9	37.2	13.1

TABLE II

Fatty Acid Composition by Chain Length (after Hydrogenation) of *P. femorata* (Total Lipids, Triglycerides, Polar Lipids, and "Free-Floating Oil") and of *M. stenolepis* (Total Lipids).

Hydrogenated Fatty acid Chain length	Weight % of total				
	P. femorata				*M. stenolepis*
	Total lipids	Triglycerides	Polar lipids	Free-floating	Total lipids
14:0	3.5	4.2	2.6	3.9	3.8
15:0	16.1	18.6	6.1	15.3	2.9
16:0	24.6	30.2	25.7	27.8	33.8
17:0	21.2	25.3	12.0	19.7	4.6
18:0	13.0	11.1	17.2	12.4	16.9
19:0	5.6	4.7	6.8	5.4	1.4
20:0	9.8	4.3	20.2	11.6	27.0
21:0	0.1	0.1	0.6	0.7	0.2
22:0	3.8	0.2	8.3	2.4	8.0
branched	0.6	1.4	0.5	0.9	0.8
total OFCA[a]	43.0	48.7	25.5	41.1	9.1

[a]Odd chain fatty acids

Lipid extractions of smelt, smelt stomach contents, and benthic fauna were carried out by the method of Bligh and Dyer (8). Samples of lipids were transesterified (9) and the methyl esters analyzed by gas chromatography on open-tubular columns as described elsewhere (10,11). Some methyl ester preparations were fractionated by thin layer chromatography (TLC) on plates coated with silicic acid impregnated with silver nitrate (12). Total lipids of *P. femorata* and the mysid *Mysis stenolepis* were fractionated into major lipid classes by gel column chromatography as described previously (13).

In sorting *P. femorata* in sea-water or formalin with forceps, a small amount of "free-floating oil" was usually released through mechanical damage.

RESULTS AND DISCUSSION

The peak time for OCFA in Jeddore Harbour smelt was known to be December-January (2). It was, therefore, of interest to find that although on Jan. 14, 1972, smelt stomachs included 92% by weight of crustacea (*P. femorata* 49%; *M. stenolepis* 34%; and *Pandalus crangon,* a shrimp, 9%), this proportion declined through February, while *P. femorata* fell to about 25% of the total (14). It was especially noteworthy that all *P. femorata* from smelt stomachs submitted for identification were terminal male instars. This findings agrees well with the anticipated visual feeding pattern of salmonid fish for whom only animals floating free in the water column are available as food. Unlike other data for smelt indicating fish as a major dietary component (15), which is partially true of Musquodboit Harbour smelt, fish were not important in the diet of Jeddore Harbour smelt (14).

A comparison of the lipid composition of *P. femorata* and *M. stenolepis,* major dietary components of Jeddore smelt on Jan. 14, 1972, shows very significant differences (Table I). Some hydrolysis of the lipids appears to have occurred in both animals, judging from the level of free fatty acids (16). This may be due in part to initial stages of digestion in the smelt stomach. More important is the observation that *P. femorata* contains twice as much lipid as *M. stenolepis,* this difference being attributable to larger amounts of triglyceride. High triglyceride levels in the amphipod may be due to the presence of oil globules in the expanded basal segments of the thoracic legs. These oil globules

are easily released by mechanical damage to the exoskeleton and were found to be almost entirely composed of triglycerides.

To facilitate comparison between the fatty acids of *P. femorata* lipid fractions and the total lipids of *M. stenolepis* (Table II), the total methyl esters from each lipid fraction examined were hydrogenated and the results, therefore, emphasize the chain length differences. The *M. stenolepis* chain length analysis is similar to that of other small crustacea such as the pelagic euphausiids *Meganyctiphanes norvegica* (17) or the benthic sand shrimp *Crangon septemspinosus* (11).

In the *P. femorata* lipids, the "free-floating oil" differed somewhat in proportions of C_{20} and C_{22} acids from the isolated triglycerides, but basically the comparison shows how this oil dominates the total lipid composition. The polar lipids had lower proportions of C_{15} and C_{17} odd chain lengths than the other lipids, but C_{19} was high, presumably reflecting a low proportion of monoethylenic C_{15} and C_{17} acids and a high proportion of polyunsaturated acids of C_{19} chain length (Table III).

The identification of polyunsaturated OCFA components in the detailed composition of *P. femorata* samples (Table III) is admittedly tentative, but is well supported by the evidence of retention-time plots and silver nitrate-TLC. The *P. femorata* fatty acids indicate the presence of all components previously identified in Jeddore Harbour smelt (1,2) as well as numerous minor components identified by Sen and Schlenk in the mullet *Mugil cephalus* (18). This evidence leads us to believe that OCFA in mullet may well be of dietary origin, although particular details are unknown (19).

The distribution of certain OCFA in *P. femorata* appears to parallel the distribution of the even chain length components longer by one carbon. Thus, the ratios of 15:0 to 15:1, and 16:0 to 16:1, for example, are both ca. 2 to 1, while 17:1 and 18:1 exceed their corresponding saturated acids by a ratio of 15 or 20 to 1. As has been pointed out earlier (20), the types of C_{19} polyunsaturated acids appear to be related to the presence of 20:4ω6 or 20:5ω3 rather than to C_{18} polyunsaturated acids.

These similarities in odd and even chain length acid distribution in *P. femorata* suggest either a common biosynthetic pathway or a common control mechanism for these components and are suggestive of biosynthesis of OCFA at this biotic level, since the occurrence of such a close similarity of odd and even chain length fatty acids at an additional but lower trophic level has a low statistical probability. On the other hand, it seems quite possible that the amphipod, browsing upon detritus in the sediment, may accumulate large amounts of materials capable of generating OCFA. One possible source, hydrocarbons (21,22), seems to be unlikely (3), as do microbial longer chain fatty acids (23-25). However, some marine species of bacteria do contain substantial proportions of longer chain OCFA (26,27). Propionate, possibly generated by microbes (23,28) or by microbial action on detritus, as well as from algae either directly (29) or from dimethyl-β-propiothetin, has been considered as a base for these acids (7,30) but is unlikely to be a precursor for the polyunsaturated acids. The close similarity of fatty acid chain length distribution patterns in *P. femorata* sampled at different locations and at different times of the year (Table III), whether male, female, mature or immature (Table IV), and in all lipid fractions also suggests a biosynthetic origin of OCFA in the amphipod.

Although the mature male *P. femorata,* the only variety of this species found in smelt stomachs, is a special morphotype, adapted for swimming freely in the water column (personal communication, E.L. Bousfield) the presence of high OCFA levels in the amphipod is evidently not a physiologically induced modification related to this special role (as the occurrence of the buoyant oil globules at first led us to suspect). It is clear from Table IV that high OCFA levels exist at all stages of maturity in both sexes of *P. femorata* and may indeed be somewhat higher in the females, perhaps as a result of their uninterrupted presence in the sediment. This data also make it quite clear that any seasonal variation in the OCFA level in smelt is not simply a reflection of changing OCFA levels in the amphipod.

The seasonally variable availability of *P. femorata* is easily explained by a closer examination of the life and reproductive cycles of the animal. Several investigations (31-33) have established that, under conditions similar to those found in Jeddore Harbour, *P. femorata* mate in early winter. Male amphipods die very shortly after reproduction and fertilized eggs appear in brood pouches of the females a short time later. Eggs hatch and young leave the brood pouch in the spring to mature during the following 18-19 months, coming to maturity, reproduction, and completion of the cycle during the winter months two years after hatching. Female amphipods die shortly after giving birth to the young. Our biological samples support this cyclic scheme in all respects.

As Figure 1 indicates, *P. femorata* is at all times of the fall and winter an important com-

TABLE III

Fatty Acid Composition (Wt. %) of *P. femorata* from Jeddore Harbour, St. Margaret's Bay, and Live-Holding Tank, Halifax

Fatty acid	Jeddore Harbour bottom sample March 23, 1973	Jeddore Harbour bottom sample May 27, 1973	St. Margaret's Bay bottom sample February 4, 1973	Live-holding Halifax April 12, 1973
14:0	2.76	1.89	2.41	1.43
I15	0.14	0.09	0.22	0.32
AI15	0.22	0.26	0.35	0.60
15:0	9.60	8.26	7.01	7.42
16:0	14.48	14.78	17.64	14.55
I17	–	0.20	0.19	0.43
AI17	–	0.23	0.17	0.44
17:0	1.10	1.28	1.32	1.84
I18	0.04	0.09	–	–
18:0	0.52	1.06	4.33	3.74
19:0	0.09	0.13	0.26	0.12
20:0	0.14	0.15	0.18	0.25
22:0	0.01	0.09	0.04	0.13
Total saturated	29.10	28.51	34.12	31.27
15:1ω8	1.31	0.72	0.59	0.42
15:1ω6+4	4.25	3.47	3.06	1.00
16:1ω7	6.68	7.53	12.04	5.47
16:1ω5	0.37	0.31	0.34	0.28
17:1ω8	20.61	18.52	14.64	13.44
17:1ω6	1.21	1.39	0.89	1.43
18:1ω9	12.01	13.12	14.09	16.06
18:1ω7	1.27	1.53	2.40	2.21
18:1ω5	0.20	0.30	0.47	0.39
19:1ω10+8	2.72	2.94	1.86	3.36
19:1ω6+4	1.15	1.40	1.25	1.33
20:1ω11+9	0.13	0.30	0.28	0.66
20:1ω5	0.04	0.01	–	–
20:1ω7	0.13	0.24	0.26	0.55
21:1ω8+6	0.75	0.65	0.42	0.81
22:1ω9	–	–	–	–
Total monounsaturated	53.01	52.43	52.59	47.46
16:2ω4	0.17	0.25	0.50	0.26
17:2ω5	2.17	1.68	1.11	1.36
18:2ω6	0.17	0.20	–	0.54
19:2ω5	0.19	0.24	0.18	–
Total diunsaturated	2.70	2.37	1.79	2.16
15:3ω4?	0.36	0.31	0.34	0.28
17:3ω4	0.88	0.83	0.56	0.13
18:3ω6	–	–	0.43	0.07
18.3ω3	–	–	0.07	0.23
20:3ω6	0.03	0.06	0.03	–
Total triunsaturated	1.27	1.20	1.43	0.71
16:4ω3	0.12	0.10	0.07	0.03
18:4ω3	0.16	0.19	0.35	–
18:4ω1	0.01	–	0.15	0.01
19:4ω5	4.32	3.66	1.77	3.32
20:4ω6	1.11	1.37	1.15	1.72
20:4ω3	0.01	0.06	0.24	0.07
21:4ω7+5	0.57	0.65	0.26	0.72
Total tetraunsaturated	6.30	6.03	3.99	5.87
17:5ω2	0.90	0.43	0.15	0.13
20:5ω3	4.22	5.58	4.52	5.42
21:5ω2	0.13	0.11	0.07	0.14
22:5ω6	0.01	0.03	0.12	0.02
22:5ω3	0.21	0.41	0.43	0.52
Total pentaunsaturated	5.47	6.56	5.29	6.23
22:6ω3	2.13	2.89	0.78	6.29
Total OCFA[a]	52.31	46.67	35.74	37.25
Calculated Iodine value	94.34	101.79	85.77	110.49

[a]Odd chain fatty acids

TABLE IV

Odd Chain Fatty Acid (OCFA) Content of *P. femorata* of Different Sexes during Various Stages of Maturity

Sample		OCFA, % of total FA
Eggs	(removed from females obtained Feb. 2, 1973)	48.5
Immature	(from bottom sample Nov. 25, 1972)	53.4
Males	(from bottom sample Dec. 9, 1972)	55.4
	(from bottom sample Dec. 20, 1972)	50.5
	(from smelt stomachs, Jan. 6, 1973)	46.4
	(from smelt stomachs, Feb. 2, 1973)	42.7
Females	(from bottom sample, Dec. 20, 1972)	55.7
	(from bottom sample, Feb. 2, 1973... egg-bearing)	53.6
	(from bottom sample, Feb. 2, 1973... without eggs)	53.8
	(from bottom sample, March 23, 1973)	51.1
	(from bottom sample, May 27, 1973)	45.4

ponent of the Jeddore Harbour benthic community. The rise in its contribution to total isolated weight of benthic fauna from October to December is probably a reflection of the rapid maturation and weight increase of individual amphipods during this time (Fig. 2). Fluctuations during January and February are an indication of the loss of male amphipods from the sediment during these months, and this is followed by a resulting decrease in the relative contribution of this species to the total benthic fauna in early spring. The "growth curve," Figure 2, shows the increase in weights of individual amphipods as maturation occurs. The levels of 30-40 mg/animal achieved in March probably represent a maximum amphipod weight for gravid females, but immature individuals were present in all bottom samples (due to overlap of succeeding year classes) and may tend to dilute and cause fluctuations in the curve.

Of greatest interest, however, is the correlation between Figure 1 and Figure 3 which indicates the occurrence of mating during December and January through the rapid disappearance of mature male amphipods from the sediment (presumably mostly into smelt stomachs) and the appearance of gravid females. By May the proportion of gravid females had dropped to a very low level (probably 5% or less) but was not readily determined due to the poor physical state of amphipods examined from that time. This state of the females (anticipated for animals having given birth) made classification difficult in many cases due to the presences of unhatched eggs and fully developed brood pouches.

The disappearance of male amphipods from the harbor bottom after mating is very sharply demonstrated for February of 1973 (Fig. 3)

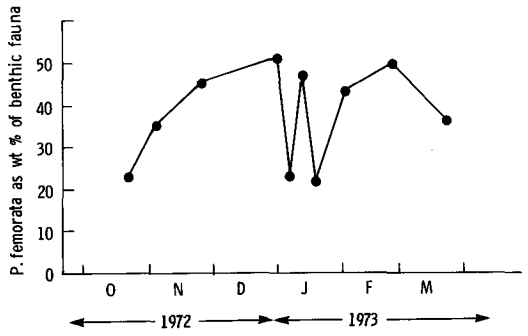

FIG. 1. Variation in levels of *P. femorata* in Jeddore Harbour bottom samples as weight % of total fauna isolated from Oct. 21, 1972, March 23, 1973.

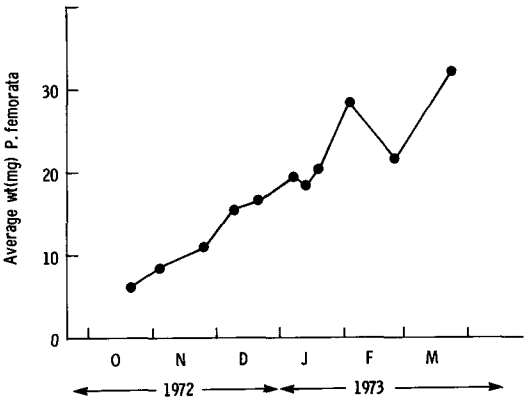

FIG. 2. Variation in average weight of individual *P. femorata* in Jeddore Harbour bottom samples from Oct. 21, 1972, to March 23, 1973.

and is indicative of their release into the water column after mating in January to become food for smelt in the ensuing weeks. The cyclic nature of this dietary component is thus re-

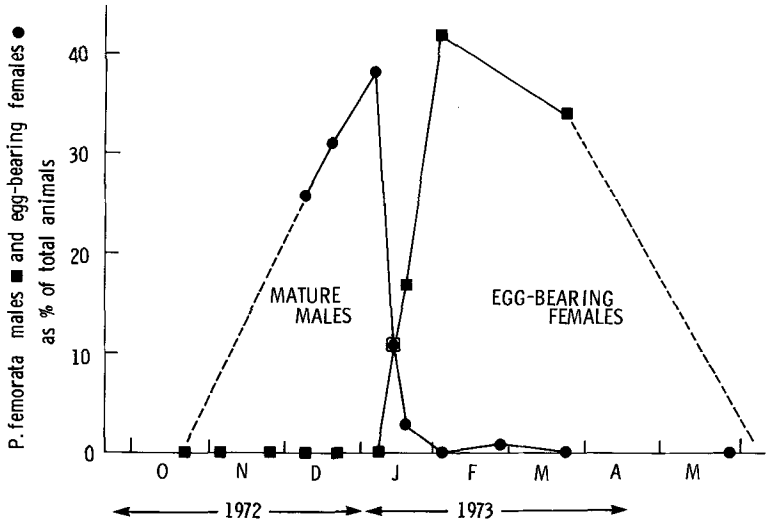

FIG. 3. Proportions of mature male *P. femorata* and of egg-bearing female *P. femorata* in Jeddore Harbour bottom samples from Oct. 21, 1972, to May 27, 1973.

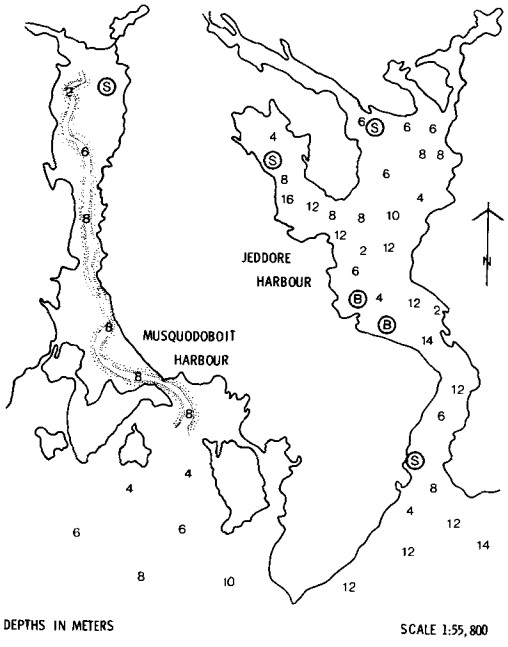

FIG. 4. Under water relief of Jeddore Harbour, showing relatively constant depth (in meters) and sampling sites for fish and bottom. Note that only the narrow channel in Musquodoboit Harbour has depths comparable to those typical of Jeddore Harbour. S = smelt fishing area, B = bottom sampling area. Approximate center of map: 44° 45'N, 63° 05'W.

sponsible for the known seasonal variation in OCFA levels in Jeddore Harbour smelt (2,14).

The timing of the reproductive cycle in *P. femorata* has been the object of several studies and appears to be under the control of the light sensitive eyespots on the anterior segments of the amphipod and to be independent of water temperature. It has thus been found that *P. femorata* will reproduce without respect to the time of year in depths of water (60 m. or deeper) which decrease incident light to a level below the sensitivity limit of the animals' visual pigment (31-33). Several factors may be expected to be of some significance in controlling the intensity of light available in a body of water such as Jeddore Harbour.

The first and undoubtedly most important is the season. Other include the average depth of the bottom, the clarity of the water and, in winter, the degree of ice and snow cover in the harbor. The depth of the bottom in Jeddore Harbour is remarkably uniform (Fig. 4), and the clarity of the water is probably smoothed by being a function of both loss of algal productivity and increased detritus (34). However, for a harbor with heavy ice and snow cover, the seasonal minima of incident light and light occulusion both occur during winter months, resulting in the regular cyclical pattern of reproductive activity peaking at the year end. The span of time over which mating and release of males into harbor waters may be expected to occur will probaly depend primarily on the range of depths experienced by *P. femorata* in the harbor. The greater the range of depths of *P. femorata* breeding grounds, especially depths greater than the visual limit of 60 m, then the broader will be the span of the time over which mating will occur. This has important ramifications in respect to the geographical limitation

of high OCFA levels in smelt. If the physical constants of a harbor are such that mating and release of male amphipods occurs over an extended period of time, then *P. femorata* may not represent a predominant dietary component of smelt at any time of the year. The effect of ingesting amphipods high in OCFA will then be diluted over a relatively long period of time and high OCFA levels may never occur in smelt. Such an explanation is necessary in light of the finding that *P. femorata* obtained from St. Margaret's Bay, Nova Scotia, also contain high levels of OCFA (Table III). This bay is some 100 km from Jeddore Harbour, and is a much larger open embayment with much greater variability in depths and overall much deeper water. On the other hand, Jeddore Harbour has a fairly uniform depth of about 8-12 m, a depth found only in a narrow channel of Musquodoboit Harbour (Fig. 4).

Amphipods transported from Jeddore Harbour to live-holding facilities in Halifax on Nov. 4, 1972 and examined some five months later on April 12, 1973 (Table IV) still showed high OCFA levels, and it thus seems likely that the phenomenon need not be restricted to a specific natural environment. Since *P. femorata* appears to be quite widely distributed along the Atlantic coast of Canada (D. Peer, private communication), the geographic limitation of high OCFA levels in smelt must be a function of harbor parameters rather than of variables in the amphipod. The task of correlating data on harbors with the reproductive cycle of *P. femorata* and OCFA levels in smelt remains as a potential goal of further studies.

It should also be of interest to examine a variety of the amphipods found along with *P. femorata* in both bottom samples and smelt stomachs from Jeddore Harbour (14), provided these did not aquire any free-floating oil from *P. femorata.* Since at least some of them may share a common diet with *P. femorata,* their content of OCFA may be an indication of whether a particular component in the amphipod diet causes high OCFA levels. The elaboration of OCFA distribution in amphipods may well answer most questions regarding the origin of these components in all higher trophic levels.

Our ability to maintain reproducing *P. femorata* under artificial live-holding conditions for a prolonged period may permit a more detailed examination of the propagation of OCFA from amphipod to smelt and the mechanisms by which the OCFA originate in *P. femorata.*

Much discussion has also taken place regarding the origin of the glacial-relict amphipod, *P. affinis* (32). This animal is the fresh water analog of *P. femorata.* It has been proposed that *P. affinis* arose from the trapping of *P. femorata* in inland waters during the recession of the penultimate ice age. Any similarity in the fatty acid composition of these closely related species, especially OCFA levels as unusual as those in *P. femorata,* would give strong support to this contention.

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Localization of a Source of Marine Odd Chain-Length Fatty Acids. II. Seasonal Propagation of Odd Chain-Length Monoethylenic Fatty Acids in a Marine Food Chain

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ABSTRACT

The unusual occurrence of elevated levels of odd chain length fatty acids (OCFA) in smelt taken during winter months in Jeddore Harbour, Nova Scotia, is due to the dietary intake of large numbers of the amphipod *Pontoporeia femorata* (Kröyer). Sampling over two winters confirms that the seasonal peak period for this amphipod in stomachs is mid-December to the end of February. The distributions of monoethylenic isomers are compared to distinguish exogenous and endogenous smelt dietary features in terms of the geographically limited phenomenon of high OCFA levels and the essential absence of biological activity for these acids.

INTRODUCTION

We have established that the lipids of an amphipod *Pontoporeia femorata* (Kröyer) are the basic source of unusual occurrences of odd chain length fatty acids (OCFA) in smelt *Osmerus mordax* taken from Jeddore Harbour (1). The geographical peculiarity involved, in that percentages of OCFA are normal in smelt from elsewhere in the same region (1-3), opens up an area which has been little investigated, that of competition between exogenous natural odd and even chain lengths in the diet of a fish. Another study has indicated that other waterborne sources of fatty acids and hydrocarbons show seasonal fluctuations in odd chain lengths (4), but not those related to OCFA in smelt. The ca. 50% OCFA content of *P. femorata* lipids is apparently constant (1), facilitating an understanding of dietary processes. The biochemistry of the monoethylenic fatty acids, one of the simpler aspects of fatty acid metabolism, is examined for smelt with and without access to OCFA.

EXPERIMENTAL PROCEDURES

Two adjacent and superficially similar tidal bodies of water (for map, see references 1 and 2) were sampled by gill net in early winter for smelt as described elsewhere (1). The smelt stomach contents were examined and the fatty acid compositions of the total lipid extracts of the eviscerated smelt bodies were compared with those of *P. femorata* collected from bottom mud samples. Particular attention was paid to monoethylenic fatty acid distributions obtained by open-tubular gas liquid chromatography (5). These will be discussed with the "ω" shorthand notation where the ethylenic unsaturation is counted from the terminal methyl group, but the results are also tabulated in the "Δ" system to facilitate comparison with literature data.

RESULTS AND DISCUSSION

Statistics of fish catches are not reported here since no correlation appeared to exist between fish size, sex, or maturity, and composition of stomach contents. It was observed that fish caught were generally large (16.5-23.5 cm), suggesting that the method of fishing by gill-net selected extra large smelt (6), and that females predominated (60-80% of total catch). The number of fish with empty or only partially-full stomachs appeared to increase during early spring months, and this may represent a change in feeding pattern in anticipation of upstream spawning runs (6).

The stomach contents of smelt taken from both Jeddore and Musquodoboit Harbours are given in Table I. It is important to note that on each of the sampling days listed, *P. femorata* was absent from the diet of smelt in Musquodoboit Harbour. There was also a rapid decrease of *P. femorata* in Jeddore Harbour smelt stomachs after the end of February, although the amphipod remains as a minor dietary component even in March. It is also evident from the table that shrimps, mysids, and other amphipods are the most regular dietary components of smelt, while polychaete worms, fish, and other components (usually copepods) are variables.

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TABLE I

Composition of Diets of Smelts from Jeddore Harbour (J) and Musquodoboit Harbour (M) from February 15 to March 15, 1972

Date	Location	Dietary components (% by weight)					
		P. femorata	Other Amphipods	Shrimp & Mysids	Polychaetes	Fish	Other
Feb. 15, 1972	J	15	22	24	33	0	7
	M	0	43	15	42	0	0
Feb. 22, 1972	J	21	12	30	27	0	11
	M	0	41	44	0	15	0
Feb. 23, 1972	J	20	18	29	22	5	5
	M	0	35	48	11	5	0
Feb. 24, 1972	J	19	23	21	26	0	11
	M	0	49	23	0	28	0
Feb. 25, 1972	J	50	19	13	0	0	19
	M	0	31	29	0	40	0
Feb. 28, 1972	J	17	38	29	17	0	0
	M	0	75	10	0	15	0
Feb. 29, 1972	J	33	0	0	67	0	0
	M	0	47	32	0	21	0
March 13, 1972	J	3	35	9	4	0	49
	M	0	41	56	0	3	0
March 15, 1972	J	0.5	6	69	0	2	16
	M	0	41	59	0	0	0

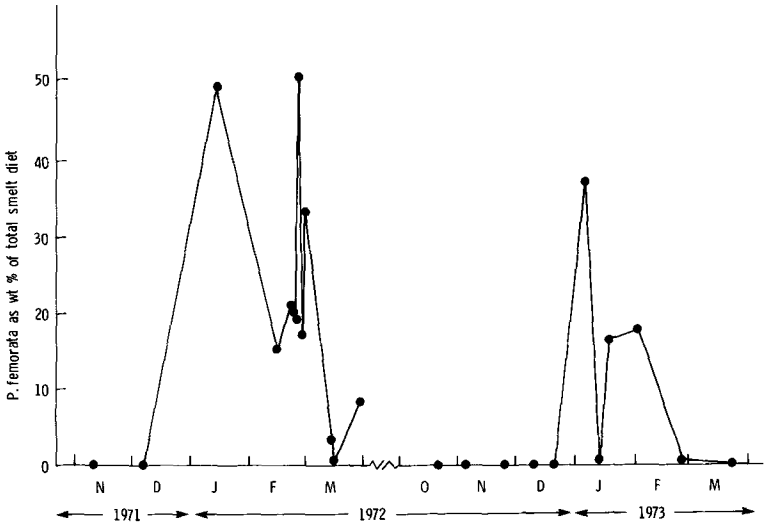

FIG. 1. Seasonal variation in levels of *P. femorata* as weight % of total stomach contents of Jeddore Harbour smelt for November 1971 to March 1973.

The most common species of shrimp and mysid appearing in the diet were tentatively identified as *Pandalus crangon* and *Mysis stenolepis*, respectively. The grouping "other amphipods" consisted of as many as a dozen different species, the most common of which were: *Gammarus oceanicus, Gammarus lawrencianus, Pontogeneia inermis,* and *Orchemenella minuta.* Amphipods were identified by Dr. E.L. Bousfield of the National Museum of Natural Sciences of Canada who further indicated that virtually all of the samples submitted were of a species or stage of maturity which could be regarded as pelagic or epibenthic.

The reason for seasonal changes in smelt OCFA levels is a very obvious variation in the seasonal intake of *P. femorata* in smelt diet (Fig. 1; see also ref. 1). Although large variations may occur from sample to sample, it is obvious from Figure 1 that at some time between December and January, *P. femorata* suddenly appear in smelt stomachs, attain maximum levels in January and February, then rapidly decrease to low or negligible amounts by early spring. It should be borne in mind that this phenomenon represents a fortunate coincidence for the smelt. If the amphipods became available only at a somewhat later date, the smelt might be in a depressed-feeding stage or could have already begun to migrate to up-river spawning grounds.

We may thus conclude, in accordance with McKenzie's findings for smelt in the Miramichi River, New Brunswick (6), that smelt moving into Jeddore Harbour in early winter in preparation for spring spawning runs encounter and consume large numbers of crustacea, and, in particular, mature male *P. femorata* floating free in the harbor waters. This dietary factor is apparently absent in the case of Miramachi (2) or Musquodoboit Harbour smelt. In the latter harbor, bottom samples of mud showed no *P. femorata* and, although this could have been a sampling problem, the results are confirmed by observations on smelt stomachs. Presumably Musquodoboit Harbour is too shallow (1) or of too low salinity to support a *P. femorata* population.

In St. Margaret's Bay, mud samples showed high levels of *P. femorata* in places (D. Peer, unpublished results). The smelt population is not as concentrated as in Jeddore Harbour; feeding competition from other open ocean and bottom-dwelling species would be very heavy; and, as outlined elsewhere, the greater depth variability would result in greater temporal diffusion of the available *P. femorata* (1).

The fatty acid chain length compositions of *P. femorata* and *M. stenolepis* lipids have been presented elsewhere (1) and provide the explanation of the high OCFA levels in smelt. The mysid maintains a normal, basically even chain length oriented distribution of fatty acids, while the amphipod manifests an extremely high level (ca. 50%) of OCFA. The high OCFA levels in *P. femorata* extend to all chain lengths and all lipid classes, including the oil globules peculiar to this species. It is of interest to note that OCFA levels are lower in polar lipids than in triglycerides. This is probably a reflection of the predominance of 15-carbon and 17-carbon saturated, and especially monounsaturated,

TABLE II

January 14, 1972 Monoethylenic Acid Isomer Distribution by Chain Lengths from *P. femorata*, Jeddore Harbour Smelt, and Musquodoboit Harbour Smelt

Fatty acid		*P. femorata*	Jeddore smelt	Musquodoboit smelt
14:1ω7	(Δ7)	64.9	-	-
14:1ω5	(Δ9)	35.1	100	100
15:1ω10	(Δ5)	tr	tr	6.6
15:1ω8	(Δ7)	23.8	29.5	49.1
15:1ω6	(Δ9)	75.7	70.5	44.3
15:1ω4	(Δ11)	0.6	-	-
16:1ω9	(Δ7)	2.6	4.1	3.4
16:1ω7	(Δ9)	95.6	94.0	94.3
16:1ω5	(Δ11)	1.9	1.9	2.3
17:1ω10	(Δ7)	-	tr	3.0
17:1ω8	(Δ9)	95.3	93.5	84.9
17:1ω6	(Δ11)	4.7	5.9	9.2
17:1ω4	(Δ13)	-	0.6	2.9
18:1ω9	(Δ9)	81.6	74.2	80.2
18:1ω7	(Δ11)	17.2	24.3	18.4
18:1ω5	(Δ13)	1.3	1.4	1.4
19:1ω10	(Δ9)	10.8	12.0	22.3
19:1ω8	(Δ11)	68.5	71.7	59.4
19:1ω6	(Δ13)	19.4	15.4	14.5
19:1ω4	(Δ15)	1.4	0.9	3.8
20:1ω11	(Δ9)	9.9	19.4	21.2
20:1ω9	(Δ11)	43.0	40.2	46.1
20:1ω7	(Δ13)	38.8	38.4	30.8
20:1ω5	(Δ15)	8.0	2.0	1.9

acids. The longer chain 19-carbon and 21-carbon polyunsaturated acids are higher in the polar lipids. The "free-floating oil" is generally similar in fatty acid distribution to triglycerides. The mysid shows somewhat elevated levels of OCFA over that expected from other crustacean analyses (7-9), but this is likely a result of contamination with the free-floating oil released by break-up of *P. femorata* in smelt stomachs. Preliminary analyses of polychaetes from Jeddore Harbour show no excessive levels of odd chain length fatty acids (R.F. Addison, unpublished results).

The efficiency with which dietary OCFA are assimilated by smelt and deposited in body fats is not known, but the assimilation is likely quite efficient, and the low body fat in smelt (2) would make the fatty acid compositions of these fish easy to modify. By a curious set of coincidences, a total fatty acid composition analysis of stomach contents (3) gave ca. 5-7% 17:1. These figures correspond closely to the range which can be calculated from the *P. femorata* content of smelt stomachs (Table I) and their 17:1 content of ca. 20% (1), and to the percentages of total fatty acids in smelt body fatty acids (2). In rearing rainbow trout on a variety of diets, Shimma and Nakada (10,11) included several petroleum yeasts with ca. 30% 17:1 in diet formulations such that their diets also contained ca. 5% 17:1. Although their fish had 6-8% lipid after 9 wk of feeding, compared to 4.9% at the start, the carcass fatty acid compositions also showed 5-6% 17:1 in all cases, including one with a dietary pellet containing 14.9% of 17:1 which would be diluted down to about the same level by other added fats. These two sets of data are suggestive of an upper limit for 17:1 accumulation in fish triglycerides, possibly governed by the proportion relative to 18:1, but as the 17:1 in the diet is limited in both cases to ca. 5% of total fatty acids, this could also be the controlling factor. The production of 17:1 from 17:0 is well documented, for example in chicks, where it was found to be especially prominent in serum sterol esters (12).

The odd chain length monoethylenic isomer distributions for Jeddore Harbour smelt sampled at or near peak OCFA accumulation appear to be remarkably consistent from year to year, as may be shown by comparison of the data in Table II for body lipids of 1972-73 fish with that published earlier for 1968-69 fish (2). This must result from the impression of a relatively high level of exogenous fatty acids, especially OFCA, on a fish composition which is basically species-oriented but susceptible to strong local dietary or environmental influences.

The close link between *P. femorata* and smelt lipids can be shown by study of OCFA monoethylenic isomer ratios [Table II; see also data of Addison and Ackman (2)]. The distribution of isomers in Jeddore Harbour smelt and in *P. femorata* is very similar. On the other hand, Musquodoboit Harbour smelt monoene isomers for 1972-73 have a different distribution pattern for OCFA components, and generally dissimilar results were also found by Addison and Ackman (2) in smelt of other origins. For example, the two major 15:1 isomers may normally be equal in smelt except when *P. femorata* is in the diet. If so, then the ratios for 15:1 isomers for Digby smelt (2) suggest some *P. femorata* in their diet. In oil of the mullet *Mugil cephalus* (13), the ratio is 15:1ω6>15:1ω8 in a ratio of 8:5, or about the same as in *P. femorata* and Jeddore Habour smelt. All monoethylenic fatty acids are, however, still qualitatively similar in composition in our studies, suggesting that OCFA arise from normal precursors by normal pathways (14,15) in both the amphipod and smelt. Presumably OCFA may pass with virtually no chemical modification from the amphipod to the smelt and become an integral part of smelt lipids. Several factors would facilitate this direct propagation: the naturally low levels of OCFA in smelt (as evidenced by low levels in Musquodoboit smelt), the predominance of *P. femorata* with its high lipid level in smelt diets during winter months (1), and the ready availability of much of the amphipod lipids as "free oil." Several researchers have also shown that OCFA are metabolized in a manner analogous to their even chain analogs and are capable of fulfilling, in part, requirements for essential fatty acids (14,15). Thus, no chemical modification of OCFA would be anticipated on this basis. In the even chain lengths, the normal biosynthetic pathways in the smelt may manipulate the exogenous 18:1 and 20:1 in a limited way to suit the localized needs of the fish since the isomer proportions for Jeddore Harbour smelt differ slightly from those for *P. femorata* for the two years when these smelt were examined. In the other locations sampled [see also Addison and Ackman (2)], there are only minor variations, but, when 18:1ω7 is low in the fish body lipids, then the related 20:1ω7 is usually also low relative to 20:1ω9. In these two cases (Pictou and Musquodoboit), 18:1ω9 is correspondingly higher and 20:1ω11 more plentiful, suggesting de novo biosynthesis of these two acids by desaturation of 18:0 and 20:0. This further suggests that Pictou and Musquodoboit smelt have a limited supply of exogenous fatty acids of the types supplied by *P. femorata*, whether of odd or even chain lengths, in Jeddore Harbour. The even chain isomer ratios in smelt differ only in relative proportions from those of many pelagic marine fish such as herring (5).

In the case of the mullet (12), the ratio for fatty acids of 18:1ω9 to 18:1ω7 was 49:41, and that of 20:1ω9 to 20:1ω7 was 37:63. In the alcohol acetates from roe (16), the ratios were both 2:3. The mullet OCFA and alcohols show the same major monoethylenic isomers as are found in Jeddore Harbour animals. The 17:1 isomers are mostly ω8, with a little ω6, and in the 19:1 alcohols (17) the isomer ratio of 19:1ω10 to 19:1ω8 is 1:7, or almost exactly the same as in the fatty acids of *P. femorata* (Table II), despite the difference in 20:1 isomer ratios and the suggestions that the next highest even chain length governs the structural types of OCFA (15,17). In the mullet oil, 15:1 totalled 1.3% and 17:1, 4.6%, almost identical to percentages in smelt (2). We are thus left with the quandary that the OCFA monoethylenic isomers are nearly identical in distribution in each fish and in *P. femorata*, whereas the even chain isomer ratios are quite different for the 18:1 and 20:0 groups. This does suggest a similar basic biochemistry, but not necessarily a related origin, for the OCFA. On the other hand, the even chain lengths, as suggested above for the smelt, are presumably subject to fairly rigid species requirements. As the mullet is basically a somewhat unusual filter feeder (18), its food may be very primary (e.g., bacteria may provide most of the fatty acids) and either be rich in 16:0 or be broken down and used to synthesize 16:0, hence the emphasis on 18:1ω7 and 20:1ω7 as successor acids to the 16:1ω7 synthesized de novo from 16:0 by desaturation.

These comparative details, and the observation that smelt apparently catabolize the OCFA readily, and possibly even preferentially, when food intake is reduced, emphasize that OCFA are basically biochemically inert in fish lipids. This makes their propagation in the marine food web of more than passing interest and suggests that they could be used in laboratory studies of dietary fat assimilation and utilization in fish along the lines published by Shimma and Nakada (10,11).

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SHORT COMMUNICATION

Fatty Acids of the Seed Oils of Alpine *Cruciferae* and Other Alpine Species

ABSTRACT

A few *Cruciferae* seed oils do not contain erucic acid but are instead rich in linolenic acid. These oils are characteristic of alpine species, and the fatty acid composition of such oils may be an adaption to an alpine habitat.

INTRODUCTION

Cruciferae seed oils are noted for the presence of C_{20}, C_{22}, and C_{24}, acids, particularly the monoene members erucic acid (22:1) and 20:1. Over 50 species have been reported (1-3), and the majority contain 25-50% erucic acid. However, some are richer in 20:1 than 22:1, and a few contain virtually no C_{20}, C_{22}, or C_{24}, acids and are instead rich in α-linolenic acid. Of the 53 species examined, 30 have erucic rich oils (Type A), 15 have 20:1 rich oils (Type B), and 8 have linolenic rich oils (Type C). Figure 1 illustrates the fatty acid profiles of these different types of oil, for both a typical species and the range which can be grouped in each class.

Types A and B differ only in the relative amounts of the component acids, but type C oils are an exception to the general rule that *Cruciferae* oils contain C_{20}, C_{22}, and C_{24} acids. The following species have type C oils:- *Alyssum saxatile* (1,2), *Arabis alpina* (1-3), *A. hirsuta* (3), *Bertheroa incana* (3), *Hesperis matronalis* (1-3), *Lepidium montanum* (1), *Matthiola bicornis* (1), *M. incana* (2). It is noteworthy that these are mainly "alpine" species. It is difficult to rigorously define an alpine species, but generally they are adapted to a habitat at high altitude (and/or latitude) with a short growing season of low average temperature. Alpine species may also grow in more temperate conditions, but temperate species will not grow under alpine conditions. The virtual absence of C_{20}, C_{22}, and C_{24} acids in the seed oils of the alpine *Cruciferae* species may be an adaption to their alpine habitat.

To test this apparent correlation, the seed oils of several other alpine *Cruciferae* species were examined. There is a scarcity of data on alpine species in general, and species from other families were also examined.

EXPERIMENTAL PROCEDURES

Seed samples were provided by St. Andrews University Botanic Gardens, except for *Alyssum montanum, Dryas octopetala,* and *Gentiana verna,* which were from commercial samples. Single samples (10-200 mg) of seeds were ground and extracted with boiling petrol (bp 40-60 C). The oil obtained on remvoing the solvent was transesterified overnight with 2% conc. H_2SO_4 in methanol (2 ml) and methyl-

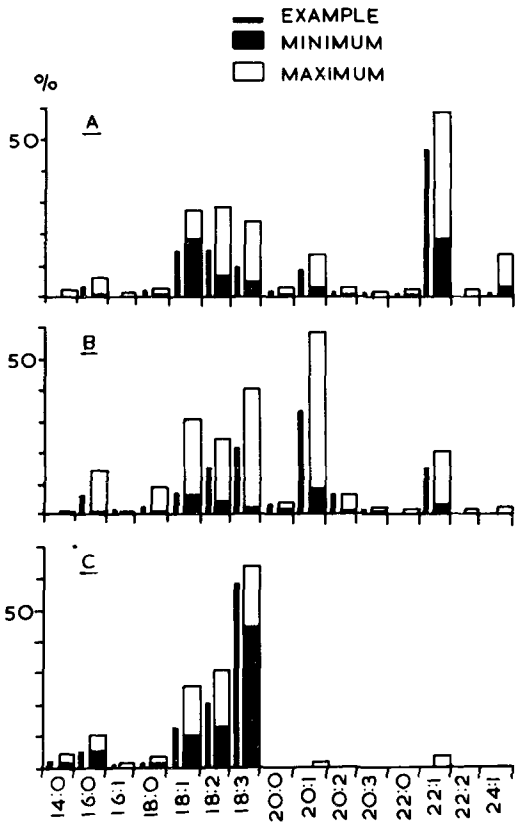

FIG. 1. Fatty acid profiles for *Cruciferae* seed oils. The examples are *Brassica campestris* (A), *Malcomia maritima* (B), and *Alyssum saxatile* (C). Data from Ref. 1.

TABLE I

Fatty Acid Composition of Alpine Seed Oils

Seed	% w/w oil in seed	Composition of Methyl Esters Wt %														
		14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:1	20:2	22:0	22:1	22:2	24:0	24:1	other
Cruciferae																
Aethionema pulchellum	20	–	7.0	0.5	1.3	11.1	12.9	67.2								
Alyssoides utriculata	6	–	6.7	tr[a]	1.6	11.0	9.5	71.1								
Alyssum montanum	11	3.7	9.8	tr	1.6	10.3	16.9	57.6								
Arabis blepharophylla	20	–	11.2	–	3.2	9.8	32.5	43.3								
Aubrieta kotschyi	5	0.6	9.2	0.7	2.6	20.4	29.7	35.2								1.6
Draba montana	12	tr	4.6	0.3	1.8	8.7	19.9	64.6								
Erysimum odoratum	13	tr	3.1	0.3	0.8	3.6	18.0	30.2	5.4	2.3	1.5	28.6	1.7	0.8	3.6	
Iberis pruitii	15	tr	2.4	0.4	0.4	19.8	14.9	7.4	5.7			43.1			5.8	
Isatis glauca	4	–	4.6	tr	0.6	11.1	13.4	29.4	10.9	1.3	1.0	23.3	0.5	0.3	3.5	
Matthiola arborescens	19	tr	7.7	–	2.9	12.5	9.4	66.4								1.0
Parrya menziesii	21	tr	5.5	0.3	1.3	7.6	12.1	30.7	11.7	1.7	1.6	26.3	0.5		tr	0.6
Gentianaceae																
Gentiana verna	14	–	6.4	–	3.1	20.5	68.7	1.3								
Leguminosae																
Oxytropis campestris	4	5.1	4.8	0.6	3.8	5.5	13.8	64.0								2.4
Rosaceae																
Dryas octapetala	7	–	5.7	tr	2.8	19.9	57.0	11.1								3.4
Saxifragaceae																
Saxifraga granulata	30	–	5.6	–	0.3	9.9	34.3	49.9								
Saxifraga nivalis	29	tr	0.9	0.1	tr	16.1	24.1	58.7								
Saxifraga nesacea	37	–	3.5	–	0.7	8.2	33.8	53.8								
Saxifraga oppositifolia	25	–	6.0	–	0.4	11.1	36.4	44.4								1.7

[a]tr = trace.

ene chloride (1 ml) at room temperature. The methyl esters were analyzed using a Pye 104 gas chromatograph, fitted with a 5 ft x ¼ in. OD column of 15% DEGS on chromosorb W AW DMCS. The component esters were identified by comparison with authentic standards.

RESULTS AND DISCUSSION

The seed oil fatty acid composition of 11 *Cruciferae* and 7 other species now examined is shown in Table I. As expected, the majority of the alpine *Cruciferae* have type C oils. The other species all have highly unsaturated oils, but they are not very different from typical temperature oils such as maize or linseed. Among the Saxifrages, *Saxifraga nivalis* and *S. oppositifolia* are high alpine species and *S. granuluta* and *S. nesacea* are more lowland species, but their oils are not significantly different.

The degree of unsaturation of an oil can be influenced by both genetic and environmental factors. Hilditch (4) noted that while unsaturated oils could be found among both tropical and temperate species, highly saturated oils were restricted to tropical species; he suggested that the oil had to be liquid at the growing temperature of the seed. Many plants, such as sunflower and linseed, produce more unsaturated oils when grown in cool conditions. This environmental effect is due to the induction of desaturase enzymes and the increased availability of oxygen at lower temperatures (5).

Riiner (6) has shown that the melting point of *Cruciferae* oils depends mainly on the amount of erucic acid present. Oils with 40-50% 22:1 melt at 2-3 C, but oils with less 22:1 and more 18:1 melt below 0 C. An oil with 30% 22:1 and 24% 18:1 melted at −6 C. None of these oils had more than 12% 18:3. Thus type C oils with only C_{18} acids will be very low melting, and those of *Erysimum odoratum, Isatis glauca,* and *Parrya menziesii* which have about 25% 22:1 and about 30% 18:3 will also melt well below 0 C. These last three are much richer in 18:3 than are normal type A oils.

Alpine plants have to germinate and make growth under cold conditions and must have a seed oil which is liquid at these temperatures. This probably requires on oil which melts a few degrees below the minimum germination temperature, i.e. an oil melting at or below 0 C. Thus the development of 18:3 rich oils, and eventually the erucic free type C oils, appears to be an adaption to the alpine habitat.

The biosynthesis of type C oils differs from that of type A oils both in the lack of chain extension beyond C_{18} and in the increased desaturation of the C_{18} acids. By contrast, selectivity bred strains of rape lack the chain extension but do not show increased desaturation, giving an 18:1 rich oil. Garden varieties of *Alyssum montanum, A. saxatile,* and *Arabis alpina* were also analyzed and contain type C oils. Such garden varieties have been bred for many generations under temperate conditions, which suggests that the oil type is genetically determined. Thus, the development of type C oils appears to be a specific adaption to an alpine habitat rather than an environmental effect on a particular plant.

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